An Investigation of a Possible Non-Neuronal Cholinergic System in the Vasculature

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka!” but ‘That’s funny…’”

Isaac Asimov

(1920-1992)

Abstract

Investigation of a possible non-neuronal cholinergic system in the vasculature

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Vascular endothelial cells express muscarinic acetylcholine receptors, which lead to NO- and endothelium-derived hyperpolarization responses that constitute major pathways for vasodilation. As evidence suggests that perivascular nerves may not represent a viable source of innervating ACh, we investigated whether suggestions of a 'non-neuronal cholinergic system' within the vasculature might act as a source of ACh that is able to influence the endothelium.

Rat mesenteric arteries were cut open and held as an *en-face* preparation with the endothelial cell (EC) layer exposed to measure EC $[Ca^{2+}]_i$. Furthermore we present the first example of EC tubes isolated from rat mesenteric arteries, used to measure EC $[Ca^{2+}]_i$. Immunohistochemistry was performed on intact arteries to demonstrate localization of important cholinergic proteins.

Direct application of rat whole blood induced increased Ca^{2+} activity in ECs that did not appear to rely on shear stress or other mechanical interaction of the blood with the endothelial cells. This response, shown to rely on IP_3 mediated Ca^{2+} release, was attenuated by the muscarinic antagonist atropine; it was concluded that blood contains or induces the release of non-neuronal ACh.

The localization of the ACh synthesizing protein, choline acetyltransferase by immunohistochemistry provides evidence for a source of ACh originating within the endothelium. In accordance with this, the blocker of high affinity choline uptake, hemicholinium-3, had a significant effect in reversing choline potentiated responses in ECs. Taken together, we proposed that ACh is released potentially via organic cation transporter proteins located in the endothelium, upon stimulation by blood in response to an as yet undetermined signal.

The ability to influence EC Ca^{2+} highlights a role for blood in the local control of these cells and the data presented suggest ACh produced locally may provide physiological relevance by exerting influence on endothelial M_3 muscarinic acetylcholine receptors.

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Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
BK_{Ca} channel	Large conductance Ca^{2+}-activated K^+ channel
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
cADPR	Cyclic adenosine diphosphate ribose
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CHT1	High affinity choline transporter 1
CICR	Ca^{2+}-induced Ca^{2+} release
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
EC	Endothelial cell
EDH	Endothelium-derived hyperpolarization
EDHF	Endothelium-derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
GPCR	G protein-coupled receptor
IEL	Internal elastic lamina
IK_{Ca} channel	Intermediate conductance Ca^{2+}-activated K^+ channel
IP_3	Inositol-1,4,5-trisphosphate
IP_3R	Inositol-1,4,5-trisphosphate receptor
L-NAME	N_ω-nitro-L-arginine methyl ester
MEGJ	Myoendothelial gap junction
MOPS	4-morpholinepropanesulfonic acid
NO	Nitric oxide
OCT	Organic Cation Transporter
OGB-1	Oregon Green 488 BAPTA-1 AM
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
ROI	Region of interest
RyR	Ryanodine receptor
SK_{Ca} channel	Small conductance Ca^{2+}-activated K^+ channel
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
Tris	Tris(hydroxymethyl)aminomethane
VAChT	Vesicular acetylcholine transporter
VGCC	Voltage-gated Ca^{2+} channel

Chapter 1

Introduction

1.1 Structure of resistance arteries

Blood flow through the capillaries provides exchange of gases and solutes between blood and tissue; a continual supply is needed which can be altered to meet the demands of the live tissue metabolism. The entirety of the circulatory system is established to efficiently execute this process; the largest arteries act principally as blood conduits whereas smaller arteries and arterioles, termed *resistance vessels*, are able to regulate peripheral arterial resistance and as such, blood flow in order to supply the tissues with oxygen and nutrients commensurate to their requirements.

The tunicae of blood vessels are three layers, consisting of: an outer layer of connective tissue interspersed with sensory-motor and adrenergic perivascular nerves (tunica adventitia), a middle layer of smooth muscle up to several cells thick (tunica media) and an inner endothelial monolayer lining (tunica intima; (Mulvany & Aalkjaer, 1990). Smooth muscle cells are circumferentially positioned around the vessel with endothelial cells oriented perpendicularly (**Figure 1.1.1**). With each endothelial cell up to ~100 μm long and underlying smooth muscle cells ~5 μm in diameter, this morphology allows for many potential points of contact between a single endothelial cell and many smooth muscle cells (Emerson & Segal, 2000; Haas & Duling, 1997). Separating the endothelium and smooth muscle however, is the internal elastic lamina (IEL), a thin layer of elastic fibres with holes in it though which the endothelial cell may project to adjoin the underlying smooth muscle. These points of contact are termed myoendothelial gap junctions and allow direct cell to cell communication via interchange of small signalling molecules as well as current (Dora *et al.*, 1997; Kansui *et al.*, 2008; Straub *et al.*, 2014).

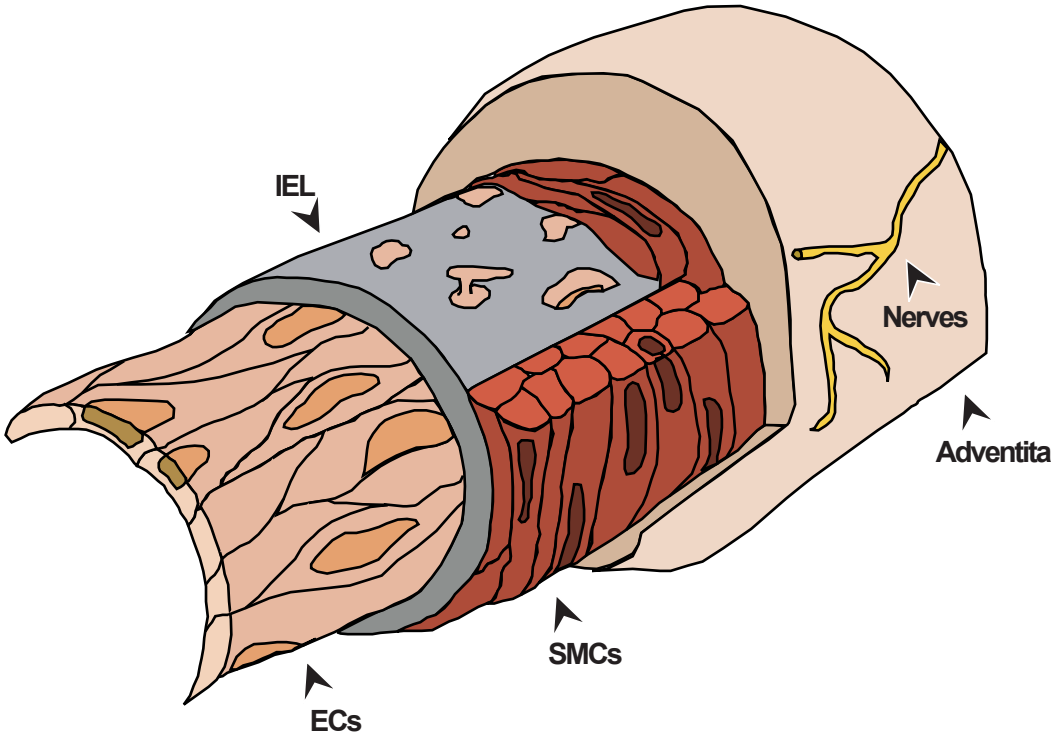


Figure 1.1.1 The structure of resistance arteries.
Schematic illustrating the structure of a resistance arteriole. The tunica adventitia is primarily formed of a matrix of connective tissue; mostly elastin and collagen and is interspersed with perivascular nerves. Dependent on the artery, the tunica media is composed of between one and several layers of smooth muscle cells (SMCs). The SMCs wrap around the vessel which partially causes occlusion of the lumen as they contract. The tunica intima consists of a monolayer of endothelial cells (ECs) that are oriented perpendicularly to the SMCs and in line with the direction of blood flow. In spite of the presence of the internal elastic lamina (IEL) between the ECs and SMCs, points of contact are formed between the two cell types through holes in the lamina, sites where the ECs can project through.

Resistance to blood flow is a major determinant of blood pressure. The relationship as stated by Pouiselle's Law between the diameter of a blood vessel and resistance to blood flow tells us that even small changes in arterial diameter have large effects on total arterial resistance; but is also influenced by the length and number of the arteries changing diameter. Thus localized dilation in one artery has less influence on arterial resistance than if the whole bed dilates, and as such conducted dilation helps reduce arterial resistance and ultimately, blood pressure.

1.2 Calcium signalling

Intracellular calcium (Ca^{2+}_i) is a ubiquitous signal that has important roles in homeostasis, cell growth, regulation of transcription and gene expression, angiogenesis and more; throughout the life of the cell Ca^{2+} is perhaps the most important intracellular messenger.

The first experiments showing Ca^{2+} as a signal demonstrated its potential for muscle contraction (Ringer, 1883) with further investigation proving the effect of Ca^{2+} was intracellular and cation specific, although it was not until much later that it became apparent that cells could actively control the buffering of Ca^{2+} and its passage across membranes by specialised proteins (Brini & Carafoli, 2000). At rest most cells have a $[Ca^{2+}]_i$ of ~100 nM but movement of Ca^{2+} between intracellular stores, the cytoplasm and extracellular space alters the speed, amplitude and spatio-temporal patterning of Ca^{2+} profiles to create signals with widely different dynamics that differentially control the numerous and hugely varied cellular processes.

1.2.i Mobilisation of Ca^{2+} from intracellular stores

Cells generate their Ca^{2+} signals by using both internal and external sources of Ca^{2+}. Release from these internal stores is controlled by various channels, of which the inositol-1,4,5-triphosphate receptor ($InsP_3R$) and ryanodine receptor (RyR) families have been studied most extensively (Berridge, 1993). The membrane systems of the endoplasmic reticulum (ER) or the equivalent organelle, the sarcoplasmic reticulum (SR) of muscle cells are important intracellular stores of Ca^{2+} which provide a sequestration point as well as a source for Ca^{2+}_i. Ca^{2+} may be released from these intracellular stores via various channels by direct activation or spontaneous leak. The first demonstration of a Ca^{2+}-mobilising second messenger was inositol-1,4,5-trisphosphate (IP_3; (Streb *et al.*, 1983), although the IP_3 receptor (IP_3R) was not identified until later (Furuichi *et al.*, 1989; Mikoshiba *et al.*, 1979; Supattapone *et al.*, 1988).

The majority of cell surface receptors are G protein-coupled receptors (GPCRs), which can be divided into three main subtypes: G_q, G_s and G_i. Activation of a GPCR coupled to a G_s or G_i subunit will stimulate or inhibit activation of adenylyl cyclase respectively, leading to altered levels of cyclic adenosine monophosphate (cAMP) which exerts its principal intracellular effects through activation of protein kinase A (PKA). In contrast, activation of a GPCR coupled to a G_q subunit will activate the membrane bound enzyme phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the messengers diacylglycerol (DAG) and IP_3. In turn, IP_3 diffuses through the cytosol to activate the IP_3R, a Ca^{2+} permeable channel found in the ER membrane. This initial rise in $[Ca^{2+}]_i$ can evoke further release of Ca^{2+} as the principal activator of the IP_3R is Ca^{2+} itself, which amplifies global $[Ca^{2+}]_i$ and facilitates the spread of the signal along the length of the cell as a 'Ca^{2+} wave'

propagated by the process of Ca^{2+} -induced Ca^{2+} release (CICR; (Boittin *et al.*, 1999). Similar in structure to the IP_3R are ryanodine receptors (RyR), stimulated to transport Ca^{2+} into the cytosol by recognising Ca^{2+} on its cytosolic side. RyRs are also activated by CICR and by another Ca^{2+}-mobilising second messenger, cyclic ADP-ribose (Berridge *et al.*, 2000).

1.2.ii Ca^{2+} influx

The extracellular concentration of Ca^{2+} is on the order of 1 mM whereas the intracellular concentration is closer to 100 nM. The opening of Ca^{2+} permeable channels in the plasma membrane results in influx of Ca^{2+} to the cytosol. Here Ca^{2+} binding proteins sequester Ca^{2+} in order to maintain the lower intracellular [Ca^{2+}] (20-100 nM) in order to avoid cytotoxic effects. Consequently, Ca^{2+} has a short diffusion distance in the cytosol (reviewed by Berridge, 1997).

In vascular smooth muscle, the predominant Ca^{2+} influx channel is the L-type voltage-gated calcium channel (VGCC). Upon depolarisation of the membrane, L-type VGCCs become activated causing influx of Ca^{2+} necessary for smooth muscle contraction. L-type VGCCs are not expressed in endothelial cells (Colden-Stanfield *et al.*, 1987). There exist many other Ca^{2+} permeable channels, including the transient receptor potential (TRP) channels present in the endothelium.

1.2.iii Ca^{2+} signalling microdomains

Within a cell, Ca^{2+} sensitive proteins may be positioned together so as to give rise to Ca^{2+} in small, localised domains such that Ca^{2+} signalling activity is sufficient to activate a particular target. These areas of spatio-temporal Ca^{2+} signalling are known as Ca^{2+} microdomains. In the vasculature these include release from Ca^{2+}

channels such as Ca^{2+} puffs (IP_3R release), sparks (RyR) and pulsars (endothelial IP_3R) and sparklets (endothelial TRPV4) (Billaud *et al.*, 2014; Ledoux *et al.*, 2008b; Sonkusare *et al.*, 2012).

1.3 Ca^{2+} signalling in vascular smooth muscle

The ability of vascular smooth muscle cells to contract and relax in response to stimuli underlies the main mechanism of arterial diameter regulation. A rise in free $[Ca^{2+}]_i$ will initiate SMC contraction. Ca^{2+} binds to the protein calmodulin and the resulting complex activates myosin light chain (MLC) kinase to trigger the contraction cascade by promoting the formation of cross bridges between myosin and actin filaments. MLC phosphatase dephosphorylates MLC thus leading to SMC relaxation. Therefore regulation of smooth muscle cell $[Ca^{2+}]_i$ and MLC kinase activity are two of the major targets in control of vascular tone. As mentioned (**section 1.2.ii**) depolarisation initiates Ca^{2+} influx through VGCCs meaning that smooth muscle cell hyperpolarisation results in closure of VGCCs and subsequently causes reduction of $[Ca^{2+}]_i$ and SMC relaxation.

1.3.i Ca^{2+} sparks

A Ca^{2+} spark arises from the activation of a cluster of ryanodine receptors, causing Ca^{2+} to be released from the SR. In vascular smooth muscle, Ca^{2+} sparks can indirectly cause vasodilation through activation and opening of closely aligned large conductance Ca^{2+} activated potassium (BK_{Ca}) channels, which cause K^+ efflux and associated hyperpolarization and vasorelaxation (Nelson *et al.*, 1995). Ca^{2+} sparks are but one example of the spatio-temporal signalling that enables calcium's versatility as a second messenger.

1.4 Ca^{2+} signalling in the endothelium

In contrast with SMC Ca^{2+} signalling, an increase in $[Ca^{2+}]_i$ in the endothelium is typically associated with vasodilation. Spontaneous Ca^{2+} events in the endothelium may be mediated by IP_3Rs, or Ca^{2+} influx via TRPV4 (Bagher *et al.*, 2012; Kansui *et al.*, 2008). Although their exact functional relevance is unclear, they may be important in local Ca^{2+} signalling microdomains where they can initiate vasodilation by production of autacoids or opening of K_{Ca} channels (Kruse *et al.*, 1994; Ledoux *et al.*, 2008b). Although VGCC are absent in ECs (Colden-Stanfield *et al.*, 1987; Johns *et al.*, 1987), Ca^{2+} rises in SMCs can pass through myoendothelial gap junctions to give rise to EC Ca^{2+} (Dora *et al.*, 1997). The myoendothelial junction is itself also an important Ca^{2+} signalling microdomain where increases in Ca^{2+} can activate intermediate conductance Ca^{2+} activated potassium (IK_{Ca}) channels (Bagher *et al.*, 2012; Ledoux *et al.*, 2008b).

1.4.i Endothelial Ca^{2+} mediates vasodilator pathways.

Initially thought of only as an inert barrier that acted as a passive filter to permit the passage of water and small molecules across the blood vessel wall, the endothelium has come to be thought of as a key component in the regulation of vascular tone. The seminal finding came when it was noted that acetylcholine would potently dilate isolated rabbit aorta in preparations with an intact endothelium (Furchgott & Zawadzki, 1980) which led to the discovery that many agonists would cause the production of the then unknown ‘endothelium derived relaxing factor’ (EDRF; Furchgott *et al.*, 1984) that would later be identified as nitric oxide (NO; Ignarro *et al.*, 1987).

1.4.ii Nitric oxide: the first EDRF

NO was the first and the best characterized EDRF. The free radical NO is produced by the enzyme NO synthase (NOS), which has three different isoforms: inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS). As eNOS is the dominant and constitutive isoform within the endothelium, it is responsible for the majority of NO production in the vasculature under normal conditions. When the intracellular calcium concentration is increased resulting from receptor activation, calcium binds calmodulin (CaM) to form a Ca^{2+}/CaM complex. The binding of eNOS to this complex and its co-factor tetrahydrobiopterin, (BH4) permits the dimerization of eNOS. Then the active homodimer of eNOS catalyzes the conversion of L-arginine to L-citrulline to produce NO (Fleming & Busse, 2003). Once NO is generated it can diffuse into the underlying smooth muscle cells and activate soluble guanylyl cyclase (sGC), in turn catalyzing the production of cyclic guanosine monophosphate (cyclic GMP) to cause smooth muscle relaxation.

1.4.iii Endothelium derived hyperpolarization

Whilst the vasorelaxant response to increased EC $[Ca^{2+}]_i$ in larger conduit arteries via NO production is significant, in the small resistance arteries the response to EC Ca^{2+} principally activates endothelium derived hyperpolarization (EDH; (Garland *et al.*, 2011; Nagao *et al.*, 1992).

The scavenging of NO with methylene blue and haemoglobin was noted not to totally inhibit relaxation and hyperpolarisation to ACh (Chen *et al.*, 1988) leading to the determination that EDH was an independent pathway to the classic NO EDRF. The importance of endothelial Ca^{2+}-activated potassium (K_{Ca}) channels was later determined when the hyperpolarisation to ACh was found to be attenuated by use of

apamin (a small conductance (SK_{Ca}) channel blocker) or charybdotoxin (a large (BK_{Ca}) *and* intermediate (IK_{Ca}) channel blocker) and abolished when used in concurrently (Waldron & Garland, 1994). This was followed up by the discovery that iberiotoxin (a blocker for BK_{Ca} channels only) was ineffectual at blocking the hyperpolarization response. Thus the importance of endothelial SK_{Ca} and IK_{Ca} channels in generation of EDH and its role in control of vascular tone in resistance arteries was confirmed (Edwards *et al.*, 1998).

As mentioned previously (**section 1.1**), myoendothelial gap junctions exist between smooth muscle cells and endothelial cells, which allows for the bidirectional spread of hyperpolarization between the two cell types (Kansui *et al.*, 2008; Straub *et al.*, 2012). Thus hyperpolarization that is initiated through binding of an agonist at an endothelial cell surface receptor can relax the underlying smooth muscle and reduce vascular resistance.

Locally acting responses in the endothelium are also able to initiate conducted vasodilation, an important occurrence in the microvasculature that allows for vasodilation up or downstream, away from the site of the initial agonist action. Conducted vasodilation is not mediated by propagation of EC Ca^{2+} (Duling & Berne, 1970) but the spread of hyperpolarization bi-directionally through neighbouring ECs (Emerson & Segal, 2000) which spreads radially to underlying smooth muscle to generate vasodilation up or downstream, even in the opposite direction to flow.

1.5 Cholinergic signalling

1.5.i Acetylcholine

Acetylcholine (ACh) was first identified by Sir Henry Dale for its actions on heart tissue in 1915, with Otto Loewi six years later recognizing ACh as the first identified neurotransmitter, which he termed 'vagusstoff' due to its release from the vagus nerve. The two men would share the 1936 Nobel Prize in Physiology or Medicine for their contributions. This makes it a little over a century ago that the study into ACh began, but perhaps fittingly for the first identified neurotransmitter, it appears that ACh is amongst the oldest signaling molecules phylogenetically, possibly functioning as an early local mediator as well as a neurotransmitter (Horiuchi *et al.*, 2003).

Acetylcholine receptors can be classified into two groups: muscarinic acetylcholine receptors (mAChRs) are GPCRs that exist as 5 molecular subtypes (M_1-M_5). The odd numbered members couple with G_q while M_2 and M_4 act through G_i. Nicotinic ACh receptors (nAChRs) on the other hand, are ligand gated ion channels. All nAChRs are pentameric structures that form a receptor-channel complex through five subunits, of which 16 different members have been identified. It is on M_3 receptors acetylcholine acts upon in endothelial cells to initiate vasodilation.

1.5.ii Neuronal cholinergic system

In neurons, ACh is synthesized by the enzyme choline acetyltransferase (ChAT) from the substrates choline and acetyl-CoA. The majority of choline used for ACh synthesis is recycled from the synaptic cleft, but choline can also be obtained from

the degradation of choline rich phospholipids, via diet, and freely circulating choline that exists at high concentrations (Bligh, 1952).

Choline readily crosses the blood-brain barrier and is taken up by cholinergic nerve terminals by the high-affinity choline transporter, which is temperature-, energy- and sodium-dependent. This system is the primary means by which choline needed for the synthesis of acetylcholine is transported into the neuron and thus is the rate-limiting step in the production of the neurotransmitter (Ray *et al.*, 2012). However, under certain circumstances when metabolic demands for acetylcholine are exceptionally high, a low-affinity choline uptake system will assist in transporting additional choline into the neuron (Meyer *et al.*, 1982). The nature of the high affinity uptake system guarantees that cholinergic neurons will always receive priority access to all available choline. Thus, only when the acetylcholine requirements of these neurons have been met, choline can be transported into non-cholinergic cells.

After its synthesis, acetylcholine is packaged into secretary vesicles by the vesicular acetylcholine transporter, an antiporter carrier protein on the vesicle membrane. Once packaged in a vesicle, acetylcholine is stored at the nerve ending until an action potential arrives and allows for its release into the synaptic cleft (Arvidsson *et al.*, 1997). Acetylcholine will bind to postsynaptic receptors to initiate a cholinergic signaling pathway or be rapidly degraded by acetylcholinesterase into the inactive metabolites choline and acetate.

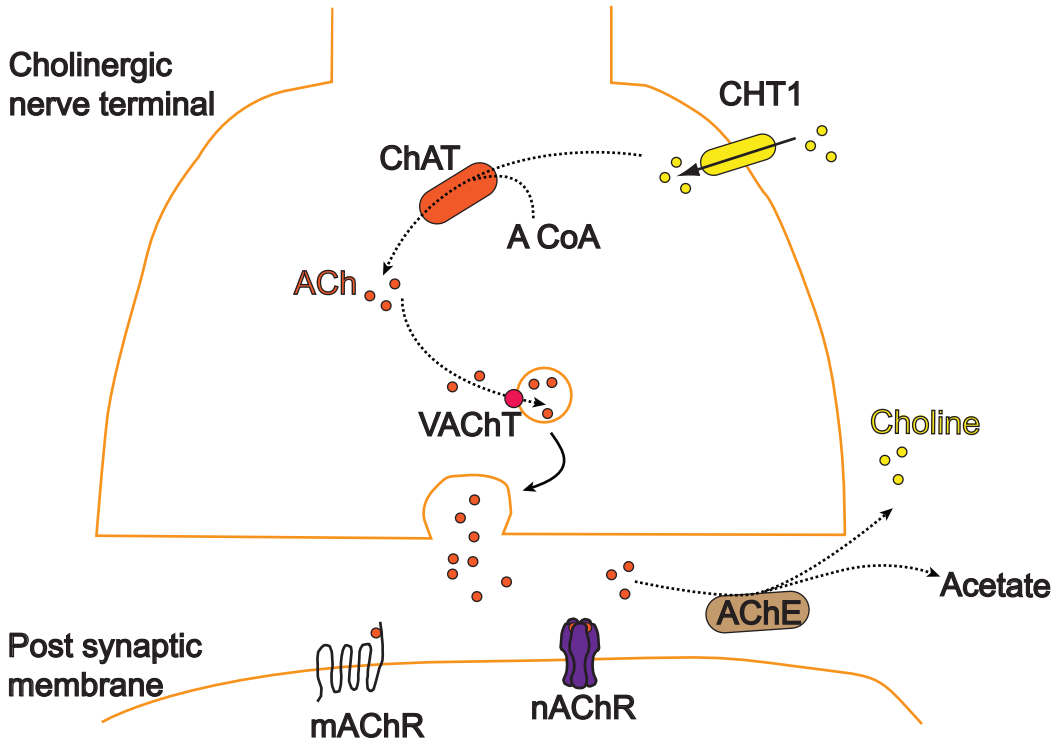


Figure 1.5.1 Basic cholinergic signaling pathway.
Neuronal cholinergic signalling is mediated by the neurotransmitter acetylcholine (ACh; orange), which is synthesised from choline (yellow) and acetyl-CoA (A CoA) by the enzyme choline acetyltransferase (ChAT). Free cytosolic ACh is scarce in the nerve terminal as it is packaged into vesicles by the enzyme vesicular acetylcholine transporter (VAChT) where it is stored until necessary. Upon receipt of an action potential stimulus and subsequent Ca^{2+} influx (not pictured), the vesicles fuse with the synaptic membrane and expel their contents into the synaptic cleft. ACh diffuses the gap to act upon postsynaptic receptors (either nicotinic or muscarinic ACh receptors; nAChR/mAChR respectively) before it is rapidly hydrolysed and inactivated by the enzyme acetylcholinesterase (AChE). Free choline is recycled by uptake via choline transporter enzymes (CHT1) to be synthesised into another molecule of ACh.

1.5.iii Non-neuronal cholinergic system

As discussed in **section 1.4**, the activation of endothelial muscarinic receptors can act to influence vascular tone through Ca^{2+} dependent pathways. With the award of a Nobel Prize for the work which first gave rise to these observations (Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1987) and the plethora of studies that followed, it is perhaps surprising then that the physiological relevance of these receptors is uncertain as they lack direct parasympathetic innervation.

Despite its recognised role as a classical neurotransmitter, a growing body of evidence suggests that ACh and corresponding cholinergic components are present in an array of non-neuronal cells and tissues; a concept termed the 'non-neuronal cholinergic system' (NNCS; Kawashima & Fujii, 2008). The list of cell types found to contain extraneuronal ACh is extensive and covers numerous species, including human tissue (Fujii *et al.*, 1995; Wessler *et al.*, 1998). In fact the NNCS is often referred to as being "nearly ubiquitous" (Wessler *et al.*, 1999) with evolutional studies finding ACh expression, as well as functional synthesising enzymes and receptors, well established in evolutionarily primitive life including invertebrates, plants, fungi and bacteria as well as mammals and other complex multicellular organisms (Horiuchi *et al.*, 2003; Wessler *et al.*, 1999). This widespread expression points to likely auto- and paracrine regulatory roles for ACh outside of the nervous system as well as its established neurotransmitter action.

1.5.iv Non-neuronal cholinergic system in the vasculature

Since Furchgott and Zawadzki (1980) seminally demonstrated the vasodilatory effects of acetylcholine (ACh), it has since become something of an agonist of choice in testing endothelial cell function and vascular reactivity via M_3 mAChRs

located on the endothelium; however there is no clearly recognised source of innervating ACh endogenously. The sparsity of perivascular cholinergic nerves (Brayden & Bevan, 1985) and low probability that they penetrate the artery wall mean they are an unlikely source, with ACh having to diffuse the entirety of the vessel wall, avoiding cholinesterases in the adventitia and media (Thompson & Tickner, 1953; Walch *et al.*, 1997), to reach the endothelium intact (Burnstock, 1990). Furthermore, a recent study concluded that perivascular cholinergic nerves innervating rat mesenteric arteries do not directly mediate vascular responses (Tangsucharit *et al.*, 2012).

It therefore seems likely that endothelial cell M_3-AChRs are influenced, not by perivascular nerves, but by local, non-neuronal sources of vascular ACh, originating from the blood or endothelium, acting in either an auto or paracrine manner. The presence of distinct receptor populations on the luminal surface of endothelial cells (Rivers & Duling, 1992; Tsukahara *et al.*, 1989) would highlight an ACh source originating in the blood. Consistent with this, it has previously been shown that blood of numerous species, including rats and humans, contains measurable quantities of ACh in nanomolar levels in rat blood, although this has been shown to vary with species (Fujii *et al.*, 1995; Hasegawa *et al.*, 1982; Kawashima *et al.*, 2012; Watanabe *et al.*, 1986).

Taken together, there exists a large body of evidence to suggest that vascular cells express elements of the cholinergic system that are functionally expressed independently of cholinergic innervations, however the characterisation of the vascular NNCS is as yet, incomplete. It is therefore of great interest to investigate the capabilities of vascular components to synthesize, store, transport and secrete

ACh that may act upon endothelial cells to further moderate vascular tone by local regulatory loops.

The aims of the experiments in this thesis are to study the role that blood plays in influencing endothelial cell Ca^{2+} signalling pathways and whether there is a contribution by non-neuronal ACh acting to stimulate endothelial cell muscarinic receptors.

Chapter 2

General Methodology

2.1 Tissue isolation and preparation

2.1.i Rat mesenteric artery isolation

Mesenteric arteries of male Wistar rats (225-250 g, Charles River, UK) were used for all experiments requiring blood vessels described in this thesis. Rats were killed by cervical dislocation and exanguination according to approved Schedule 1 methods of the Animals (Scientific Procedures) Act 1986. The mesentery was removed and immediately placed in ice-cold MOPS buffered physiological solution (see **section 2.5**). An arcade of mesentery was transferred to a Sylgard coated Petri dish filled with fresh ice-cold MOPS buffered solution and pinned, ready for dissection (**Figure 2.2.1**).

Second or third order branches of the superior mesenteric artery were dissected free of adherent connective tissue under a dissecting microscope. Tissue in the dissection dish was stored at 4°C for later use if necessary and the solution changed approximately every 2 h. Segments of the arteries used in pressure myography experiments were cut to a final length of ≥2mm which contained no visible side branches. Artery segments used in *en-face* experiments had a small 'v-shaped' incision made on the upper, outer wall of the artery as not to cut through the whole cross section. The artery was cut open longitudinally along the top with care taken to avoid damaging the endothelial layer on the bottom. Sections ≥2mm were cut off to include an intact section to aid in orienting and manipulating the vessel.

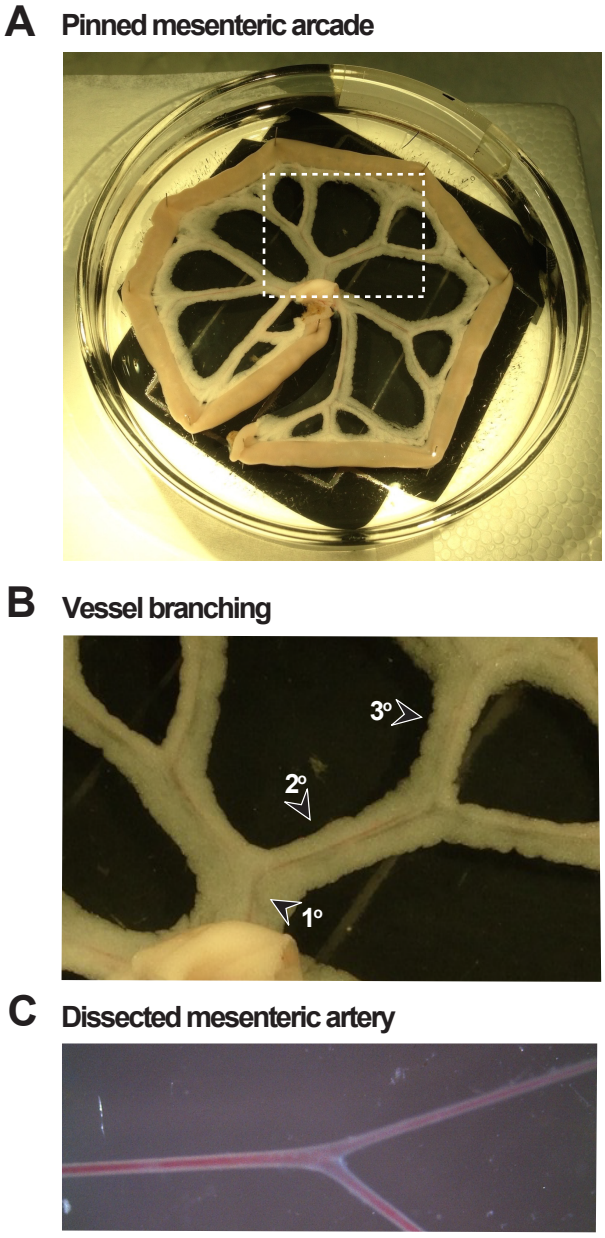


Figure 2.1.1 Rat mesenteric artery dissection.
(**A**) A section of a rat mesenteric artery arcade pinned into a Sylgard lined dissection dish. (**B**) Enlarged image of dotted section in (A) illustrating the branching of the mesenteric bed with first (1^o), second (2^o) and third (3^o) order arteries indicated. (**C**) Second order artery with bifurcation into two third order branches, dissected free from adherent perivascular adipose tissue and connective tissue, prepared for experimental use.

2.1.ii Blood collection

Blood samples were collected into a syringe containing 1:9 4% sodium citrate as an anticoagulant and various blockers or enzymes indicated in the relevant methods sections. Various components of the collected blood were separated by centrifugation at 1000 x g for 20 mins in a benchtop centrifuge (MSE Micro Centaur; Sanyo, UK) to yield platelet rich plasma, buffy coat or erythrocyte layers, with platelet rich plasma undergoing further centrifugation to yield pure plasma (repeated as above x3). Components were aspirated and re-spun to ensure separation, then afterwards stored separately. Erythrocytes were further washed three times in a wash buffer (section 2.5 and as previously described: Sprague *et al.*, 1996) as detailed in **section 4.2.ii**.

Blood used experimentally was withdrawn from the animal for use with a corresponding mesenteric artery, which originated from the same animal. Following collection and preparation as above, blood samples were kept in eppendorf tubes for later use, up to a maximum of 6 hours. The potential impact of these conditions are discussed later in **section 3.4.iv.**

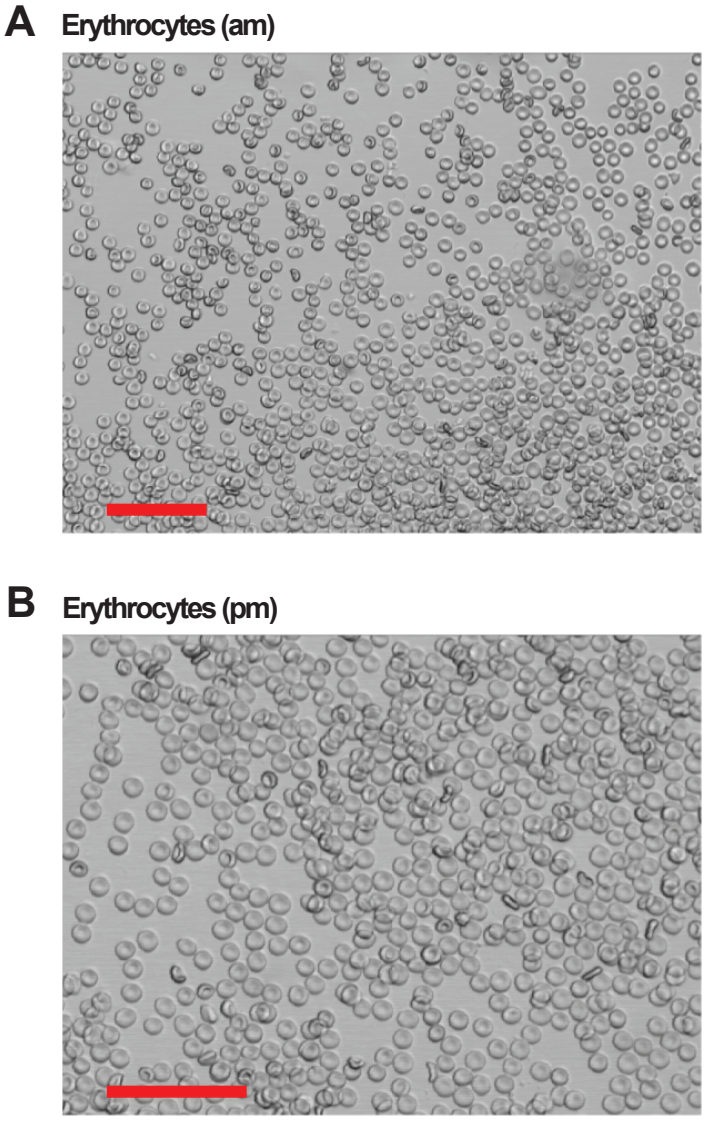


Figure 2.2.1 Whole blood samples

A sample of 20 μl rat whole blood in recording chamber approximately 30 min after collection (**A**) or after the conclusion of experiments (**B**) approximately 7 hours later. Note the overwhelming proportion of erythrocytes that make up the sample, characterised by their rounded and biconcave morphology. Hours later the cells remained intact and had not lysed or deformed into 'erythrocyte ghosts'.
Scale = 50 μm.

2.1.iii Rat cremaster muscle isolation

Male Wistar rats (225-270 g, Charles River, UK) were killed by cervical dislocation and exsanguination according to approved Schedule 1 methods of the Animals (Scientific Procedures) Act 1986. The cremaster muscle was exteriorized, removed and immediately placed in ice-cold MOPS buffered solution (see **section 2.5**). The cremaster muscle was pinned in a Sylgard-coated dissection Petri dish and dissected free of adherent tissue.

2.2 Detection of EC Ca^{2+} activity in an *en-face* preparation

2.2.i Preparing the en-face vessel

Sections of a second order branch of mesenteric artery (≥2mm in length) cleaned of surrounding tissue (see **section 2.1.i**, **Figure 2.1.1C**) were used for these experiments. A small 'v-shaped' incision was made on the upper, outer wall of the artery along which the artery was cut longitudinally, opening the vessel. Shallow cuts were made, with care taken to avoid damaging the endothelial layer on the bottom.

The open vessel was then isolated and transferred to a 2 ml heated recording chamber (RC27 chamber and PH-6 heating platform, Warner Instruments, Conneticut, USA; **Figure 2.2.1A**) in 4 °C MOPS buffered solution. The artery was moved to the base of the chamber and pinned onto the coverslip at the corners using sealed glass pipettes to hold the vessel down in an *en-face* preparation, with the luminal side (endothelial cells) open to the bath (**Figure 2.2.1B**). The vessel was left to equilibrate by 20 min continual superfusion (flow rate 2 ml min^{-1}) at 37ºC and the stage transferred to an inverted laser scanning confocal microscope with

FluoView software (IX70 or IX81, Olympus, Japan) or a spinning disc microscope (Olympus IV70 with Andor IQ 1.8.1).

2.2.ii Measurement of EC $[Ca^{2+}]_i$

After an equilibration period, the chamber solution was replaced with filtered (0.22 mm pore) MOPS buffered solution containing a cell permeable Ca^{2+}-sensitive fluorescent dye Oregon Green 488 BAPTA-1 AM (OGB-1, 10 μM; dissolved in DMSO with 0.02% Pluronic F-127) for 30 min to selectively load endothelial cells, as described previously (Kansui *et al.*, 2008). The dye was washed out with MOPS buffered solution for 30 min to allow for de-esterification. OGB-1 was excited at 488 nm and emitted fluorescence intensity was collected at >505 nm using a 40x water immersion objective (UAPO-W, Olympus, Japan). Endothelial cells were visualised by adjusting the focal plane to the top of the artery preparation and fluorescence intensity captured using FluoView software (Olympus, Japan) at ~3 Hz. A region was selected for recording/acquisition with at least 10 cells that were in a shared focal plane. Images were analysed offline using MetaMorph software (see **section 2.2.iv**).

2.2.iii Experimental protocol

Drugs were typically applied to a static bath and mixed using trituration with a pipettor. Blood was applied directly over the exposed endothelial cells. Drugs were washed out for 20 mins. Blood was washed by exchanging the chamber solution a minimum of 5 times followed by a 20 min wash. Pre-incubations were 20 min in the chamber unless stated otherwise.

Changes in endothelial cell $[Ca^{2+}]_i$ were investigated in response to agonists and rat blood, in the absence or presence of various antagonists. Cell reactivity was assessed by testing with 0.1 μM ACh; only vessels in which typical endothelial cell $[Ca^{2+}]_i$ were observed in >90% of the cells in view were used, signifying healthy endothelium. A baseline recording period was observed for 60-90 s before addition of drug in all runs.

There was a gradual decrease in cell brightness over the duration of data collection; for this reason, we chose to analyse responses to various treatment conditions primarily via effects on the frequency of Ca^{2+} transients, and not their amplitude. Furthermore, data included in the analysis were from preparations that maintained the ability to respond to ACh (or ADPβS when atropine was present) at the end of experiments.

2.2.iv Event detection and analysis

Endothelial cell Ca^{2+} imaging data were analysed offline using MetaMorph software (v6.1, Molecular Devices, USA). Whole-cell regions were used to identify the borders of endothelial cells on the time series. Small, subcellular circular regions of interest (ROIs) were then placed manually at sites within each individual endothelial cell that appeared to show a Ca^{2+} event, seen as a peak in the average fluorescence trace over time (**Figure 2.2.1**). These potential events were then assessed by eye using movie playback and only events confirmed both by eye and fluorescence trace were included. This ensured that changes in fluorescence intensity due to the movement of cells were avoided.

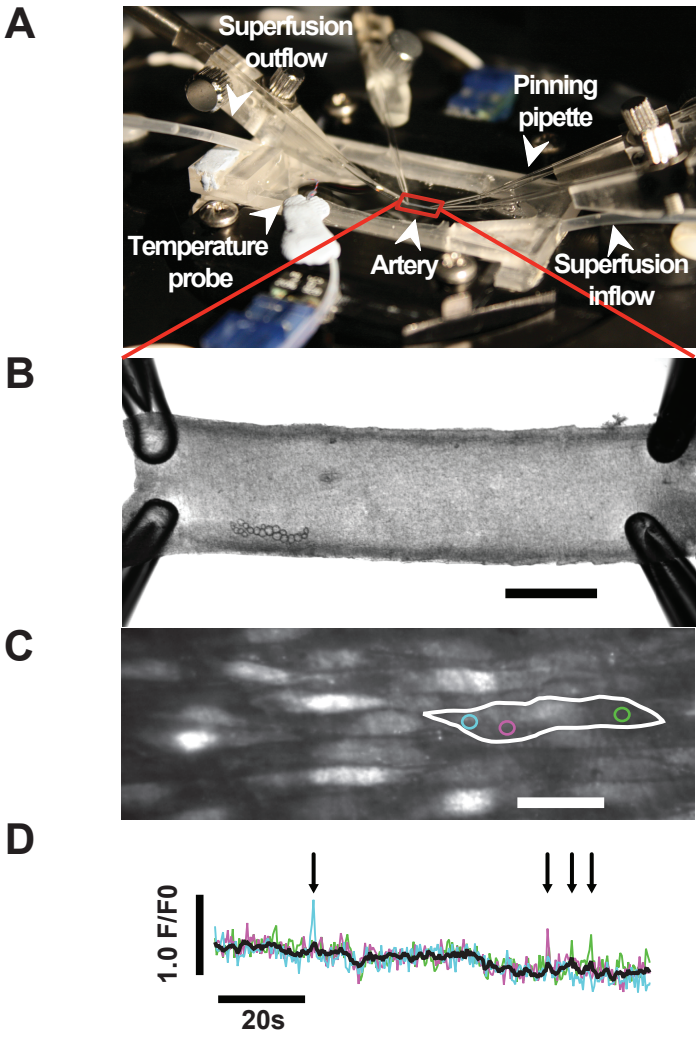


Figure 2.2.1 ***En-face*** **artery setup for detection of EC Ca^{2+} activity**
Isolated rat mesenteric arteries were cut open longitudinally and transferred to a heated perfusion chamber (**A**) in MOPS buffered solution. The artery was held as an *en-face* preparation with the endothelium exposed to the bath (**B**; Scale = 500 μm). Endothelial cells were loaded with the calcium sensitive fluorescent dye, Oregon Green 488 BAPTA-1 AM (**C**) and Ca^{2+} activity was captured using confocal laser scanning microscopy (Scale = 40 μm). Whole cell (white) or subcellular active sites (circles) were used to measure average fluorescence intensity changes. Spontaneous basal Ca^{2+} events (**D**) tended to be localised to subcellular regions within cells (arrows; coloured traces correspond to average intensity in regions of interest (ROIs) in **C**) not detectable by whole cell analysis (black trace, corresponds to average intensity in white ROI in **C**).

2.3 Immunohistochemistry

2.3.i Pinned immunohistochemistry

Dissected *en-face* artery sections were pinned into a Sylgard-lined 24-well plate fixed with 2% PFA for 10 min at 37ºC. The samples were washed 3x 5 min in PBS at 37ºC, then pre-incubated in blocking buffer (1% BSA + 0.1% Tween 20 in PBS) before incubating overnight at 4ºC in primary antibody dissolved in blocking buffer applied at room temperature. After washing with PBS, tissue segments were incubated for 2 h with secondary antibody, propidium iodide (45 µM) and Alexa Fluor 633 hydrazide (1 µM) dissolved in blocking buffer (1% BSA and 0.01% Tween 20 in PBS; as previously described by Bagher *et al.*, 2012). After washing with PBS, the segments were mounted on VectaShield Mounting Medium for Fluorescence H-1000 (Vector Laboratories Inc., USA) on microscope slides and sealed with colourless nail varnish under a 10 mm diameter glass coverslip.

2.3.ii Immunohistochemistry in a pressurised artery

A section of mesenteric artery was dissected as in **section 2.1.i** and cannulated at each end to polished glass pipettes (external diameter ~200 μm) and tied with sutures. The artery was positioned at the bottom of a 2 ml recording chamber (RC 27 + PH-6 heating platform, Warner Instruments) taking care not to touch the bottom of the chamber, on the stage of a laser scanning confocal microscope. After heating to 37^{o}C for about 10-15 min (flow rate 2 ml min^{-1}), the vessel was pressurised to physiological pressure of 70 mmHg. The vessel was tested to ensure there were no side branches or leaks. Reactivity of arteries was assessed by contraction to 1-3 μM phenylephrine and by relaxation to 0.1-1 μM ACh. Only vessels exhibiting maximal relaxation to ≤1μM ACh, reflecting undamaged endothelium were used.

Once pressurised, arteries were fixed in 2% PFA for 10 min at 37^{o}C at 70 mmHg, then washed with PBS 3x5 min. Arteries were then pre-incubated in blocking buffer (1% BSA + 0.1% Tween 20 in PBS) for 1 hour at 37^{o}C, 15 mmHg before incubating (not pressurised) in primary antibody dissolved in blocking buffer at 4^{o}C overnight. Antibodies were applied by luminal pumping as well as abluminally. After washing with PBS (3x5 min) the next day including intraluminal wash for 8 min, arteries were incubated for 2 hours with secondary antibody dissolved in blocking buffer (1% BSA + 0.01% Tween 20 in PBS) applied luminally. Propidium iodide (45 μM) and Alexa Fluor 633 hydrazide (1 μM) were applied to the bath for 20 min to stain nuclei and internal elastic lamina respectively. After washing with PBS, the artery was ready for imaging.

2.3.ii Immunohistochemistry imaging.

Imaging was performed on a laser scanning confocal microscope with FluoView software (Olympus, Japan). Fluorescence at various planes throughout the vessel wall were excited at 488 nm, 543 nm and 633 nm and the emission at 505-550 nm, 560-620 nm and 655-755 nm (to capture 488 secondary antibody, propidium iodide and Alexa Fluor 633 hydrazide signals respectively) were acquired sequentially through a 40x water immersion objective. Z-Stacks through the artery wall were obtained in 0.1-0.5 μm increments and reconstructed offline using Imaris software (Bitplane, Switzerland).

Protein	Primary Antibody	Immunogen	Secondary Antibody
ChAT	• 1:200 • ab68779 • Rabbit polyclonal • Abcam	Peptide sequence: GLFSSYRLPGHTQDTLVAQKSS	• 1:200 • A-11008 • Goat anti-rabbit • Life Tech
VAChT	• 1:200 • ab68984 • Rabbit polyclonal • Abcam	Synthetic peptide (sequence not given), from rat VAChT, conjugated to an immunogenic carrier protein	• 1:200 • A-11008 • Goat anti-rabbit • Life Tech
CHT1	• 1:200 • ab170546 • Rabbit polyclonal • Abcam	(Synthetic peptide (sequence not given) conjugated to Keyhole Limpet Haemocyanin	• 1:200 • A-11008 • Goat anti-rabbit • Life Tech
OCT1	• 1:200 • ab55916 • Rabbit polyclonal • Abcam	Peptide sequence: LATNRSHLPL GPCQDGWVYD TPGSSIVVTEF	• 1:200 • A-11008 • Goat anti-rabbit • Life Tech

Table 2.4.1 Antibodies used for immunohistochemistry.
Details of primary and secondary antibody combinations used in all immunohistochemistry experiments.

2.4 Endothelial cell tubes

2.4.i Isolation of EC tubes

Working in ice cold dissection buffer (see **section 2.5**), six or seven third order branches of rat mesenteric arteries were dissected free of connective tissue (see **section 2.1.i**; **Figure 2.1.1C**), removing as much as possible as excess connective tissue and perivascular adipose tissue will interfere with dissociation of smooth muscle cells. Artery sections were cannulated onto a micropipette (120-160 μm outer diameter) and the lumen flushed of blood cells to further improve access of the digestion enzymes and prevent blood cells from being trapped in the isolated EC tube. The arteries were cut into sections ≥1700 μm whilst ensuring damaged end pieces were removed. Arterial pieces were transferred to microtubes (max three to four per microtube) using a pasteur pipette, then placed on ice until all samples were prepared.

When ready for dissociation, the microtubes were allowed to come to room temperature (~10 min). For every microtube containing samples, a new microtube was prepared with room temperature dissociation buffer (see **section 2.5**) containing papain (0.62 mg ml^{-1}), Dithioerythritol (1.0 mg ml^{-1}) and collagenase (1.5 mg ml^{-1}) and then incubated at 37°C for 30 min. The room temperature dissection buffer was then carefully aspirated from the arterial pieces before gentle washing in room temperature dissociation buffer. Finally, the room temperature dissociation buffer was delicately aspirated and replaced with the dissociation buffer containing digestion enzymes and returned to a heat block to incubate at 37°C for 25 min.

At cessation of enzymatic digestion, arterial pieces were gently transferred to a culture dish prepared with fresh dissociation buffer without enzymes. Using a microsyringe pump (Nanoliter 2010 & SYS-Micro4 controller, WPI), a micropipette (120-160 μm inner diameter) was used to gently triturate an arterial piece until all smooth muscle cells had fallen away and been removed from the endothelial cell tube. The endothelial cell tube was then transferred into an imaging chamber (RC27 chamber and PH-6 heating platform, Warner Instruments, Conneticut, USA; **Figure 2.2.1B**; as in **section 2.2.i**) in dissociation buffer and pinned at each end using rounded end glass pinning pipettes (120-140 μm outer diameter; **Figure 2.4.1C**), then carefully stretched to a near physiological length. Once the EC tube was secured, the chamber solution was changed to perfusion buffer (see **section 2.5**) and equilibrated for 15 min.

2.4.ii EC tube Ca^{2+} imaging

*The method and equipment used for Ca^{2+} imaging of EC tubes was the same as that used in **section 2.2.il** with the following exceptions:*

EC tubes were loaded with the cell permeable Ca^{2+}-sensitive fluorescent dye OGB-1 but in perfusion buffer (**section 2.5**), instead of MOPS buffered solution. The solution remained filtered and containing 0.02% Pluronic F-127.

EC tubes were maintained at a temperature up to 34 °C, not at 37 °C as EC tubes isolated from rat mesenteric arteries were liable to separate at higher temperatures, similarly recorded in EC tubes prepared from other species (Socha *et al.*, 2011).

Experiments visualising EC tubes were carried out using a 60x water immersion objective (UAPO-W, Olympus, Japan) rather than at 40x.

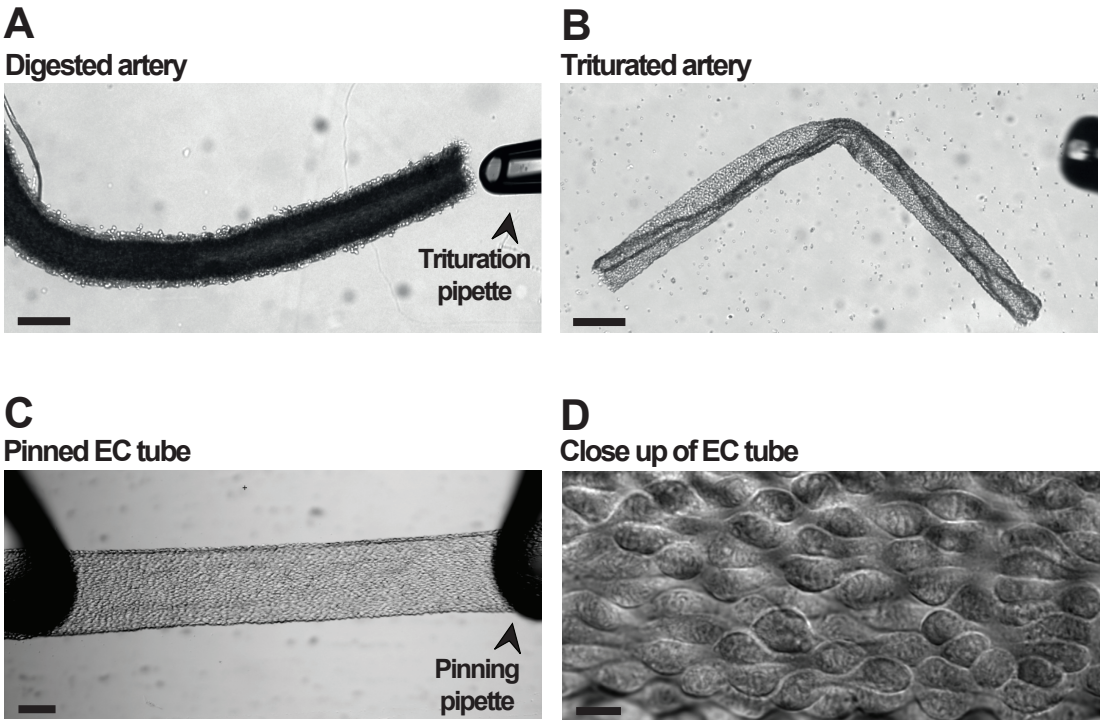


Figure 2.4.1 Endothelial cell tube isolation and preparation.

An enzymatically digested section of an artery is positioned near a trituration pipette (**A**) and gently aspirated to dissociate surrounding adventitia and smooth muscle cells which fall away to leave an intact length of coupled endothelial cells: the endothelial cell tube (**B**). The endothelial cell tube is then pinned under two round ended glass pinning pipettes and gently stretched to approximate physiological length (**C**). This allows intact and coupled endothelial cells to be studied in isolation (**D**).

Scale bar = 100 μm, except (**D**) where scale = 10 μm.

2.5 Solutions and drugs

MOPS buffered solution contained (in mM): NaCl, 145.0; KCl, 4.7; $CaCl_2$ 2.0; $MgSO_4.7H_2O$ 1.17; MOPS, 2.0; NaH_2PO_4, 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, 2.75; pH adjusted to 7.40 ± 0.02 at 37ºC.

For experiments utilising a Ca^{2+} free solution, $CaCl_2$ was replaced with additional NaCl to maintain osmolarity.

EC tube dissection buffer contained (in mM): NaCl, 137; KCl, 5.6; $MgCl_2$, 1.0; HEPES, 10; glucose, 10; plus 10% BSA, and 10 μM SNP, with pH adjusted to 7.40 and osmolarity adjusted to 285-305 mOsm.

EC tube dissociation buffer contained (in mM): NaCl, 137; KCl, 5.6; $CaCl_2$, 0.1; $MgCl_2$, 1; HEPES, 10; glucose, 10; plus 10% BSA, with pH adjusted to 7.40 and osmolarity adjusted to 285-305 mOsm.

EC tube perfusion buffer contained (in mM): NaCl, 137; KCl, 5.6; $CaCl_2$, 2.0; $MgCl_2$, 1; HEPES, 10; glucose, 10; with pH adjusted to 7.40 and osmolarity adjusted to 285-305 mOsm.

Erythrocyte wash buffer contained (in mM): NaCl, 140.5; KCl, 4.7; $CaCl_2$, 2.0, $MgSO_4$, 1.2; glucose, 5.5; tris, 21.0; with 0.5% BSA and pH adjusted to 7.40.

pH was adjusted using NaOH or HCl.

Osmolarity was adjusted using DI water or glucose.

All salts were purchased from Sigma-Aldrich, UK, except NaCl, KCl, and glucose which were obtained from Fisher Scientific, UK. Details of suppliers of drugs and chemicals are given in **Table 2.5.1**.

Drug / Chemical	Product code	Supplier	Stock conc.	Stock solvent	Final conc.
Acetylcholine	A6625	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	1 μM
Alexa Fluor 633 Hydrazide	A-30634	Thermo Fisher Scientific	N/A	N/A	1 μM
Apyrase	A6237	Sigma-Aldrich (UK)	10 Units ml^{-1}	Milli-Q	1 Unit ml^{-1}
Atropine	A0132	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	1 μM
Bovine serum albumin (BSA)	A3059	Sigma-Aldrich (UK)	N/A	N/A	*Various, see text*
Carbenoxolone	C4790	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	100 μM
Choline chloride	C7017	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	100 μM, 300 μM
Collagenase	C8051	Sigma-Aldrich (UK)	N/A	N/A	1.5 mg ml^{-1}
Corticosterone	27840	Sigma-Aldrich (UK)	10^{-2} M	EtOH	100 μM
Dimethyl sulfoxide (DMSO)	276855	Sigma-Aldrich (UK)	N/A	N/A	*<1:1000*
Dithioerythritol (DTE)	D8255	Sigma-Aldrich (UK)	N/A	N/A	1.0 mg ml^{-1}
Hemicholinium-3	H108	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	10 μM
Heparin sodium	H0200000	Sigma-Aldrich (UK)	N/A	Milli-Q	10 Units ml^{-1}
Methyllycaconitine citrate	1029	Tocris Bioscience	10^{-2} M	Milli-Q	10 μM
MRS 2179	0900	Tocris Bioscience	10^{-2} M	Milli-Q	3 μM
Neostigmine bromide	N2001	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	10 μM
NF449	13-911-0	Tocris Bioscience	10^{-2} M	Milli-Q	1 μM
Nifedipine	N7634	Sigma-Aldrich (UK)	10^{-2} M	EtOH	1 μM
Oregon Green 488 BAPTA-1 AM (OGB-1)	06807	Life Technologies (UK)	2 mM	DMSO	10 μM
Papain	P4762	Sigma-Aldrich (UK)	N/A	N/A	0.62 mg ml^{-1}
Paraformaldehyde (PFA)	*157-2*	Electron Microscopy Sciences (UK)	2%	Distilled water	2%
Pluronic F-127	P3000MP	Life Technologies (UK)	20% (w/v)	*supplied in DMSO*	0.05%
Propidium iodide (PI)	P4170	Sigma-Aldrich (UK)	4.5 mM	Milli-Q	45 μM
PNU 282987	2303	Tocris Bioscience (UK)	10^{-2} M	DMSO	1 μM
Quinine	22620	Sigma-Aldrich (UK)	10^{-2} M	Milli-Q	300 μM
Sodium citrate	C7254	Sigma-Aldrich (UK)	N/A	Milli-Q	4%
Sodium Nitroprusside	228710	Sigma-Aldrich (UK)	N/A	N/A	10 μM
Xestospongin C	BML-CA409-0050	Enzo Life Sciences	10^{-2} M	Chloroform	10 μM

Table 2.6.1 Details of drugs and chemicals.
Details of drugs and chemicals used in experiments including their sources.

2.6 Statistical analysis

All data are expressed as mean ± S.E.M. from *n* experiments. Experiments were analysed using GraphPad Prism 5.0 software. One-way or repeated measures ANOVA was performed as appropriate for comparing multiple treatments, with a Bonferroni post-test to analyse specific sets as indicated in the figures. For single comparisons, a two-tailed Student's *t*-test was performed. $P<0.05$ was considered statistically significant. All comparisons were made in matched observations, except where stated.

Chapter 3

Stimulation with blood activates endothelial cell Ca^{2+} events

3.1 Introduction

As discussed in chapter 1, the role of resistance arteries, enacted by their endothelial and smooth muscle interactions, is to regulate vascular tone, and hence, blood flow. The regulation of the flow of blood is in accordance with the metabolic demands of the live tissues being supplied in order to continually cover its oxygen and nutrient needs. The ability for the blood vessel to detect signals and messengers from the circulation that relay the need to alter vessel diameter, and thus resistance to blood flow, is therefore of considerable importance. Being the innermost lining of blood vessels, endothelial cells have a unique importance in their interaction with the circulating blood by having to detect and respond to all the changes occurring within the blood. Endothelial cells can detect and respond to changes in haemodynamic forces (Falcone *et al.*, 1993), hypoxia (Michiels *et al.*, 2000), and intravascular pressure (Bagher *et al.*, 2012) as well as a myriad of circulating vasoactive factors.

The endothelium is known to be a semi-permeable barrier that regulates the transfer of molecules between blood and surrounding tissue; their ability to pass depends on their molecular size (Mehta & Malik, 2006) or lipid solubility (Lew *et al.*, 1989). Therefore the majority of circulating molecules are acting upon luminal endothelial cell receptors in order to exert their action. One interesting paradigm is the existence of mAChRs on the luminal surface of endothelial cells (Rivers & Duling, 1992; Tsukahara *et al.*, 1989) and the reported presence of ACh within the blood of various species (Fujii *et al.*, 1995; Hasegawa *et al.*, 1982), yet no action of this endogenous ACh at these receptors has been clearly described. As discussed in the introduction (**section 1.5.iii**) it is likely there exists a local source of this ACh within the vasculature termed a non-neuronal cholinergic system. Our initial aim was

to explore whether interaction between the blood and endothelium could reveal an action of this blood ACh on endothelial mAChRs and if so, subsequently investigate possible sources and mechanisms of this cholinergic system within the vasculature.

Our investigation required access to endothelium to examine interactions between the blood and endothelial cells. Intraluminal pumping of the blood was not a valid option due to issues with clot formation and washing out. Therefore we developed a technique that allowed us to cut open the vessel and be held in an *'en*-face' preparation to allow direct access to the endothelium.

The aims of the experiments in this chapter were to test the viability of an *en-face* preparation as a means of direct endothelial access, to test the hypothesis that stimulation with blood could increase endothelial cell Ca^{2+} via activation of cell surface receptors or mechanical interaction, and to characterise the profile of the response using pharmacological tools to investigate the mechanism of activation.

3.2 Methods

*Refer to **Chapter 2: General methodology** for full details of the methods used in this chapter. Details relevant specifically to this chapter are given below.*

3.2.i Protocols for imaging Ca^{2+} in ECs.

Second order rat mesenteric arteries were set up in an *en-face* preparation (as described in **section 2.2.i**), loaded with the Ca^{2+} sensitive dye OGB-1 for Ca^{2+} imaging (**section 2.2.ii**). EC tubes were set up as described in **section 2.4**. Drugs were applied to the side of a static bath and mixed by trituration with a pipettor. Blood was applied as a 20 μl bolus directly over the ECs. Blood was washed out immediately following the run and drugs were washed out for 20 min.

Nifedipine (1 μM), NF449 (1 μM), MRS 2179 (1 μM) and atropine (1 μM) were all pre incubated in the recording chamber for 20 min prior to recording. Xestospongin C in the recording chamber for 30 min prior to recording. Apyrase (1 Unit ml^{-1}) was present in the blood since collection

3.2.ii Blood centrifugation.

To separate the blood into its various fractions, blood was spun by low speed centrifugation as described in **section 2.1.ii**.

3.3 Results

Owing to the necessity for access to the endothelium in order to directly stimulate ECs with blood, we first determined that due to issues with clotting and washing out, intraluminal pumping of blood into a pressurised artery was problematic, and so the adeptness of an opened, *en-face* rat mesenteric artery as a preparation was tested. The viability of the endothelium was confirmed by the presence of localised spontaneous Ca^{2+} events and the maintained ability to generate on-going, oscillatory Ca^{2+} responses to 0.1 μM ACh and above. Endothelial cells displayed classic longitudinally elongated and aligned morphology and maintained their coupling to adjacent cells when an appropriate amount of tension was used to pin the open artery.

As discussed in the introduction (**section 3.1**), endogenous circulating factors such as hormones, peptides and small molecule transmitters could all reasonably exert effects on endothelial cells. We observed that application of a 20 μl bolus of rat whole blood directly onto *en-face* endothelial cells generated pronounced elevations in endothelial cytosolic Ca^{2+} that were transient (~30-40 s on average) and largely oscillatory before returning to values close to the baseline (**Figure 3.3.1B**). The raised level of $[Ca^{2+}]_i$ manifested as a significantly increased overall frequency of intracellular Ca^{2+} events *per cell* (**Figure 3.3.1C**) as well as an increase in the number of responsive cells from baseline levels (**Figure 3.3.1D**). Periods of basal activity were more typically associated with discreet, localised Ca^{2+} events ('local'). Stimulation with rat whole blood dramatically increased the ratio of those events which propagated along the longitudinal axis of the cell to generate Ca^{2+} waves that act to more prominently increase global EC Ca^{2+} indicating an activated endothelium

(**Figure 3.3.1E**). The succession of several Ca^{2+} waves gave an overall repetitive rise and fall in whole cell Ca^{2+} (oscillatory), however the amplitude and interval between these oscillations could vary within a given cell to often give rise to local events that occurred in between waves. This is typically uncommon with most tested agonists but very frequent in the response to whole blood, potentially indicative of an intensely activated endothelium. Furthermore EC Ca^{2+} activity occurred independently of adjacent cells. This asynchronous signalling between even neighbouring cells (cyan (cell 1) and magenta (cell 2) traces versus highlighted cell 3, **Figure 3.3.1A/B**) suggests each cell was independently activated.

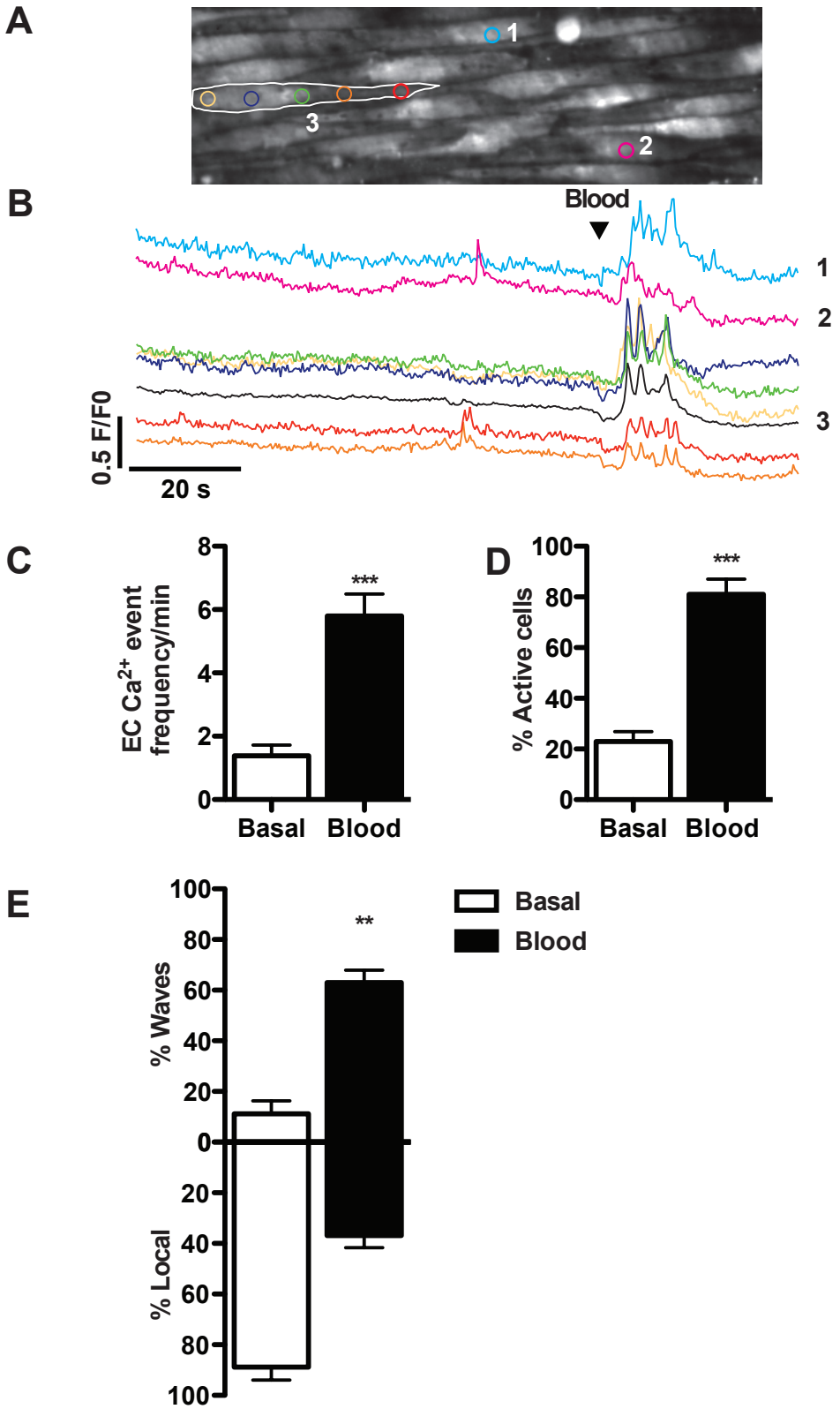


Figure 3.3.1 **Whole blood causes transient endothelial cell calcium events.**

Figure 3.3.1 Whole blood causes transient endothelial cell calcium events.
Fluorescent images of ECs loaded with the Ca^{2+} indicator OGB-1 (**A**; scalebar = 50 μm) and representative fluorescence intensity traces (**B**; corresponding to coloured ROIs in panel A) of EC $[Ca^{2+}]_i$ upon adding a bolus (20 μl, unmixed; arrow) of whole blood directly to the EC surface. Analysis of multiple subcellular regions (coloured ROIs) shows blood gave rise to transient, asynchronous Ca^{2+} events that propagated along the endothelial cell to generate waves.
Summary data showing direct application of rat whole blood onto *en-face* ECs caused an increase in Ca^{2+} activity, seen as an increase in the frequency of events per cell (**C**) as well as the % of cells exhibiting changes in $[Ca^{2+}]_i$ (**D**). The change between the proportions of local to cell wide wave Ca^{2+} events is shown in panel **E**. Paired experiments, *n*=8; *P=<0.05, significantly different from basal by paired t test.

To investigate the action of whole blood on endothelial cell Ca^{2+}, we first proposed to confirm whether the response was potentially receptor mediated or could be explained by mechanical stimulation. Typically agonists were applied to the side of the bath so as not to create undue stresses or disruption to the experimental artery. In the case of blood, it was observed that addition of whole blood (20 μl) to the side of the bath did not stimulate EC Ca^{2+} activity. The application of MOPS buffered solution (20 μl as an inert control for volume), directly over the ECs in a similar manner to blood application failed to alter endothelial cell $[Ca^{2+}]_i$ (**Figure 3.3.2A**). Furthermore the presence of superfusate flow (at 2 ml min^{-1}) over the exposed surface of the *en-face* endothelium was also unable to initiate a Ca^{2+} response on the scale of the response to application of whole blood (**Figure 3.3.2B**).

Vehicle alone (4% sodium citrate anti-coagulant) had no effect on endothelial cell $[Ca^{2+}]_i$ (**Figure 3.3.3A**), and blood collected in heparin, another widely used anti-coagulant, also increased EC Ca^{2+} events (**Figure 3.3.3B**).

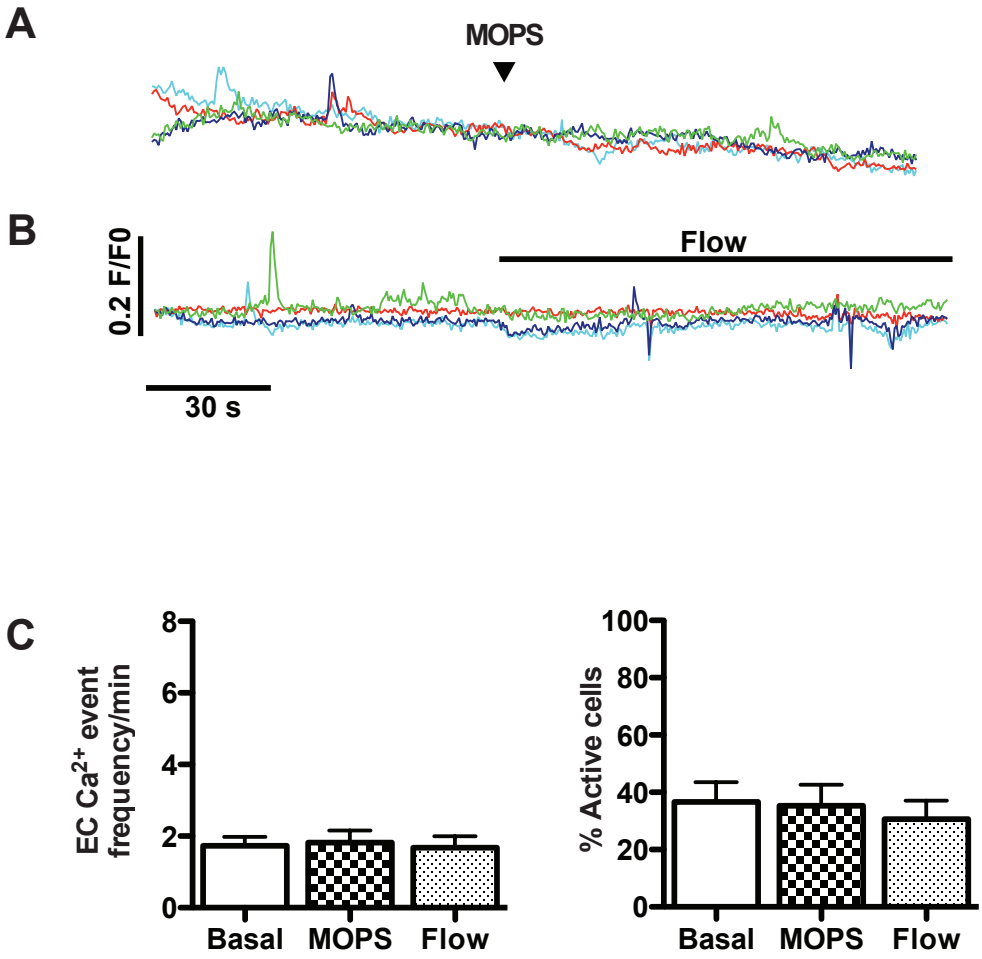


Figure 3.3.2 Flow forces are not responsible for increased EC Ca^{2+} activity.
Direct application of MOPs buffered solution (20 μl to mimic blood; **A; arrow**) and exposure to superfusate flow (2 ml min^{-1}; **B**) was not sufficient to generate a Ca^{2+} response in endothelial cells.
Summary data (**C**) shows with no change from the level of spontaneous basal event frequency, nor were the numbers of cells exhibiting Ca^{2+} activity altered. Paired experiments, *n*=3; *P=<0.05, significantly different from basal by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

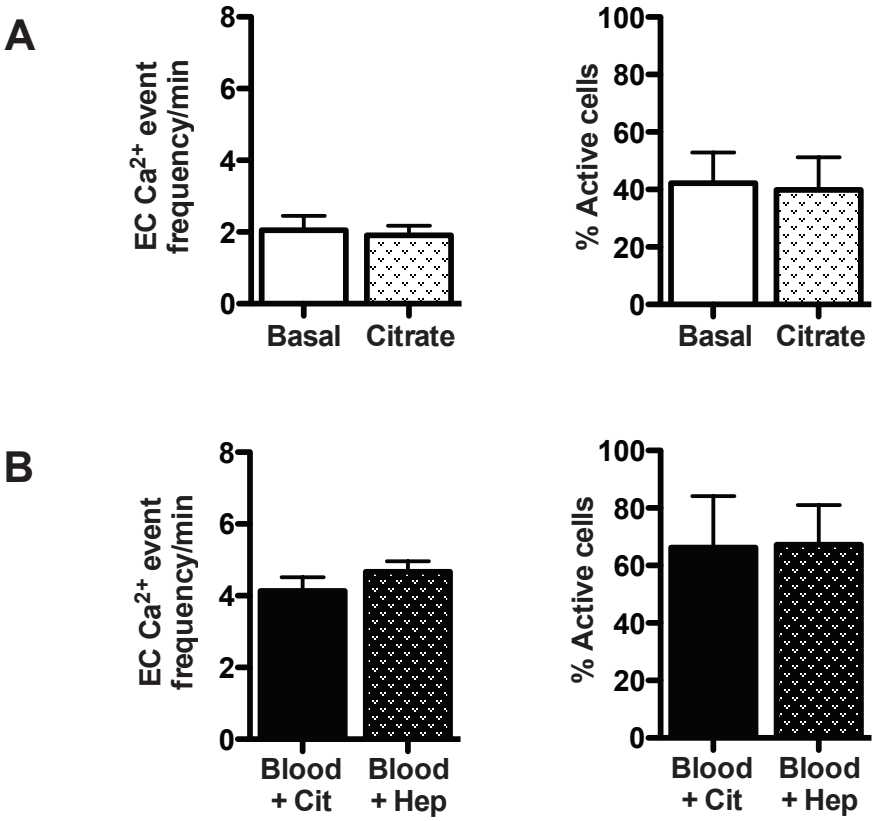


Figure 3.3.3 Sodium citrate anticoagulant is not responsible for increased EC Ca^{2+} activity.

Addition of 4% sodium citrate (**A**) had no stimulatory effect on endothelial cell $[Ca^{2+}]_i$ with no change from the level of spontaneous basal event frequency, nor were the numbers of cells exhibiting Ca^{2+} activity altered.

There was also no difference between blood samples (**B**) collected in 'standard' 4% sodium citrate anticoagulant compared with those collected using 10 U/ml heparin as anticoagulant, in eliciting an EC Ca^{2+} response when applied to *en-face* ECs. Paired experiments, *n*=3; *P=<0.05, significantly different from basal by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

Since blood appeared to be activating endothelial cells in the *en-face* preparation where interactions with underlying smooth muscle cells could potentially influence endothelial cell signalling, we investigate whether blood could elicit a Ca^{2+} response in freshly isolated endothelial cell tubes (**Figure 3.3.4**). Endothelial cell tube preparations were isolated freshly in order to study native endothelial cells in the absence of smooth muscle cells. Rat whole blood (20 μl) caused an increase in Ca^{2+} activity in virtually all endothelial cells in the tube, in much the same way as in the *en-face* preparation. Again, ACh (1 μM) was employed as a positive control for endothelial cell viability, with a characteristically 'healthy' preparation exhibiting cell-wide oscillating Ca^{2+} events in essentially every cell. Any preparations that did not elicit typical responses when challenged with ACh were not taken further in experimental protocols. The response to blood was strong and reproducible and interestingly comparable with that in *en-face* artery preparations.

Whole blood was separated into constituent components by centrifugation; in this way, plasma, erythrocytes, and platelets were assessed as with whole blood. All components significantly raised endothelial cell calcium above baseline conditions (**Figure 3.3.5**) but to variable extents compared with whole blood. There were no differences between any set of blood components and all increased the percentage of waves occurring as the predominant form of Ca^{2+} activity.

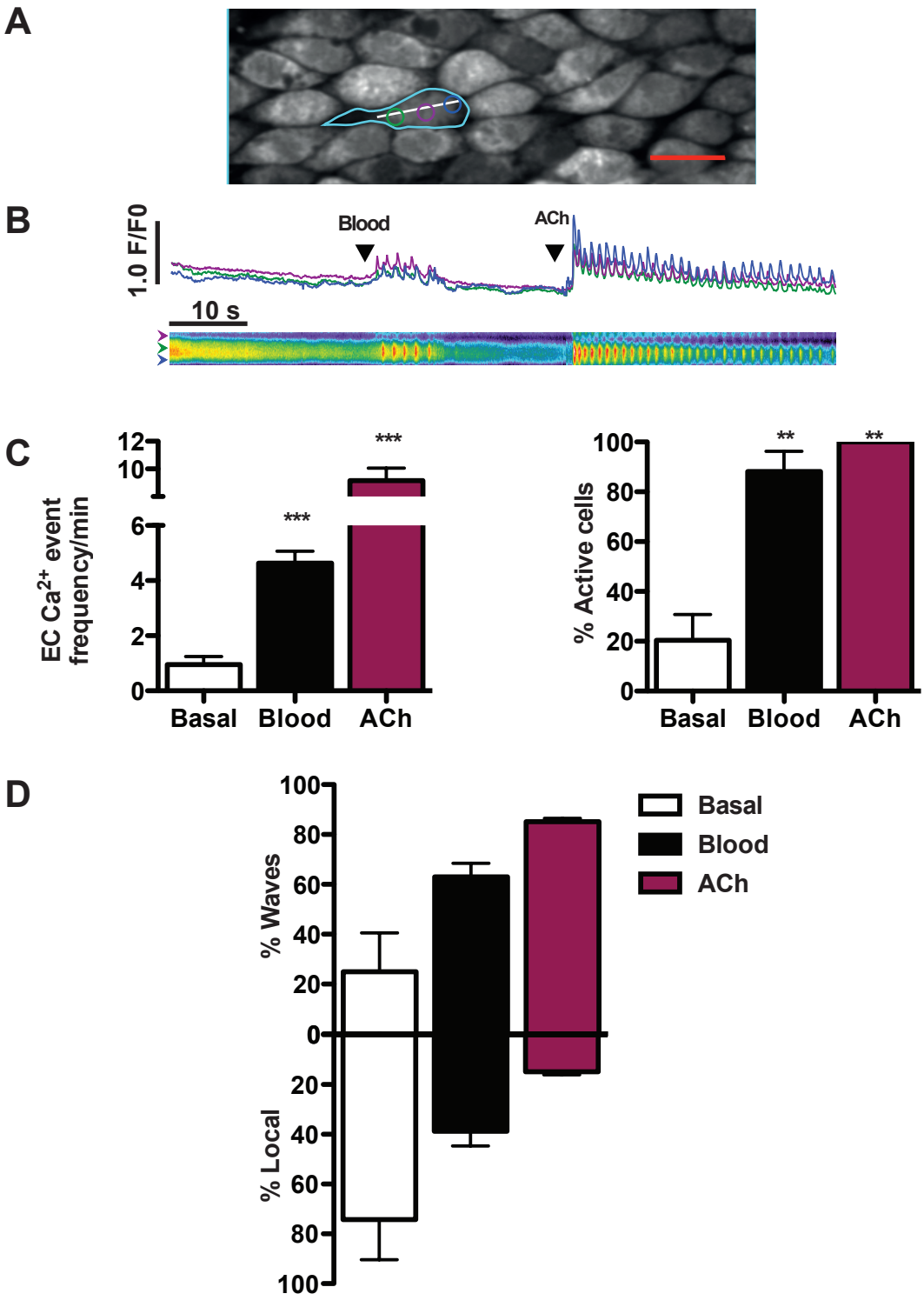


Figure 3.3.4 **Whole blood causes EC Ca^{2+} events in isolated endothelial cell tubes.**

Figure 3.3.4 Whole blood causes EC Ca^{2+} events in isolated endothelial cell tubes. An endothelial cell tube isolated from rat mesenteric artery loaded with the Ca^{2+} indicator OGB-1 (**A**). Representative fluorescence intensity traces (corresponding to coloured ROIs in panel B) and linescan (corresponding to white line in panel B) of EC $[Ca^{2+}]_i$ upon adding a bolus (20 µl, unmixed) of rat whole blood directly to the endothelial cell surface. Exogenous ACh (1 µM) was tested to confirm EC tube viability. Summary data (**C**) showing the effects of blood on the frequency of EC calcium activity per cell as well as the % of cells exhibiting changes in $[Ca^{2+}]_i$ after stimulation with blood (black) and ACh (red). The shift between the proportions of local Ca^{2+} events and cell wide waves is shown in panel **D**. Paired experiments, *n*=5; *P=<0.05, significantly different from basal (white) by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

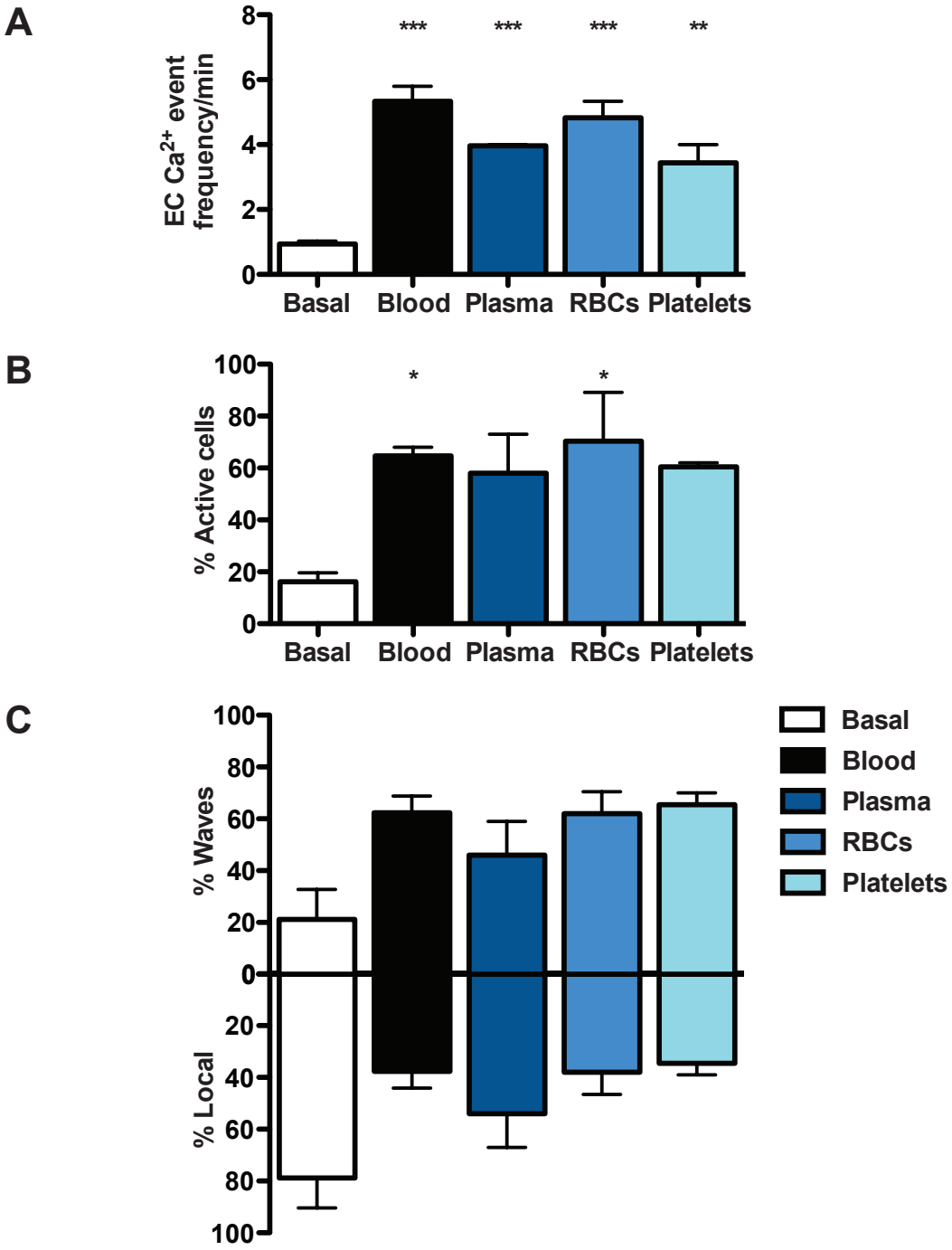


Figure 3.3.5 Separated blood components increase EC $[Ca^{2+}]_i$.
Samples of blood were centrifuged to yield separate fractions, which were applied to *en-face* ECs as with whole blood. Plasma, red blood cells (RBCs), and platelets all increased EC Ca^{2+} frequency (**A**) and the number of cells (**B**) responding above baseline levels. The increased activity was displayed as an increase in the percentage of propagating Ca^{2+} waves in all conditions (**C**). Unpaired experiments, *n*=3; *P=<0.05, significantly different from basal (white) by one way ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

To determine the mechanism of EC activation by whole blood we tested whether the predominant pathway of increased EC cytosolic Ca^{2+} in response to blood was one of extracellular Ca^{2+} influx or intracellular store mediated release. We investigated whether ECs could still elicit a response to stimulation with blood under removal of external Ca^{2+} by switching to MOPS buffer containing 0 mM Ca^{2+}. It was discovered that the response elicited by whole blood was not dependent on extracellular Ca^{2+} (**Figure 3.3.6**) with no significant reduction in the amount of Ca^{2+} activity within ECs. Inhibition of L-type calcium channels with nifedipine (1 μM; **Figure 3.3.7**) similarly had no effect on EC Ca^{2+} activity induced by stimulation with whole blood.

The IP_3 receptor blocker Xestospongin C (Xe C; 10 μM) essentially abolished EC Ca^{2+} activity to blood. Vehicle alone (chloroform) had no effect on endothelial cell Ca^{2+} activity. ACh was employed to ensure successful block of IP_3 receptors, with Xe C also fully preventing the typical EC Ca^{2+} response resulting from muscarinic receptor activation (**Figure 3.3.8A/B**). Xe C significantly reduced the frequency of EC Ca^{2+} events to blood (**Figure 3.3.8C**) and abolished increases stimulated with 1 μM ACh. The number of cells responsive to blood returned to levels similar to baseline (**Figure 3.3.8D**). In accordance, Ca^{2+} events that occurred as propagating waves in response to blood dropped in favour of local events under block with Xe C (**Figure 3.3.8E**), similar to spontaneous basal activity.

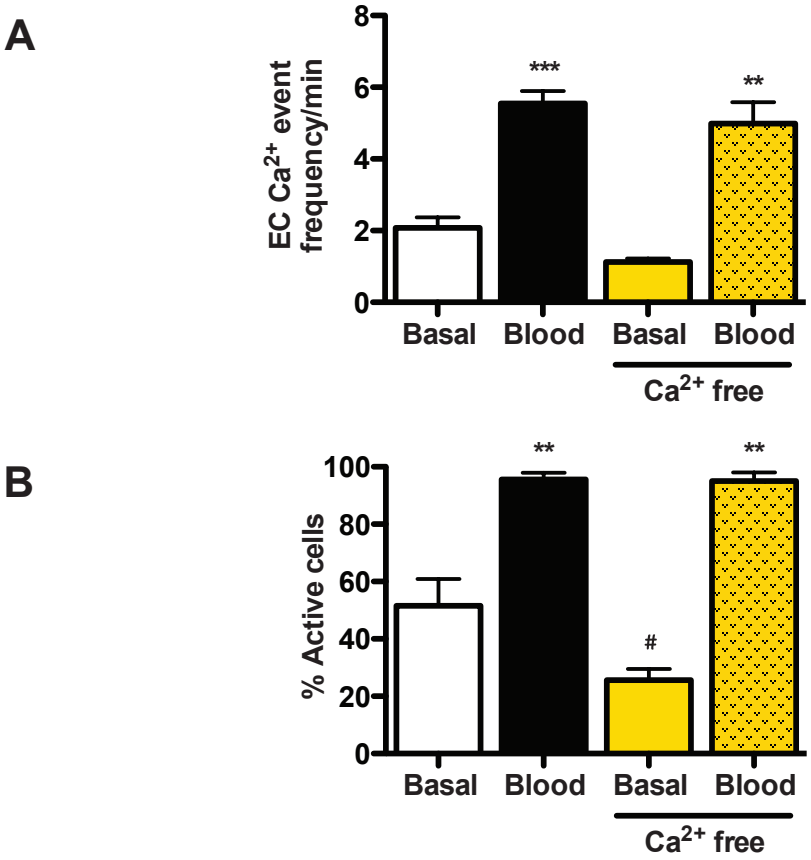


Figure 3.3.6 Increase in endothelial cell Ca^{2+} to blood stimulation but not spontaneous basal activity is independent of Ca^{2+} entry.
Summary data of Ca^{2+} event frequency (**A**) or % of active cells (**B**) showing removal of extracellular Ca^{2+} (20 min, yellow bars) had no effect on blood response but spontaneous basal activity is at least partly dependent on Ca^{2+} entry. Paired experiments, *n*=5, *P<0.05 by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

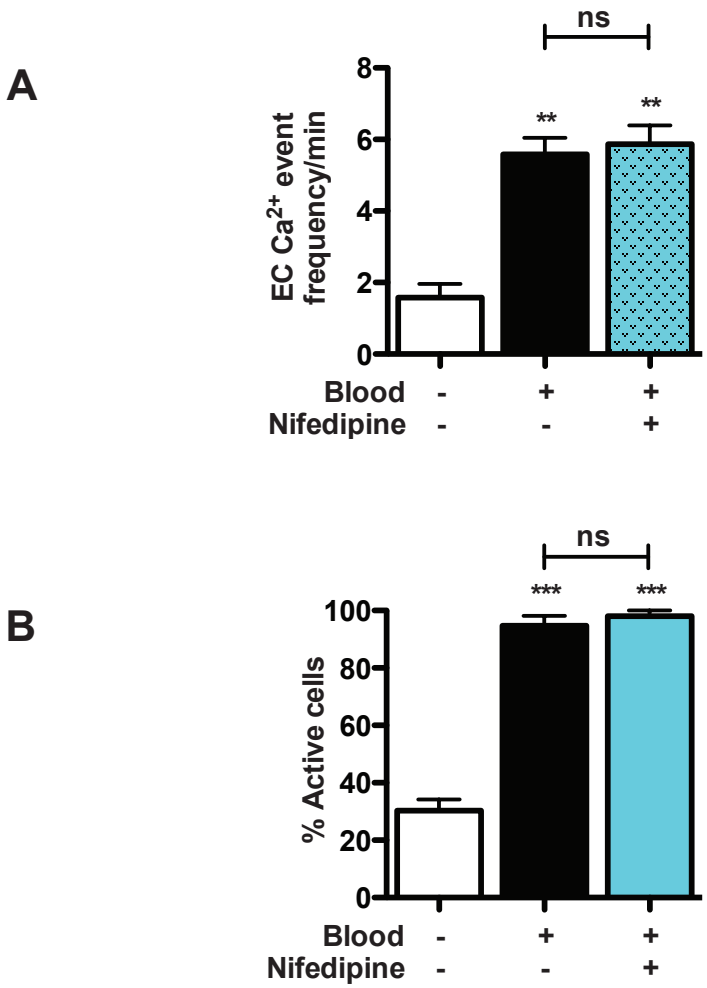


Figure 3.3.7 Inhibition of L-type VGCC does not affect whole blood evoked EC Ca^{2+} events.

Summary data of Ca^{2+} event frequency (**A**) or % of active cells (**B**) in *en-face* ECs showing block of L-type voltage gated calcium channels with nifedipine (1 μM; 20 min; blue) had no effect on the response to stimulation with whole blood. Paired experiments, *n*=3, *P<0.05 by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

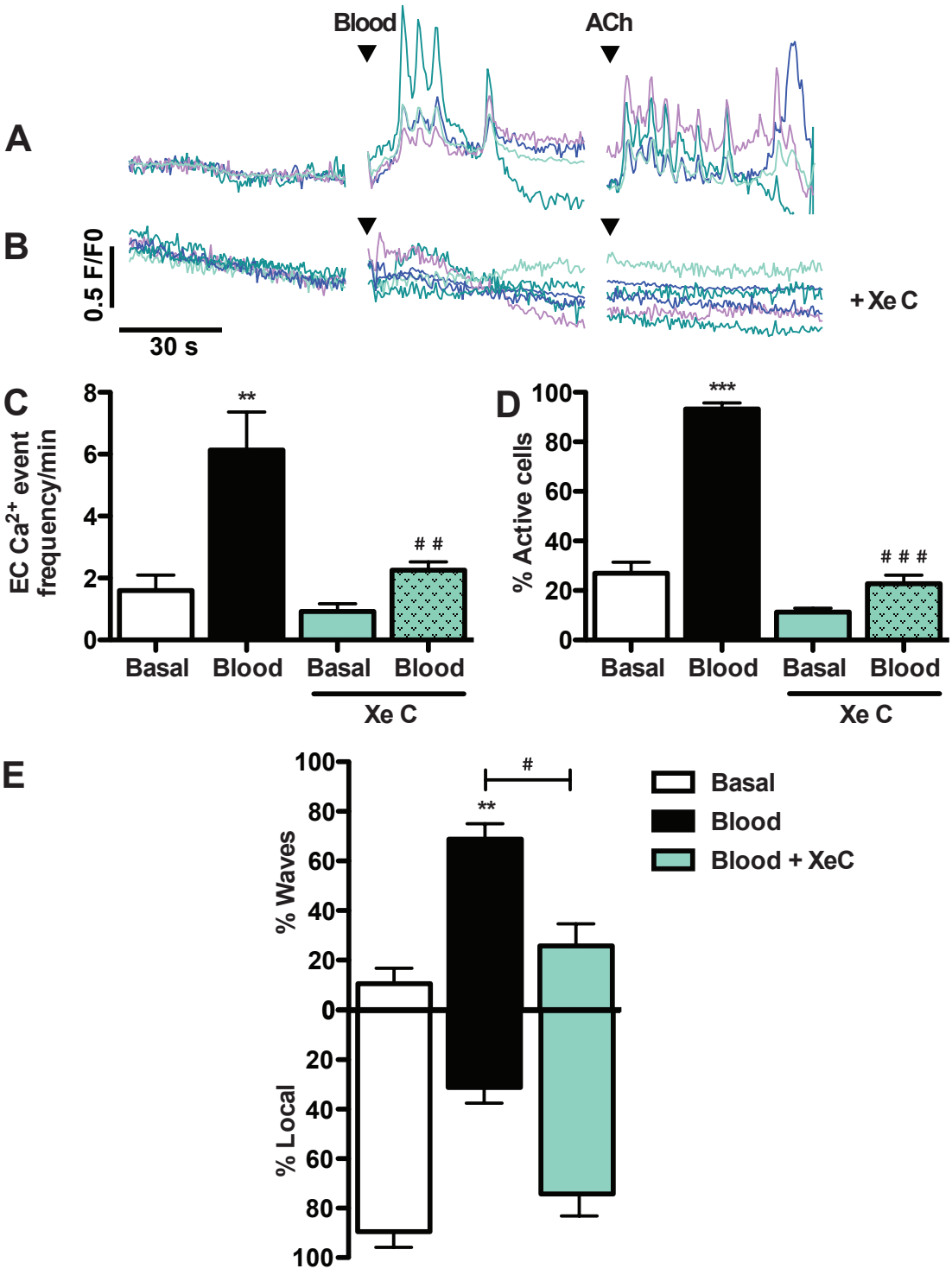


Figure 3.3.8 Increase in endothelial cell Ca^{2+} to blood stimulation is prevented by inhibition of IP_3R signalling.

Figure 3.3.8 Increase in endothelial cell Ca^{2+} to blood stimulation is prevented by inhibition of IP_3R signalling.

(**A**) Representative fluorescence intensity traces to show EC Ca^{2+} activity in basal, blood and ACh conditions (arrows) and (**B**) the same regions after block of IP_3 receptors with xestospongin C (Xe C). Summary data of EC Ca^{2+} event frequency (**C**) and % of active cells (**D**) in response to blood before (black bars, 20 μl bolus) and after incubation of Xe C (10 μM, 30 min; green bars) in *en-face* rat mesenteric arteries. (**E**) Cells that maintained a response to blood in the presence of Xe C exhibited predominantly local events; a reversal from control that indicated a return to basal signalling. Paired experiments, *n*=4, *P<0.05 significantly different from basal; #P<0.05, significantly different from matched data by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

Because previous studies established that ATP can be released from erythrocytes under hypoxic conditions or due to mechanical deformation (Sprague *et al.*, 1996; Sridharan *et al.*, 2010) and ATP raises EC $[Ca^{2+}]_i$ in rat mesenteric arteries (Rodriguez-Rodriguez *et al.*, 2009), we sought to investigate whether circulating or released ATP could form part of the observed response to whole blood. Endothelial cell Ca^{2+} responses could be generated by the introduction of 3 μM ATP; this could be blocked by pre incubation of the artery with 3 μM MRS2179, an antagonist selective for the endothelial purinergic $P2Y_1$ receptor, but not by 1 μM NF449, an antagonist for P2X receptors found on smooth muscle cells (**Figure 3.3.9A**). EC Ca^{2+} responses to 3 μM ATP could also be abolished by incubation with 1 unit ml^{-1} apyrase, an enzyme that catalyses the hydrolysis of ATP (**Figure 3.3.10A/B**) but neither treatment altered the EC Ca^{2+} event frequency or % active endothelial cells in response to stimulation with whole blood (**Figure 3.3.9B; Figure 3.3.10C**).

As reports have placed circulating ACh in a range from 1-300 nM in rat (Fujii *et al.*, 1995; Hasegawa *et al.*, 1982), we investigated the ability of the muscarinic receptor antagonist atropine (1 μM) to influence the EC Ca^{2+} response to whole blood. ADPβS (1 μM), a non-hydrolysable analogue of ADP was employed as a positive control for EC viability in place of ACh when pre incubated with atropine. Whilst the effect of ACh was abolished by atropine (**Figure 3.3.11A**), the response to blood was not blocked, although a significant attenuation in the frequency of EC Ca^{2+} events was observed. The number of responsive cells was also reduced significantly, but many remained active (**Figure 3.3.11B**).

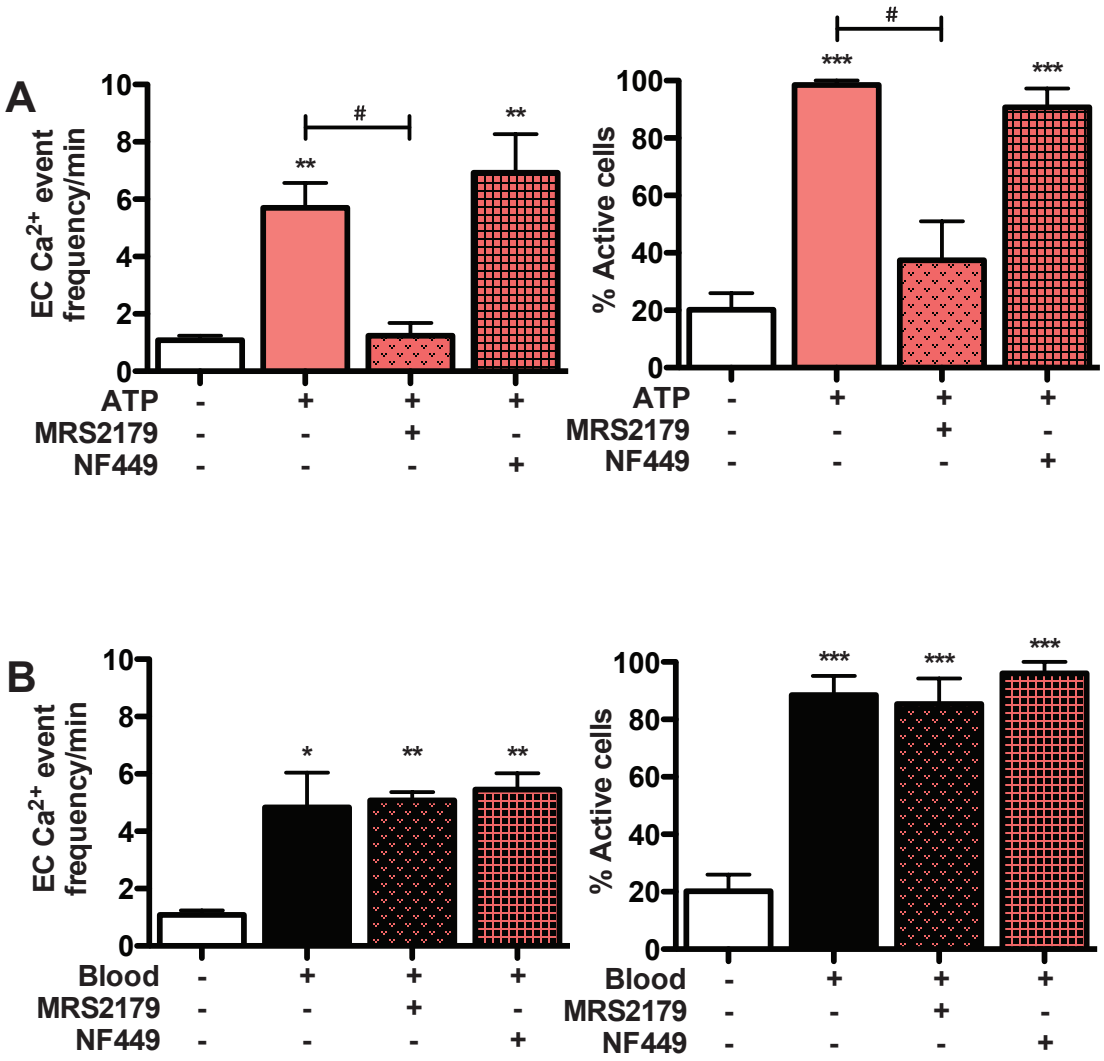


Figure 3.3.9 Increases in endothelial cell Ca^{2+} to ATP but not to whole blood is purinergic receptor mediated.

(**A**) ATP (3 μM; pink) increased endothelial cell Ca^{2+} from control conditions basally (white) which could be prevented by pre incubation with MRS2179 ($P2Y_1$ receptor selective, 3 μM, 20 min; dotted) but not with NF449 (P2X receptor selective, 1 μM, 20 min; hatched). The EC Ca^{2+} response induced by blood (**B**, black) was not altered by pre incubation of MRS2179 or NF449. Data are pooled from separate series of experiments: 1) ATP + whole blood ± MRS2179, *n*=3; 2) ATP + whole blood ± NF449, *n*=3. Unpaired data, *P<0.05 significantly different from basal; #P<0.05, significantly different from matched data by one way ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

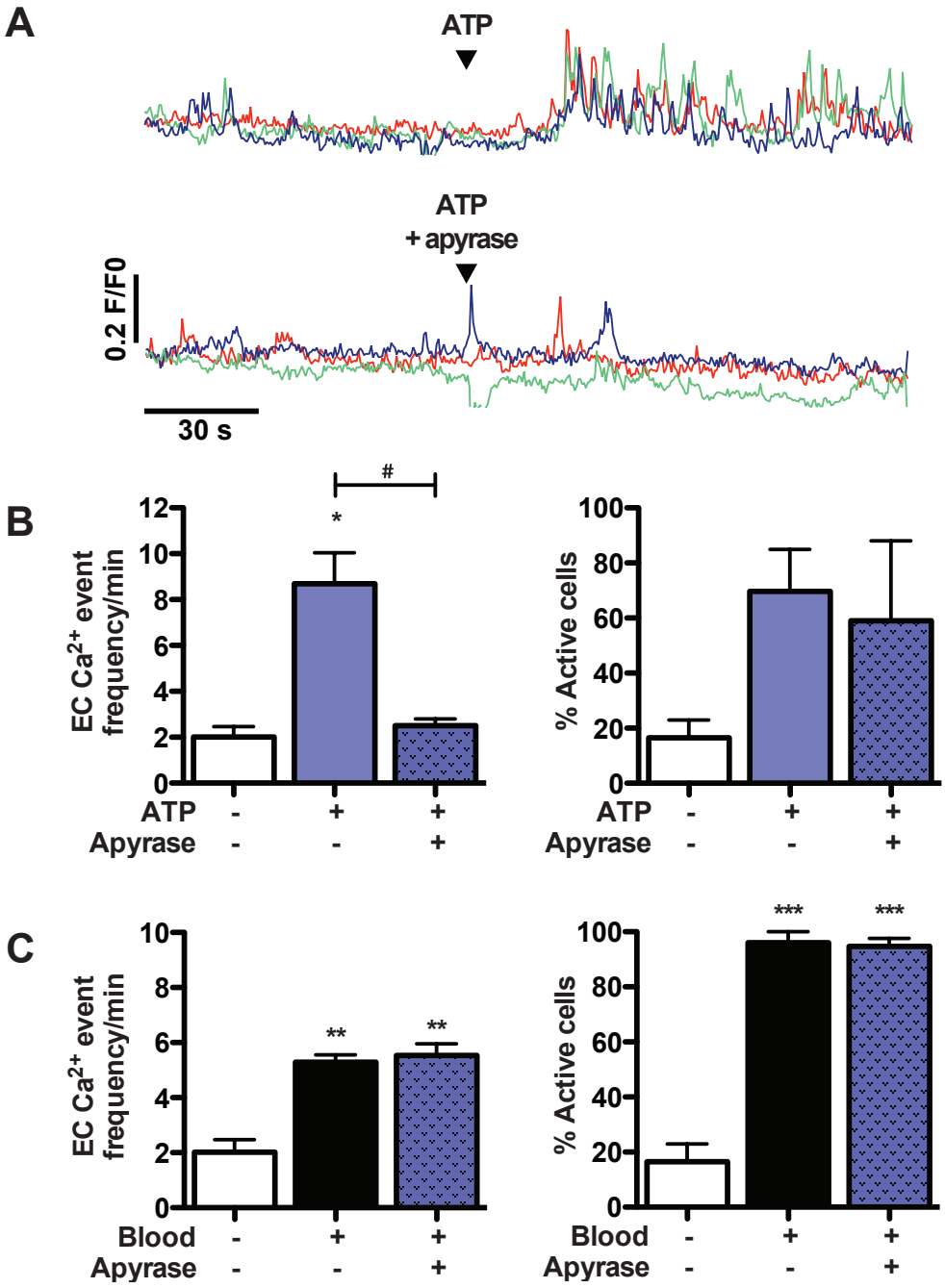


Figure 3.3.10 Apyrase reduces EC Ca^{2+} to stimulation with ATP but not whole blood. (**A**) Representative fluorescence intensity traces demonstrating the effect of ATP (3 μM; arrow) on EC Ca^{2+} before and after (**top**, **bottom** respectively) addition of 1 unit ml^{-1} apyrase. Summary data illustrating apyrase reduced ATP mediated EC Ca^{2+} responses (**B**) but not those caused by whole blood (**C**). Paired experiments, *n*=3, *P<0.05 significantly different from basal (white); #P<0.05, significantly different from matched data by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

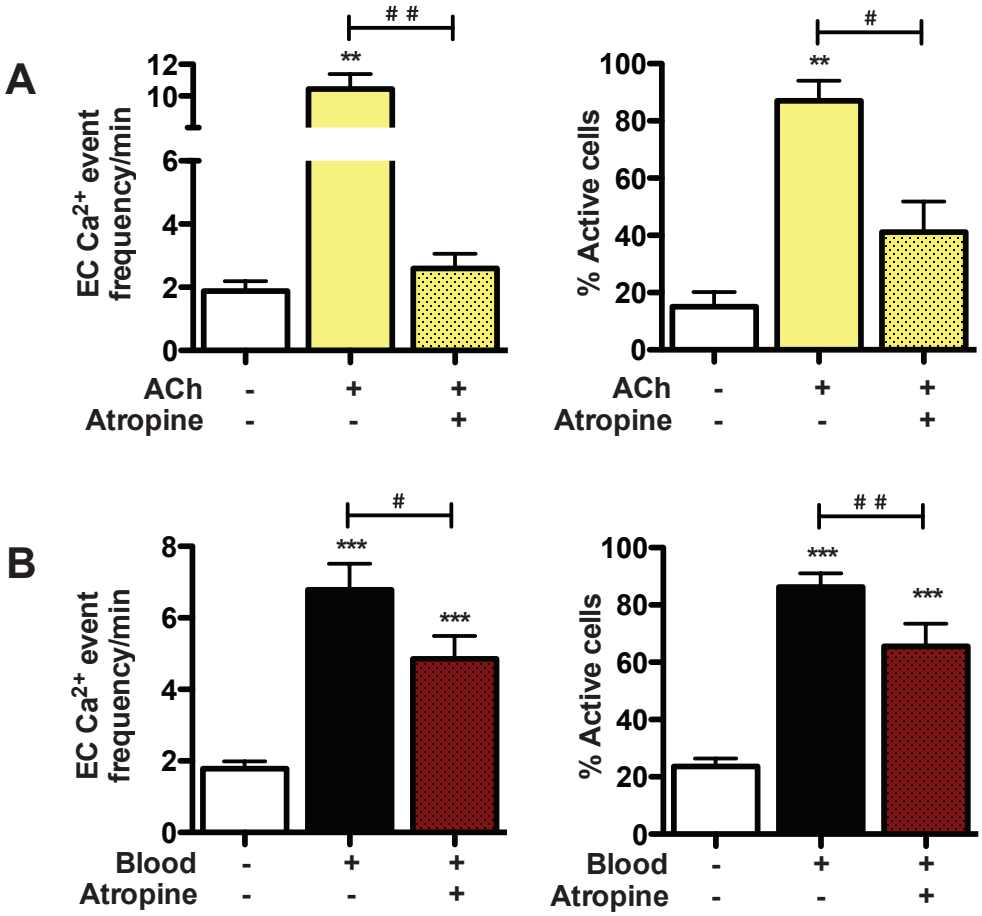


Figure 3.3.11 Atropine reduces EC Ca^{2+} to stimulation with ACh and whole blood. Summary data showing the effect of ACh (Yellow) on EC Ca^{2+} and its inhibition by the muscarinic receptor antagonist atropine (**A**; 1 μM, dotted). Pre incubation with atropine attenuated but did not block the effect of whole blood on raising EC Ca^{2+} (**B**). Paired experiments, *n*=8, *P<0.05 significantly different from basal (white); #P<0.05, significantly different from matched data by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

3.4 Discussion

The data presented in this chapter demonstrate that:

1) Blood activates endothelial cells, which gives rise to a pronounced Ca^{2+} response.
2) This increase in EC Ca^{2+} may reflect a biochemical, ligand based activation that mobilises Ca^{2+} via IP_3R signalling.
3) At least part of the observed EC Ca^{2+} response stimulated by blood is acting upon EC mAChR, suggestive of non-neuronal ACh within the blood.
4) Endothelial cell tubes are a valuable model for studying endothelial cells in isolation and were demonstrated for the first time from rat mesenteric arteries.

3.4.i *En-face* arterial preparation for measurement of EC $[Ca^{2+}]_i$.

Owing to the necessity for direct access to the endothelium, an *en-face* preparation was developed for rat mesenteric arteries as previous studies in mouse had proven Ca^{2+} imaging of a healthy opened mesenteric artery was possible (Ledoux *et al.*, 2008a). It was possible to cut open these arteries and pin them in place without dealing too much damage to the central endothelial cells, as shown by characteristic responses to endothelial stimuli such as ACh and the occurrence of low frequency, spontaneous Ca^{2+} events in the absence of stimuli, making the Ca^{2+} handling and responses observed akin to those in a pressurised mesenteric artery (Bagher *et al.*, 2012; Kansui *et al.*, 2008).

Spontaneous EC Ca^{2+} activity occurs during basal conditions devoid of agonist stimulation. These Ca^{2+} events can propagate to form Ca^{2+} waves and have been demonstrated in various vascular beds (Bagher *et al.*, 2012; Kansui *et al.*, 2008;

Ledoux *et al.*, 2008b). Although the direct functional role of these spontaneous events is unclear, it is known that their frequency is inverse to intraluminal pressure (Bagher *et al.*, 2012) and they may play key roles in regulating important Ca^{2+} sensitive proteins such as eNOS and K_{Ca} channels found in spatially localised Ca^{2+} signalling microdomains that have important implications in control of vascular tone (Kruse *et al.*, 1994; Ledoux *et al.*, 2008b). We also report these spontaneous Ca^{2+} events during un-stimulated, resting baseline periods in our *en-face* rat mesenteric artery preparation (**Figure 2.2.1**; basal, white bars in all Ca^{2+} summary figures). These details taken together indicate that the intra- and inter-cellular signalling of the *en-face* preparation was maintained.

3.4.ii Stimulation with blood raises EC $[Ca^{2+}]_i$.

The application of blood directly over *en-face* ECs generated pronounced and reproducible rises in $[Ca^{2+}]_i$ that were formed predominantly by cell wide propagated Ca^{2+} waves. These responses were transient, lasting up to 40s on average, although in some instances some individual cells could remain active over a more prolonged period of time. The transient nature of the response could reflect a few possibilities, such as the presence of EC agonists may be diminished through degradation by tissue enzymes. Alternatively the attenuation of the response to blood may reflect rapid equilibration throughout the recording chamber to an overall lower, inactive concentration. Despite the diversity of potential signals originating from the blood that may increase EC Ca^{2+}, the observed response is remarkably consistent.

Sodium citrate anticoagulant was also applied over *en-face* ECs as a vehicle control and failed to generate a Ca^{2+} response (**Figure 3.3.3A**). In corroboration with this,

blood samples collected instead in heparin, another widely used anticoagulant, were no different in exhibiting a Ca^{2+} response to samples collected in sodium citrate (**Figure 3.3.3B**). In addition to ruling out vehicle artefacts in the observed response this had the benefit of confirming that citrate was not upregulating the synthesis of vasoactive mediators including acetylcholine, as has been demonstrated through its incorporation as an alternative source of acetyl coenzyme A substrate, the precursor to ACh synthesis (Lipton & Barron, 1946; Sterling & O'Neill, 1978).

The activation of EC Ca^{2+} does not appear to be associated with, or released from, one particular blood cell type upon application (**Figure 3.3.5**). Whether vasoactive agents potentially acting upon EC surface receptors are circulating prior, or are released upon application of the blood, it is likely they originate from a local source being either endothelial cells or blood cells. From which cell type and the mechanism for how any agonists are released remains the subject of further investigation. Of particular importance is the ability of plasma to raise EC Ca^{2+}, further suggesting that not only are the increases in Ca^{2+} likely to be independent of mechanical or shear stresses, but that indeed the active agents (or their precursors) are likely to already be present upon application, and not a result of stimulated release from blood cells during interaction with ECs. An *in situ* study of endothelial cell Ca^{2+} transients reported data in accordance with our study, noting that a high frequency of spontaneous endothelial cell calcium transients are markedly decreased during occlusion of blood flow, but returned following flow recovery. These findings were attributed to “a depletion of luminally available endogenous Ca^{2+} agonists” and not due to mechanical, or shear stress mechanisms (Duza & Sarelius, 2004).

In endothelial cells, $[Ca^{2+}]_i$ has roles in the control of vascular tone via release of nitric oxide (NO), prostacyclin, EETs and regulation of endothelium-derived hyperpolarization (EDH). These represent significant vasorelaxant pathways that may be activated following the rise in EC Ca^{2+} in response to blood. Throughout the response to blood (and ACh), local Ca^{2+} events propagated and were observed as waves that appear to reflect amplification due to Ca^{2+} release from intracellular stores.

3.4.iii Blood raises EC Ca^{2+} independent of mechanical stresses.

Changes in endothelial $[Ca^{2+}]_i$ are known to occur in response to receptor activation and in response to mechanical stimuli. Activation of endothelial cells by shear stress also may generate increases in $[Ca^{2+}]_i$ and may be mediated by influx of extracellular Ca^{2+}. The passage of extracellular Ca^{2+} is suggested to be through two predominant shear stress dependent ion channels, namely P2X purinergic receptors and transient receptor potential channels (Ando *et al.*, 1988; Kohler *et al.*, 2006; Mendoza *et al.*, 2010; O'Neil & Heller, 2005; Yamamoto *et al.*, 2000).

However we noted no reduction in EC Ca^{2+} during $P2X_1$ receptor antagonism by NF449 during stimulation with blood (**Figure 3.3.9B**). In accordance with this the dependency of the EC Ca^{2+} response to blood did not appear to rely on fluid motion caused by either the application of an inert 20 μl bolus, or superfusate flow (at 2 ml min^{-1}) over the *en-face* ECs (**Figure 3.3.2**). Furthermore, the absence of cells in plasma, yet persistent EC Ca^{2+} responses (**Figure 3.3.5**) suggests a lack of mechanical signalling involvement in the response studied.

3.4.iv Blood raises EC Ca^{2+} predominantly through IP_3 receptor signalling.

The response to whole blood was noted for its strong effect on EC Ca^{2+} (**Figure 3.3.1**). As mentioned in **section 1.2.i**, IP_3 as well as cyclic ADP-ribose and nicotinic acid dinucleotide phosphate (NAADP) can trigger release of Ca^{2+} from intracellular stores. However, EC Ca^{2+} may also be influenced by influx of extracellular Ca^{2+}, and coupled smooth muscle cells (Dora *et al.*, 1997; Kansui *et al.*, 2008; Mendoza *et al.*, 2010). The effect of whole blood on EC Ca^{2+} was not prevented by removal of extracellular Ca^{2+} (**Figure 3.3.6**), indicating that influx of extracellular Ca^{2+} is not a significant pathway activated by EC stimulation with blood. Although endothelial cells do not express L-type voltage gated calcium channels (VGCC; Colden-Stanfield *et al.*, 1987; Johns *et al.*, 1987), they are a major Ca^{2+} influx channel in SMCs. Considering Ca^{2+} and/or IP_3 can pass between SMCs and ECs (Dora *et al.*, 1997) it is possible that VGCC can indirectly influence EC Ca^{2+}. We therefore examined the effect of nifedipine on EC Ca^{2+} as part of the response to blood stimulation, however found no evidence for involvement of VGCC (**Figure 3.3.7**).

Xestospongin C, an IP_3 receptor blocker, reduced the effect of whole blood, fully inhibiting the response in three of four performed experiments and markedly reducing events in the fourth, which amounted to a minor response, akin to baseline activity (**Figure 3.3.8A&B**). Thus the graph demonstrating event frequency infers some cells may still respond to blood, but remaining events were of lower magnitude and occurred in a lower number of cells compared with control. To reflect this, the predominance of wave events initiated by whole blood was shifted from mostly waves in favour of a return to local activity when blocked with

xestospongin C (**Figure 3.3.8C**) implicating a near total abolition of the control response to blood. Xestospongin C was chosen to investigate Ca^{2+} mobilisation from ER stores due to demonstrable off target effects of U73122, a phospholipase C inhibitor (Heemskerk *et al.*, 1997), and issues caused by SERCA inhibition. Specifically, depleting the ER Ca^{2+} stores in this way increases cytosolic [Ca^{2+}], which may confound Ca^{2+} imaging as well as affect Ca^{2+} sensitive proteins in the cells (Crane *et al.*, 2003).

It is of note that removal of extracellular Ca^{2+} lowered overall level of basal Ca^{2+} activity (**Figure 3.3.6**) by significantly reducing the number of cells displaying spontaneous Ca^{2+} events. The amount of basal activity was also slightly reduced by xestospongin C suggesting that spontaneous Ca^{2+} release via IP_3 receptors may represent a secondary mechanism underlying basal activity, either through spontaneous store release or activation of IP_3 receptors mediated by Ca^{2+} influx. This is in line with previous reports that indicate a reliance on extracellular Ca^{2+} and spontaneous release via IP_3 (Bagher *et al.*, 2012; Kansui *et al.*, 2008).

Taken together these data indicate that blood raises EC Ca^{2+} via a non shear stress mechanism (**Figure 3.3.2**), is endothelial cell specific (**Figure 3.3.4**), and acts predominantly through IP_3R signalling. Given the prior evidence for blood components to interact with EC membrane receptors, it seemed likely at this point that blood was acting directly on receptors to raise IP_3, most likely $G_{q/11}$ coupled GPCRs.

Agonists carried in the blood stream that may have an influence on endothelial cells can originate from circulating blood cells directly. Adenosine triphosphate (ATP) is

known to be present in circulating blood, even reaching micromolar concentrations in humans (Rosenmeier *et al.*, 2004). Purinergic receptors are grouped into two types: P1 and P2 for their sensitivity to adenosine and ATP respectively (Burnstock, 1978), the latter further being split into both P2X and P2Y families (Burnstock & Kennedy, 1985). The P2X family of purinergic receptors comprise a family of ligand gated ion channels whereas P2Y consist of G-protein coupled receptors (Abbracchio & Burnstock, 1994). In the vasculature, P2X receptors are expressed on smooth muscle cells with P2Y receptors expressed on both smooth muscle and endothelial cells (Burnstock, 2006; Gitterman & Evans, 2001; Vulchanova *et al.*, 1996; Wang *et al.*, 2002). Activation of endothelial P2Y receptors in rat mesenteric arteries stimulates dilation and rises in EC Ca^{2+} (Rodriguez-Rodriguez *et al.*, 2009; Winter & Dora, 2007). Vascular sources of ATP include smooth muscle, endothelial cells and platelets (Gordon, 1986), but ATP is also known to be contained in large amounts in erythrocytes (Miseta *et al.*, 1993). Upon release, erythrocyte derived ATP has been shown to influence endothelial cell production of nitric oxide (Sprague *et al.*, 1996) demonstrating that agents produced by circulating cells must also be considered in the larger model of vascular tone regulation.

Our results suggest that the effects of ATP on endothelial purinergic receptors (**Figure 3.3.9**) do not form part of the observed response to whole blood (**Figure 3.3.10**). ATP has been demonstrated to be released via pannexin channels on the erythrocyte membrane in response to hypoxia and mechanical deformation (Sprague *et al.*, 1996; Sridharan *et al.*, 2010). Hypoxia and mechanical deformation are two triggers for ATP release, although these do not seem to be factors in our observed blood response. It would be interesting to investigate whether hypoxic conditions could potentiate the response seen to blood via ATP signalling, although preliminary experiments whereby the rat was killed by an overdose of CO_2 had

seemingly no effect on the blood response. Nonetheless, although our results indicate that ATP did not have a significant effect on the EC Ca^{2+} response, it may be important to consider that the storage of blood could mean that samples have a low pO_2 level and that this may affect the presence or absence of signalling molecules associated with hypoxia.

The reduction of EC Ca^{2+} to whole blood by atropine (**Figure 3.3.11**) indicates blood is mediating at least part of its effect via mAChRs, suggesting that ACh may be present within the blood or is released from a local cell type as part of the response to blood. The origin of this ACh remains to be fully elucidated and is the subject of the following chapter.

None of the antagonists fully abolished the response to blood alone, instead reducing the response in some cases. This could be expected through use of the blood to stimulate the endothelium, as there are likely to be multiple agonists that continue to signal during the blockade of another.

3.4.v Endothelial cell tubes: a valuable isolated EC model.

Although previously demonstrated in hamster cremaster and mouse abdominal feed arterioles (Cohen & Jackson, 2005; Socha *et al.*, 2011), the current work represents the demonstration of endothelial cell tube isolation from rat mesenteric arteries for the first time. The isolated EC tube model allows these cells to be studied intact and without influence from surrounding smooth muscle cells or perivascular nerves. Previous investigations on the nature of Ca^{2+} signalling in isolated ECs have largely been through cell culture. Compared with those freshly obtained from isolated arteries, cultured ECs often exhibit altered phenotypes that may include the loss of

muscarinic receptors (Brunner & Kukovetz, 1991; Tracey & Peach, 1992). EC tubes however respond reliably and in typical fashion to ACh (**Figure 3.3.4**). The ability to pin EC tubes to approximate physiological length is advantageous in maintaining the flat, elongated morphology of ECs akin to those in intact preparations, opposed to the rounded, 'cobblestone' morphology of isolated EC sheets (McSherry *et al.*, 2005). Alterations in cell shape may disrupt Ca^{2+} handling by shifting the arrangement of intracellular stores or proteins.

Through use of this technique we confirmed stimulation by whole blood in the role of EC $[Ca^{2+}]_i$ regulation is an endothelial cell specific response. Our experiments in freshly isolated endothelial cell tubes indicate that the response to blood may be principally directed by EC receptor activation raising Ca^{2+} and not via transmission of IP_3 or other Ca^{2+} mobilising signals transferring from SMCs to ECs, as the frequency of Ca^{2+} events to blood is much the same in intact *en-face* endothelial cells as it is in EC tubes, where surrounding smooth muscle cells are lost. One difference however, is the reduction of spontaneous basal activity in EC tubes than *en-face* arteries. This most likely results from the loss of myoendothelial junctions, as they are important microdomains for Ca^{2+} signalling in ECs (Bagher *et al.*, 2012; Straub *et al.*, 2014).

3.5 Conclusion

Data in this chapter demonstrate that blood can increase EC $[Ca^{2+}]_i$ as both an increased frequency of events as well as a greater number of cell wide waves that reflects endothelial cell activation. Endothelial cell tubes were isolated from rat mesenteric arteries for the first reported time, allowing these cells to be studied intact and in isolation without influence from surrounding smooth muscle cells. The results obtained in EC tubes confirm that blood was having a direct action on endothelial cells.

A dominant involvement of diffusible blood borne factors as opposed to mechanical activation is likely due to the ability of several components of the blood including plasma to produce the same response. This is further corroborated by the lack of an observed Ca^{2+} response to flow stresses.

Endogenous circulating factors such as hormones, peptides and small molecule transmitters could all reasonably exert effects on endothelial cells, and we have demonstrated the ability for blood to raise endothelial cell $[Ca^{2+}]_i$, highlighting a possible role for blood in the local control of these cells. The demonstration that the blood response may be in part cholinergic, as shown through attenuation with atropine, has interesting implications in the regulation of vascular tone. In light of this we aimed to further study the EC Ca^{2+} response to blood in order to better understand where ACh may be originating from.

Chapter 4

Investigation of possible non-neuronal acetylcholine from blood cells

4.1 Introduction

As discussed (**section 1.5.iii**) cholinergic signalling has been extensively studied in tissues lacking parasympathetic innervation and data from the previous chapter suggest that a vascular non-neuronal cholinergic system may explain the physiological role for luminal muscarinic ACh receptors on endothelial cells. Alongside exposing the cell type of origin, another key uncertainty is under what conditions ACh is released: whether ACh is tonically released into the circulation and regulated by erythrocyte acetylcholinesterase, or rapidly as part of a stimulated response during blood cell activation or interaction with ECs.

Various investigations offer numerous suggestions as to the cell type of origin. It has been demonstrated that the ACh content of blood cells is around 25 times greater than that of plasma in rabbits (Kawashima *et al.*, 1989). Furthermore, administration of nicotine induced significant increases in plasma [ACh] whilst decreasing that of blood cells, indicating not only that plasma ACh may originate from blood cells but suggests their capacity for ACh storage. Human erythrocytes have been studied for both the presence of functional acetylcholine receptors (Huestis & McConnell, 1974), and ACh synthesis (Holland & Greig, 1952) implicating them as a potential source of non-neuronal ACh, in much the same way that ATP originating from erythrocytes can act upon ECs. Release of signalling molecules from erythrocytes are generally thought to be via channel mediated release as under physiologoical conditions, mature erythrocytes are vesicle free (Locovei *et al.*, 2006). Pannexins represent a recently discovered family of gap junctional proteins which are distinct from connexins (Panchin *et al.*, 2000). Pannexin 1 can form non-junctional membrane channels that provide a passageway from the cytoplasm to the extracellular space for molecules in the size range of second messengers

(Bruzzone *et al.*, 2003) including ATP (Sridharan *et al.*, 2010). As pannexin channels likely form large channels that connect the erythrocyte cytoplasm with the extracellular space, they are a prime candidate for the release of erythrocyte derived signalling molecules including ATP; which is reduced when blocked with carbenoxolone (Locovei *et al.*, 2006; Lohman *et al.*, 2012; Sridharan *et al.*, 2010).

Platelets are known to store an impressive pool of neurotransmitters in their dense granules, which are released during activation and cause positive feedback activation (Anfossi & Trovati, 1996; Boullin & Green, 1972; Whiteheart, 2011). It has been proposed that ACh may be among the signalling molecules contained within platelet stores, and released upon activation of platelets via nAChα7 receptors (Schedel *et al.*, 2011; Wessler *et al.*, 1998). The entire components of the cholinergic system (ChAT, ACh, ACh receptors) are found in cells of the immune system (Kawashima & Fujii, 2000; Kawashima *et al.*, 2012) with 60% total human blood ACh content detected in mononuclear leukocytes (Kawashima *et al.,* 1993).

In most of these cases, roles have been suggested for this ACh that suggest complete, local cholinergic systems confined to blood cells that may reflect more auto- than paracrine roles (de Almeida & Saldanha, 2010; Huestis & McConnell, 1974; Kawashima *et al.*, 2012). These have been suggested to be independent, self contained non-neuronal cholinergic systems, particularly in immune cell. However the possibility that this endogenous ACh derived from cells of the blood can influence the endothelium, but also that it may in fact come from more than one source with multiple roles in relation to its functions within the vasculature.

The aim of the experiments in this chapter was to investigate the atropine sensitivity of particular fractions of blood samples to test the possibility that ACh acting upon the endothelium (Figure 3.3.11) was derived from blood cells. In addition certain pharmacological tools were utilised to attempt to attenuate or augment the cholinergic component of the response to blood by investigating pathways suggested by the literature (section 4.1).

4.2 Methods

*Refer to **Chapter 2: General methodology** for full details of the methods used in this chapter. Details relevant specifically to this chapter are given below.*

4.2.i Protocols for imaging Ca^{2+} in ECs

Second order rat mesenteric arteries were set up in an *en-face* preparation (as described in **section 2.2.i**), loaded with the Ca^{2+} sensitive dye OGB-1 for Ca^{2+} imaging (**section 2.2.ii**). Drugs were applied to the side of a static bath and mixed by trituration with a pipettor. Blood was applied as a 20 μl bolus directly over the ECs. Blood was washed out immediately following the run and drugs were washed out for 20 min. The protocol for using washed erythrocytes began immediately after wash was complete. Atropine (1 μM) was pre incubated for 20 min in the chamber. Carbenoxolone (100 μM), and methyllycaconitine (1 μM) were incubated in blood samples from the point of collection and replenished during any wash stages. PNU 282987 (1 μM) and neostigmine were incubated in samples of the blood for 20 min prior to commencement of the recording. Plasma and platelet rich plasma samples were obtained as described in **section 2.1.ii**.

4.2.ii Erythrocyte wash procedure

Whole blood was centrifuged at 500 x g for 10 min in a benchtop centrifuge (MSE Micro Centaur; Sanyo, UK). The supernatant (platelet rich plasma) was aspirated and cell wash buffer added to the erythrocyte pellet. The wash procedure was repeated for a total of three washes of the erythrocytes. This purity of erythrocyte preparations by this procedure has been confirmed in rabbit, human and rat erythrocytes (Dietrich *et al.*, 2000; Hanson *et al.*, 2008).

4.3 Results

As reviewed in chapter 3, application of blood stimulates a rise in EC $[Ca^{2+}]_i$ that acts predominantly through IP_3 receptor signalling and is most likely mediated by activation of EC surface receptors. At least a component of this response appears to be mediated through EC muscarinic receptors due to its sensitivity to the muscarinic receptor antagonist atropine. Given the evidence discussed in **section 4.1** that certain cells of the blood are implicated in the synthesis or storage of ACh, we investigated whether we could determine that the cholinergic portion of our observed response was through ACh originating from the blood.

Since increased EC Ca^{2+} by stimulation with blood was not associated with a particular cell type (**Figure 3.3.5**) but could represent involvement of various different agonists, each potentially associated with a separate cell type, we investigated whether sensitivity to atropine was shared by other fractions of the blood. Being devoid of blood cells, a lack of cholinergic response from plasma samples would indicate a possible reliance on stimulated ACh release from a particular blood cell type, whereas the presence of atropine sensitivity would indicate the shared distribution of a signal equilibrated throughout both the blood and plasma. We therefore stimulated *en-face* ECs with plasma samples (20 μl, bolus) in the presence and absence of the muscarinic receptor antagonist atropine. Plasma raised an EC Ca^{2+} response comparable in profile to stimulation by whole blood, which was also sensitive to pre incubation of the vessel with 1 μM atropine (**Figure 4.3.1**). Atropine significantly reduced the frequency of EC Ca^{2+} events as well as the number of cells responding to plasma.

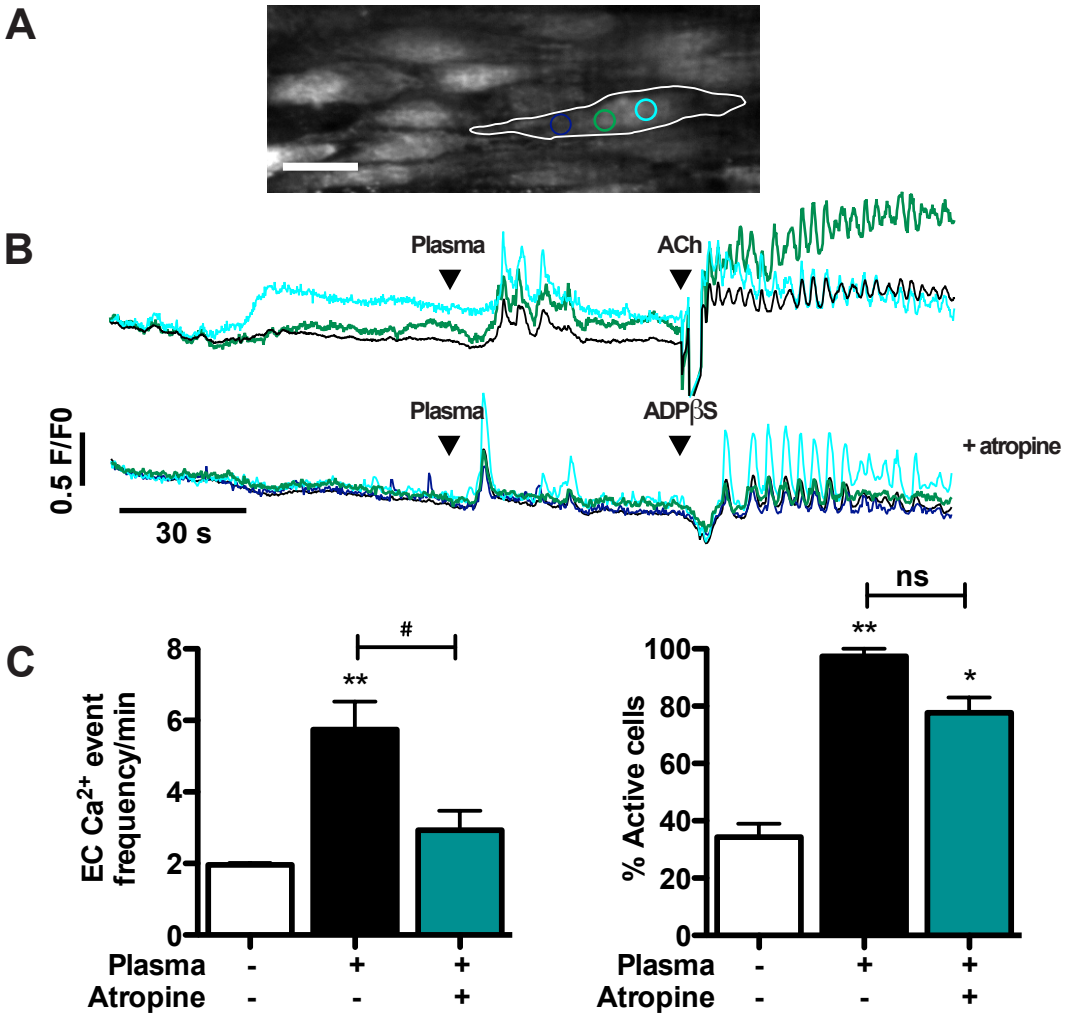


Figure 4.3.1 Plasma raises atropine sensitive EC Ca^{2+} events.
En-face ECs loaded with the Ca^{2+} indicator OGB-1. Scale = 50 μm (**A**). Representative fluorescence intensity traces (B; corresponding to coloured ROIs in panel A) of EC $[Ca^{2+}]_i$ upon adding a bolus (20 μl, unmixed) of rat plasma directly to the endothelial cell surface and ACh (1 μM) or ADPβS (1 μM). Pre-incubation with atropine (1 μM, 20 min; **bottom**) reduced the increase to blood. Exogenous ACh, or ADPβs (1 μM) was tested to confirm EC viability. Summary data (**C**) showing the effects of blood on the frequency of EC calcium activity per cell as well as the % of cells exhibiting changes in $[Ca^{2+}]_i$ in the absence and presence (green bars) of atropine. (n=3; *P=<0.05, significantly different from basal (white); #P=<0.05, significantly different from matched data).

The atropine sensitive (cholinergic) component of the response to plasma suggests that ACh was not being released by activation of blood cells during stimulation of ECs, instead implying that ACh may be present in the sample beforehand and spread between blood fractions including plasma. We therefore moved to determine whether cells of the blood could be activated or otherwise induced to further release their contents, which have been suggested to include ACh.

We tested samples of platelet rich plasma (PRP) for their ability to release ACh upon activation, which simultaneously accounted for the unlikely event that platelets remained in plasma samples in previous experiments (**Figure 4.3.1**). Samples containing platelets activated by the nAChRα7 agonist PNU 282987 (PNU; 1 μM) were used to stimulate *en*-face ECs which exhibited a large increase in Ca^{2+} activity that did not display sensitivity to the muscarinic receptor antagonist atropine (1 μM; **Figure 4.3.2A**). Following this, in separate experiments platelet samples alone were used to stimulate *en*-face ECs, which exhibited a smaller increase in Ca^{2+} activity than activated by PNU 282987 but still raised EC Ca^{2+} above basal activity (**Figure 4.3.2B**). There was no difference when platelets had been antagonised from collection with the nAChRα7 antagonist methyllycaconitine (1 μM).

We also tested samples of blood for their ability to pass vasoactive agonists through pannexin channels expressed on their membranes. The non-specific pannexin (and connexin) channel blocker carbenoxolone (100 μM) was shown to have no effect on the ability of the blood to stimulate rises in EC Ca^{2+} activity (**Figure 4.3.3**) when pre incubated in blood samples.

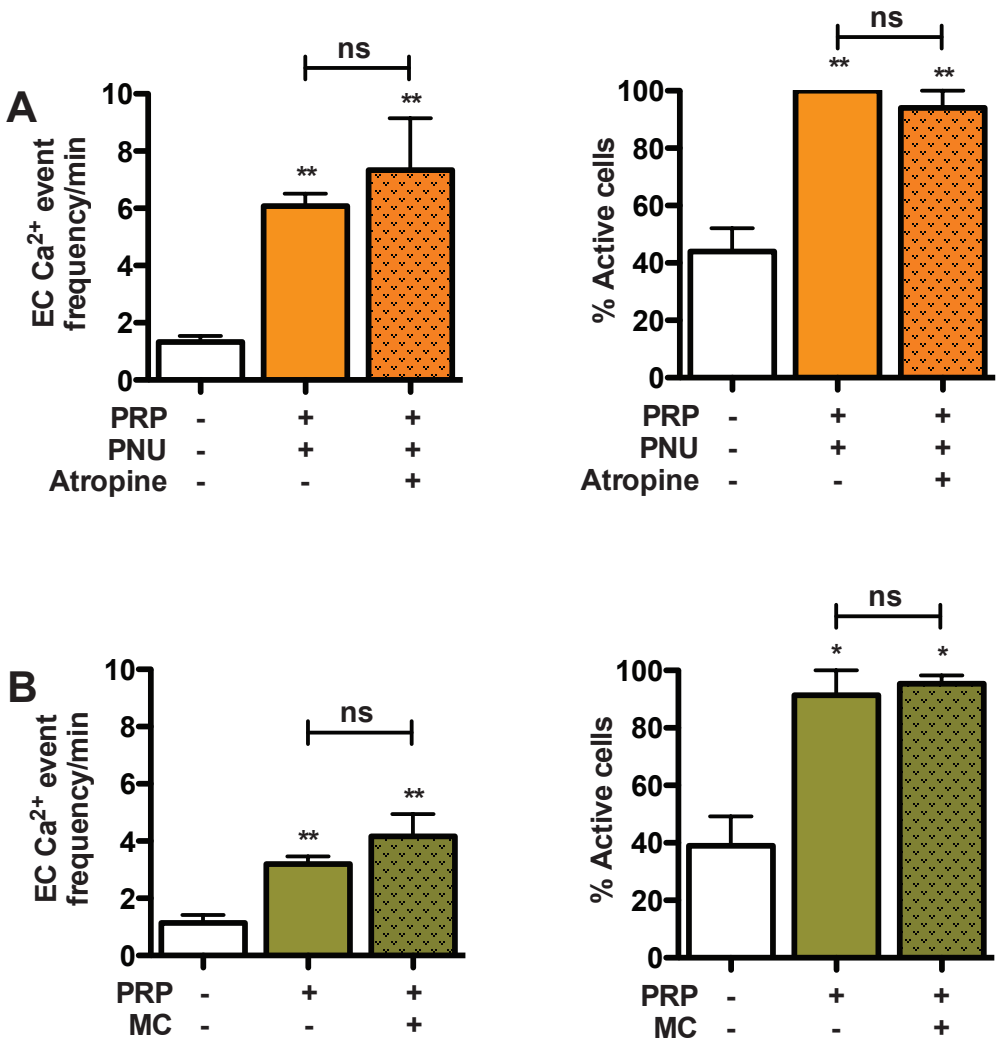


Figure 4.3.2 Activated platelets did not exhibit a cholinergic response.
(**A**) Summary data to show the frequency of EC Ca^{2+} events and % of ECs exhibiting Ca^{2+} activity in response to stimulation with platelet rich plasma (PRP) incubated with the nAChRα7 agonist PNU 282987 (PNU; orange). No alteration of the response was seen when ECs were pre incubated in the presence of the muscarinic receptor antagonist atropine (1 μM; dotted; *n*=3).
(**B**) Summary data to show PRP alone (olive) stimulated EC Ca^{2+} events with no difference when PRP was incubated with the nAChRα7 antagonist methyllycaconitine (dotted; 1 μM).
Data are displayed from separate experiments: 1) PRP + PNU ± atropine, *n*=3; 2) PRP ± methyllycaconitine, *n*=3. Each are paired experiments, *P<0.05 significantly different from basal (white) by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

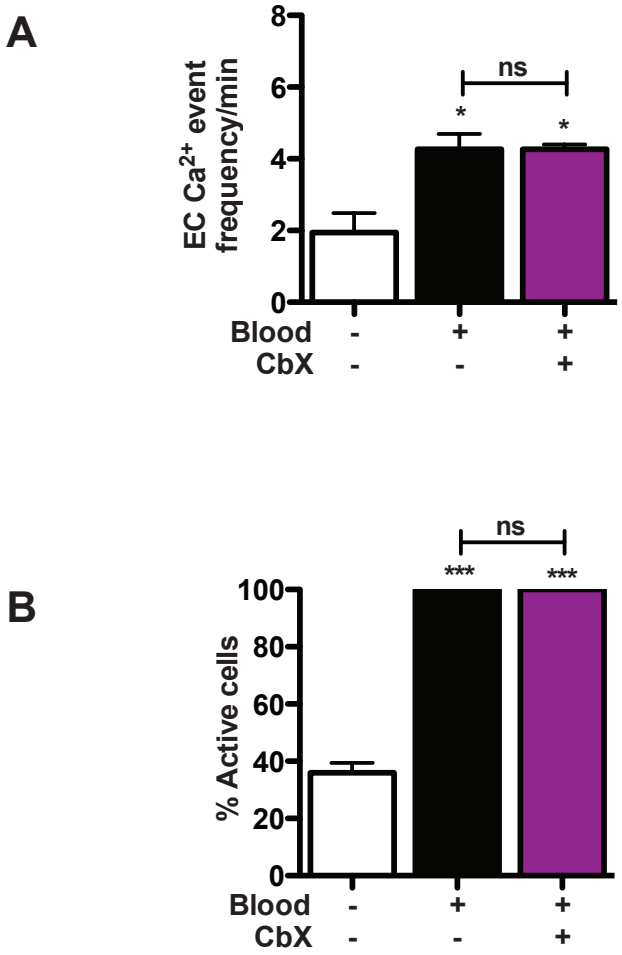


Figure 4.3.3 Erythrocyte pannexin channel blockade did not affect EC Ca^{2+} activity. Summary data to show the frequency of EC Ca^{2+} events (**A**) and % of cells exhibiting Ca^{2+} activity (**B**) to whole blood (black) and blood pre-incubated with the pannexin channel blocker carbenoxolone (100 μM; purple). Blood with and without carbenoxolone raised EC Ca^{2+} activity above basal levels but exhibited no difference in treatment with the pannexin channel blocker. Paired experiments, *n*=3, *P<0.05 by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

Previous experiments with plasma (**Figure 4.3.1**) had suggested that if originating from the blood cells, ACh was most likely being released into the sample prior to stimulation of ECs. Consequently we considered that by washing erythrocyte samples prior to inducing EC Ca^{2+} responses with blood, we may wash out accumulated EC Ca^{2+} agonists. Experimentally we still detected a significant EC Ca^{2+} response upon application of washed erythrocytes essentially identical to whole blood (**Figure 4.3.4A**); furthermore we still identified sensitivity to EC muscarinic antagonism with atropine (1 μM) in our samples of washed cells (**Figure 4.3.4B**).

As acetylcholinesterase (AChE) is known to be expressed on erythrocyte membranes, the use of the AChE inhibitor neostigmine (10 μM) was employed in samples of whole blood. Across the same timeframe as control, a general but insignificant increase in the strength of the response was observed (**Figure 4.3.5**), with only one in seven experiments acting to prolong the duration of the response period.

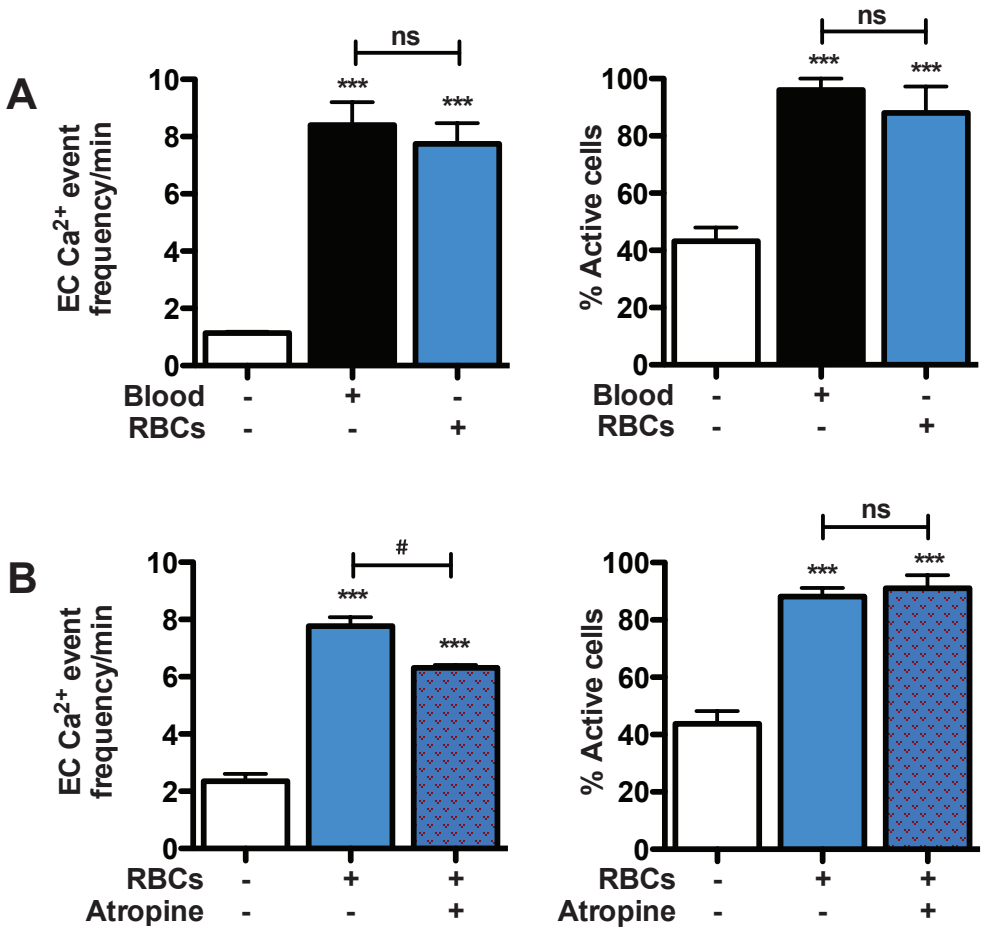


Figure 4.3.4 Washed erythrocytes raised atropine sensitive EC Ca^{2+} responses.
(**A**) Washed erythrocytes (RBCs; 20 μl, unmixed; blue) and whole blood (black) both increased endothelial cell Ca^{2+} from control conditions basally (white) but were not different from one another. (**B**) Washed erythrocytes (blue) displayed a reduced capacity to increase EC Ca^{2+} when ECs were pre-incubated with the muscarinic receptor antagonist atropine (1 μM; dotted). Data are from paired, but separate experiments: 1) whole blood vs. washed erythrocytes, *n*=5; 2) washed erythrocytes vs. washed erythrocytes + atropine, *n*=4. *P<0.05 significantly different from control (basal, white), by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

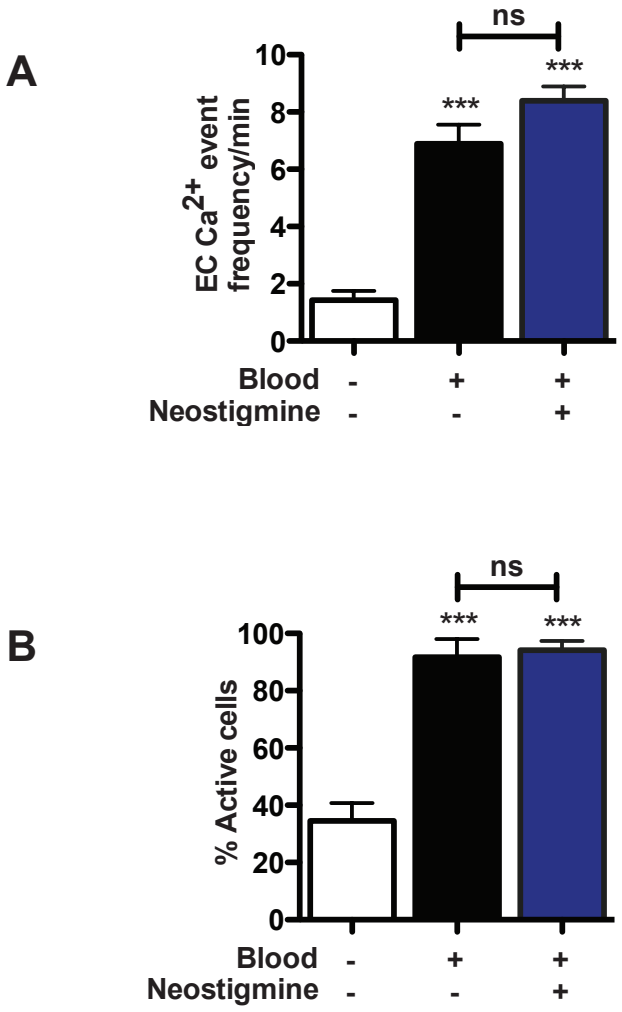


Figure 4.3.5 Inhibition of acetylcholinesterase did not potentiate the EC Ca^{2+} response to blood.

Summary data to show the frequency of EC Ca^{2+} events (**A**) and % of cells exhibiting Ca^{2+} activity (**B**) to blood (20 μl, unmixed; black) and blood treated with the acetylcholinesterase inhibitor neostigmine (10 μM; navy). Blood with and without neostigmine raised EC Ca^{2+} activity above basal levels but exhibited no difference between treatments. Paired experiments, *n*=7, *P<0.05 by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

4.4 Discussion

The data presented in this chapter demonstrate that:

1) The ability to raise EC Ca^{2+} via muscarinic receptors is not dependent on the presence of any blood cells suggestive of free ACh in blood samples or a messenger that acts to mobilise ACh from ECs.
2) Platelets activated through a non-neuronal nAChR increased EC Ca^{2+} but not via the release of ACh.
3) Despite washing to remove signals, erythrocytes retained the ability to raise EC Ca^{2+}, in part via muscarinic receptors, suggesting involvement in the synthesis of either free ACh or a shared messenger with plasma that acts to mobilise ACh.
4) Inhibition of acetylcholinesterase did not potentiate the EC Ca^{2+} response, suggesting ACh does not accumulate within blood samples and instead may be quantally released from ECs.

Our initial focus was based upon investigating the ability of free ACh within the blood to influence endothelial cells. Previous investigations detected varying amounts of ACh in the blood of several species (Fujii *et al.*, 1995) and our previous observation that atropine could reduce an EC Ca^{2+} response to application of blood seemed to support this (**Figure 3.3.11**). One of the major questions arising from this observation was which cell type presented the source of this endogenous ACh, as centrifugation had previously revealed a role for all fractions of the blood (**Figure 3.3.5**). Another pertinent question raised was whether ACh was released from a cell locally in a quantal manner (possibly in response to activation during EC stimulation with blood), or whether ACh was circulating and therefore contained within our sample. We proposed to test whether certain blood cells could be

influenced to alter the observed response. We also submitted that involvement of free ACh would mean it was distributed throughout the blood including the plasma, and may initiate EC Ca^{2+} activity in the absence of blood cells.

4.4.i Plasma raises EC Ca^{2+}, partially through mAChR activation in a blood cell independent manner.

That plasma was responsible for a similar atropine sensitive response (**Figure 4.3.1**) suggests that ACh acting on ECs is not associated with any blood cell type in particular and further supports that the mechanical action of erythrocytes and other cell types on the endothelial surface is not responsible for the increase in EC Ca^{2+}. This advocated that ACh is unlikely to be being released from a blood cell becoming 'activated' during the bolus application, and that ACh is either pre distributed throughout the blood including the plasma, or that some other messenger that is also present in the plasma is available to stimulate ECs into ACh synthesis and/or release. To determine between possible free ACh within the sample and some alternative signal that triggers downstream release of ACh, future experiments involving cholinesterase enzymes would prove useful. Addition to a plasma sample would theoretically hydrolyse any free ACh present, preventing the muscarinic component of the stimulated blood response due to any free plasma ACh and help to elucidate between these two suggested pathways.

4.4.ii Examination of platelet activation by, and release of, non-neuronal ACh.

We tested samples of platelet rich plasma (PRP) for their ability to release ACh from their intracellular granules, which simultaneously accounted for the unlikely event that platelets remained in plasma samples in previous experiments (**Figure 4.3.1**).

Platelets are known to store a remarkable pool of neurotransmitters in their dense and alpha granules, including ADP, ATP, histamine and serotonin. These signalling molecules are released during platelet activation and cause positive feedback activation (Anfossi & Trovati, 1996; Boullin & Green, 1972; Whiteheart, 2011). Because platelets have been proposed to contain ACh (Schedel *et al.*, 2011; Wessler *et al.*, 1998), we investigated whether platelet activation could lead to ACh release and activation of ECs. We found no clear evidence that activation of platelets through nAChRα7 mediated stimulation elicited a cholinergic response. A significant EC Ca^{2+} response was still observed, which may be due to some of these other signalling molecules contained in the platelet releasate, but there was no effect of atropine to indicate any increase in ACh levels from control (**Figure 4.3.2A**). In consideration that platelets in our previous blood samples may have already been activated some point during their isolation, separate experiments were performed to examine platelet samples without activating them with PNU, and attempted to block their activation from the moment of collection by having a specific nAChRα7 antagonist (methyllycaconitine) present. However we again saw no change from control (**Figure 4.3.2B**). We noticed PRP activity alone was lower than +PNU 282987, suggestive of Ca^{2+} agonist release upon platelet nAChRα7 activation possibly reflecting granular contents. This was not shown to include a cholinergic component by lack of atropine sensitivity. The lack of difference between PRP and +methyllycaconitine indicates that nAChRα7 activation is not occurring in our blood samples, but the EC Ca^{2+} activity that was still observed (lower than +PNU 282987) may signify activation by other receptors that are not nAChRα7. Alternatively this could reflect platelet-endothelial interactions mediated by adhesion molecules (Siegel-Axel & Gawaz, 2007).

Alongside exploring whether ACh from platelet granules could influence ECs, these experiments provided additional evidence for vascular non-neuronal cholinergic systems, by demonstrating an effect of nAChR activation in platelets by the specific agonist PNU 282987 (**Figure 4.3.2A**).

4.4.iii Erythrocytes influence EC mAChRs but not by involvement of pannexin channels.

Previous reports have suggested measurement of ACh within erythrocytes (Kawashima *et al.*, 1989) and our prior data demonstrate erythrocytes as being able to influence EC Ca^{2+} (**Figure 3.3.5**) and that ACh or some ACh mobilising signal may be constitutively released into the blood plasma (**Figure 4.3.1**). We resolved to investigate any potential contribution of erythrocyte derived ACh or other EC Ca^{2+} agonists. Shown to exist in erythrocytes, pannexins form transmembrane channels that principally connect the intracellular and extracellular space to allow the passage of ions and small molecules and therefore are ideal for potential passage of ACh or other EC Ca^{2+} agonists. Pre incubation of blood samples with the pannexin channel blocker carbenoxolone (100 μM) did not affect the ability of blood to raise a Ca^{2+} response in ECs (**Figure 4.3.3**) suggesting involvement of another release mechanism. Additional experiments using inhibitors for other suggested channels would be informative in this regard. In addition, use of the choline acetyltransferase inhibitor bromoacetlycholine in samples of washed erythrocytes would help elucidate whether the cells are producing ACh directly.

In further experiments we also pre incubated ECs with carbenoxolone (100 μM) prior to blood application with similar effect (data not shown).

As experimental runs were performed immediately following the final wash procedure in our erythrocyte samples, the timescale between wash out of accumulated EC Ca^{2+} agonists and the apparent stimulation of EC Ca^{2+} by erythrocytes was very short (up to a few min). This implies that synthesis of any signal from erythrocytes responsible for the response in ECs is rapid. If the identity were to be proven as ACh, we would therefore expect that inhibition of erythrocyte AChE would result in accumulation of greater [ACh] in blood samples. Interestingly, a substantial portion of the response existed in spite of the presence of atropine and apyrase. These data suggest that erythrocytes are potentially a source of other agonists or signals that can convey an increase in EC Ca^{2+} and can be replenished by the erythrocytes upon their removal.

4.4.iv ACh does not accumulate from the blood under inhibition of AChE.

In spite of the presence of erythrocyte membrane bound AChE (Lawson & Barr, 1987; Paleus, 1947), numerous studies have demonstrated ACh within the blood, at levels capable of stimulating local cells (Fujii *et al.*, 1995; Hasegawa *et al.*, 1982; Kawashima & Fujii, 2000). In our hands, neostigmine, an AChE inhibitor, was only able to insignificantly increase the intensity of the EC Ca^{2+} response to blood and without potentiation in the duration of response. However this imitates the results of previous studies in which only insignificant increases in blood [ACh] were observed in the presence of neostigmine (Kawashima *et al.*, 1989). Although this paradoxical relationship is still the subject of investigation, several hypotheses have been proposed. One such suggestion is the AChE contained within the blood is a different isoform with different sensitivity to neuronal AChE (Wright & Plummer, 1973); it may allow for a considerable blood [ACh] but hydrolyses any excess, maintaining a

constant blood [ACh]. One group has suggested that whilst erythrocyte AChE is capable of hydrolysing sufficient quantities of ACh, this may not be its main function, instead being involved in the alteration of erythrocyte cell properties (de Almeida & Saldanha, 2010) as well as moderation of AChE enzymatic activity by band 3 phosphorylation status, showing significant decreases in enzymatic activity (Carvalho *et al.*, 2009). That neostigmine did not potentiate the response (**Figure 4.3.5**) would favour earlier suggestions of ACh being released in response to other stimuli from either the blood or endothelial cells rather than a constitutive release which would presumably see the concentration of ACh rise over time under AChE inhibition.

5.5 Conclusion

Data outlined in this chapter demonstrate that plasma samples, devoid of blood cells, raised EC Ca^{2+} at least in part by muscarinic receptors, demonstrable by the reduction in response caused by atropine antagonism. In order for ACh to be acting on EC mAChRs it is either present in the sample, having been released from blood cells, or some other signal is present that acts to later release ACh. This hypothetical ACh mobilising signal, whilst being present in the plasma may derive from erythrocytes.

In summary, the evidence does not support a quantal release mechanism from blood cells, but rather implicates ECs as a source of non-neuronal ACh, release of which may be stimulated by a messenger coming from the blood.

Chapter 5

Endothelial cells as a source of paracrine non-neuronal acetylcholine

5.1 Introduction

Our investigations outlined in **chapter 4** established that the ability to raise EC Ca^{2+} via muscarinic receptors did not require the presence of any blood cells, yet inhibition of acetylcholinesterase did not result in a build up of ACh within our samples. This seemed to suggest that ACh implicated in the response might not originate from the blood itself.

The alternative possibility remains that blood ACh is of endothelial origin, based on the detection of ChAT and vAChT mRNA (Haberberger *et al.*, 2000) and immunohistochemical localisation of ChAT and vAChT protein in endothelial cells of selected vascular beds (Kirkpatrick *et al.*, 2003), as well as instances of synthesis and release of ACh from cells in culture (Milner *et al.*, 1990). It remains possible therefore that the endothelium is a source of auto regulatory ACh. To operate as a non-neuronal cholinergic system, the endothelium would need to express vital cholinergic proteins to synthesise and release ACh in addition to simply exhibiting cholinergic receptors.

5.1.i Synthesis of non-neuronal ACh.

The chief indicator of a cholinergic tissue would be the ability to synthesise ACh, which is typically undertaken by the enzyme choline acetyltransferase (ChAT). Evidence for non-neuronal acetylcholine synthesis exists in different cell and tissue types including airway epithelia, immune cells, placenta and the bladder (Kawashima & Fujii, 2008), all occurring through choline acetyltransferase. Besides its expression, the activity of ChAT has been detected in primary cultures of human umbilical vein endothelial cells (Kirkpatrick *et al.*, 2003; Kirkpatrick *et al.*, 2001), bovine carotid artery (Kawashima *et al.*, 1990) as well as in isolated endothelial cells

from rat (Gonzalez & Santo-Benito, 1987) and bovine (Galea & Estrada, 1991) brain capillaries. Expression of elements from the cholinergic gene locus, which have been located in endothelial cells (Haberberger *et al.*, 2000), together with expression of CHT1 and AChE, is collectively good evidence that a tissue is authentically cholinergic (Mallet *et al.*, 1998; Weihe *et al.*, 1998).

By the same token an additional important indicator of a cholinergic tissue would be the ability to uptake choline necessary for the synthesis of ACh. Choline transport is demonstrated classically in two major systems classified by the degree of affinity for choline. Low affinity choline transporters are found throughout multiple tissues and via this mechanism, primarily supply choline for synthesis of phosphatidylcholine and other phospholipids in the cellular membrane (Meyer *et al.*, 1982). In contrast the high affinity choline uptake mechanism is associated with choline supply for ACh synthesis (Yamamura & Snyder, 1973) and is therefore less widely expressed, but it has been found in accordance with several tissues displaying non-neuronal cholinergic systems (Haberberger *et al.*, 2002; Hanna-Mitchell *et al.*, 2007; Pfeil *et al.*, 2003). The plasma membrane transporter responsible for high affinity choline uptake has been cloned and termed CHT1 (Apparsundaram *et al.*, 2000). Choline plasma levels are highly regulated at around ~10 μM (Bligh, 1952) in animals and humans; therefore circulating choline is an easily accessible substrate for vascular ACh synthesis. Indeed the high levels of circulating choline could reflect the high enzymatic activity of erythrocyte bound acetylcholinesterase (AChE; (Wright & Plummer, 1973), the major enzyme responsible for the degradation of acetylcholine (also not restricted to neurons) suggesting a high rate of ACh turnover.

5.1.ii Release of non-neuronal ACh

Much remains unknown about the release of non-neuronal ACh, in contrast to the neuronal exocytosis of ACh. The release mechanisms of non-neuronal ACh seems to be an area of divergence from the classical neuronal model involving vesicular exocytosis, which has been shown to be notably absent from some non-neuronal cholinergic systems (Fujii *et al.*, 1998; Hanna-Mitchell *et al.*, 2007; Yajima *et al.*, 2011) but present in others (Hao *et al.*, 2014; Kummer *et al.*, 2008). The vesicular acetylcholine transporter (VAChT), which packs acetylcholine into vesicles where it is stored until release, has not been as widely studied for its presence in endothelial cells as ChAT has. Reports differ with regards to its detection, and none have so far demonstrated its action. Rat and human pulmonary endothelial cells express VAChT mRNA, but protein expression could not be detected (Haberberger *et al.*, 2000) whereas in cultured human umbilical vein endothelial cells VAChT was suggested to be associated with the presence of intracellular vesicles (Kirkpatrick *et al.*, 2001).

Many of the studies that reported a lack of non-neuronal VAChT proposed a small number of alternative ACh release pathways. One such mechanism involves the cholinergic mediatophore, a proteolipid complex which supports Ca^{2+} dependent, low affinity neurotransmitter release on the presynaptic membrane and on synaptic vesicle membranes (Israel *et al.*, 1984). Mediatophore mimics the physiological quantal release of acetylcholine, including from non-neuronal cells (Cavalli *et al.*, 1991; Fujii *et al.*, 2012). As our investigation suggests the observed response is not dependent on extracellular Ca^{2+} and seems to release Ca^{2+} in *response* to ACh (amongst other agonists), the Ca^{2+} dependent action of mediatophore suggests non-participation.

Members of the solute carrier family (subfamily 22), organic cation transporters (OCT) mediate the transport of organic cations across the cell membrane (Grundemann *et al.*, 1994) and are widely expressed throughout the body, with the subtype OCT1 the most commonly expressed of the major OCT subtypes (Dresser *et al.*, 1999; Grundemann *et al.*, 1994). As an organic molecule with a permanent positive net charge at physiological pH, acetylcholine is a candidate for transport via OCTs, which has been demonstrated in separate non-neuronal cholinergic systems in the airway (Kummer *et al.*, 2008; Lips *et al.*, 2005), placenta (Wessler *et al.*, 2001), and colon (Bader *et al.*, 2014). The subtypes OCT1 and OCT3 have even been shown in brain microvessel endothelial cells and localised to the luminal membrane (Lin *et al.*, 2010), although their capacity to transport ACh was not the subject of investigation.

Taken together as described above, it seems likely that endothelial cells can express components necessary for cholinergic signalling to form a non-neuronal cholinergic system potentially capable of the synthesis of its own endogenous autoregulatory ACh.

The aim of the experiments in this chapter was to investigate the presence of vital cholinergic protein expression in endothelial cells that may constitute a non-neuronal cholinergic system. Following their localisation we sought to probe their contribution to the blood stimulated EC Ca^{2+} response through manipulation using pharmacological agents and therefore determine the extent of endogenous endothelial derived ACh.

5.2 Methods

*Refer to **Chapter 2: General methodology** for full details of the methods used in this chapter. Details relevant specifically to this chapter are given below.*

5.2.i Protocols for imaging Ca^{2+} in EC tubes

Third order EC tubes freshly isolated (as described in **section 2.4.i**) from rat mesenteric arteries were loaded with the Ca^{2+} sensitive dye OGB-1 for Ca^{2+} imaging (**section 2.4.ii**). Drugs were applied to the side of a static bath and mixed by trituration with a pipettor. Blood was applied as a 20 μl bolus directly over the EC tube. Blood was washed out immediately following the run and drugs were washed out for 20 min. Pre incubation was 20 min for atropine (1 μM), hemicholinium-3 (1 μM), corticosterone (100 μM) and quinine (300 μM) with choline (100 μM) incubated for 10 mins.

Immunohistochemistry procedures

Immunohistochemistry was carried out first in positive control tissue for VAChT, ChAT and CHT1 (cremaster muscle; Pierzga & Segal, 1994) and OCT1 (ileum; Girardin *et al.*, 2012) to ascertain that primary antibodies exhibited staining in tissue known to contain its target (**Figure 5.2.1**).

Immunohistochemistry was carried out first in pinned *en-face* rat mesenteric arteries for all studied antibodies (following the protocol outlined in **section 2.3.i.**). Following successful staining in pinned preparations, choline acetyltransferase and vesicular acetylcholine were selected for additional repeats in a pressurised artery that followed the protocol as described in **section 2.3.ii**.

Secondary only controls were carried out to certify secondary antibody did not exhibit non-specific binding of native proteins and therefore ensured that fluorescence signal detected reflected labelling of bound primary antibody only. No staining was shown in any plane of the artery wall in these control experiments (**Figure 5.2.2**).

Neuromuscular junction stain

Rat cremaster muscle was prepared as described in **section 2.1.3** and rinsed in Milli-Q purified water before immersion in 4^{o}C incubation medium for 8 hours in the dark to stain neuromuscular junctions (NMJs) by their presence of cholinesterase. The thiocholine ester present in the incubation medium is a substrate for hydrolysis by cholinesterase, with the liberated thiocholine and Cu^{2+} precipitating as copper thiocholine (Karnovsky & Roots, 1964). The tissue is washed in Milli-Q and then the copper thiocholine stain developed by covering with 1% ammonium sulphide for 2 min at room temperature. The **incubation medium** contained (in mM): acetylthiocholine iodide, 7.9; acetate buffer, 65; sodium citrate, 31; $CuSO_4$, 3; $K_3Fe(CN)_6$, 0.5.

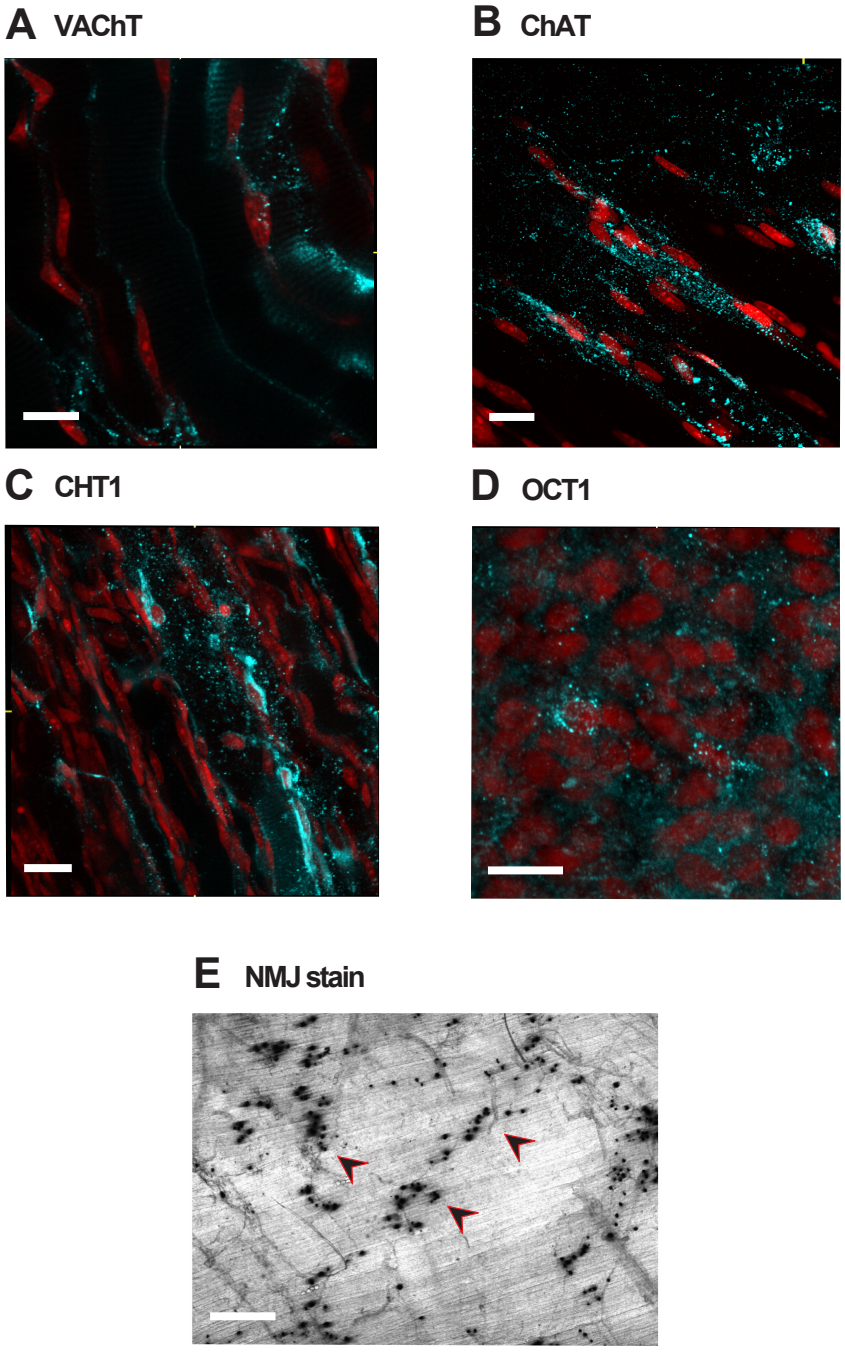


Figure 5.2.1 Positive control tissues for primary antibodies.
Staining was visible for all four primary antibodies (**A-D**; cyan) in cremaster muscle (**A-C**) and ileum (**D**). Nuclei are stained with propidium iodide (45 μM, red). Scale bars = 20 μm. Each image *n*=1. (**E**) Thiocholinesterase staining of neuromuscular junctions (dark spots; **arrows**) in rat cremaster muscle indicating cholinergic tissue. Scale bar = 500 μm, *n*=1.

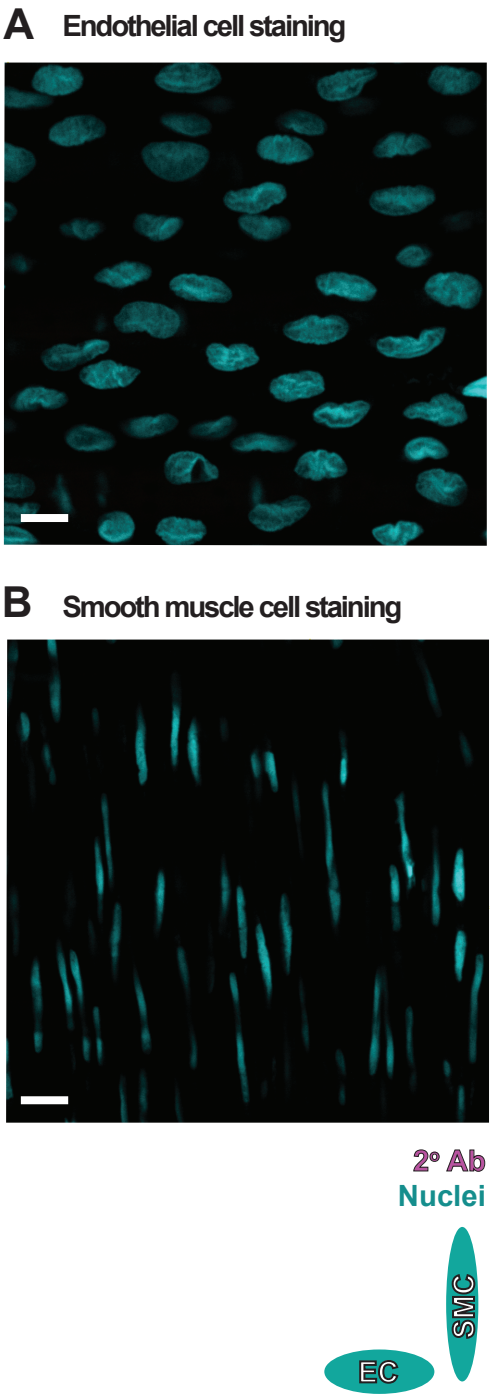


Figure 5.2.2 Lack of staining in secondary antibody alone controls.
Fluorescent signal from the secondary antibody (purple) was non-existent when applied alone in the absence of primary antibody in the endothelium (**A**) and smooth muscle (**B**) planes of rat mesenteric arteries. Nuclei are stained with propidium iodide (45 μM, cyan). Scale bars = 10 μm. Images are representative of *n*=2.

5.3 Results

In this chapter we sought to investigate whether the endothelium of rat mesenteric arteries could present a source of acetylcholine, which would then presumably act in an autocrine manner. Many studies in other vascular beds have located vital cholinergic system components to endothelial cells; thus to demonstrate the presence of such proteins in the rat mesentery, we performed immunohistochemistry to establish the existence and expression patterns of cholinergic machinery.

Freshly isolated endothelial cell tubes stimulated with blood exhibited increases in EC Ca^{2+} activity that were sensitive to atropine (**Figure 5.3.1**) confirming that this cholinergic response attributable to the blood is entirely EC specific.

To investigate the presence of ChAT in endothelial cells, immunohistochemistry was performed on pressurised rat mesenteric arteries using an antibody raised against the protein. As a positive control the primary antibody was first used in sections of rat cremaster muscle, which expresses ChAT within its skeletal neuromuscular junctions (**Figure 5.2.2B,E**). In rat mesenteric arteries, ChAT staining was visible in the endothelial cell layer (**Figure 5.3.2A**) and not the smooth muscle (**Figure 5.3.2B**). The staining was heterogeneous in its expression between cells with some cells exhibiting a modest amount of ChAT expression with others presenting considerably more (**Figure 5.3.2A, below, markers 1&2 respectively**). However staining in all cells seemed to be strongest surrounding the nucleus. An overlay of the internal elastic lamina (IEL) illustrates that the majority of staining occurs outside of IEL holes, suggesting that the protein is located within the main cell body and not strongly associated with myoendothelial signalling domains.

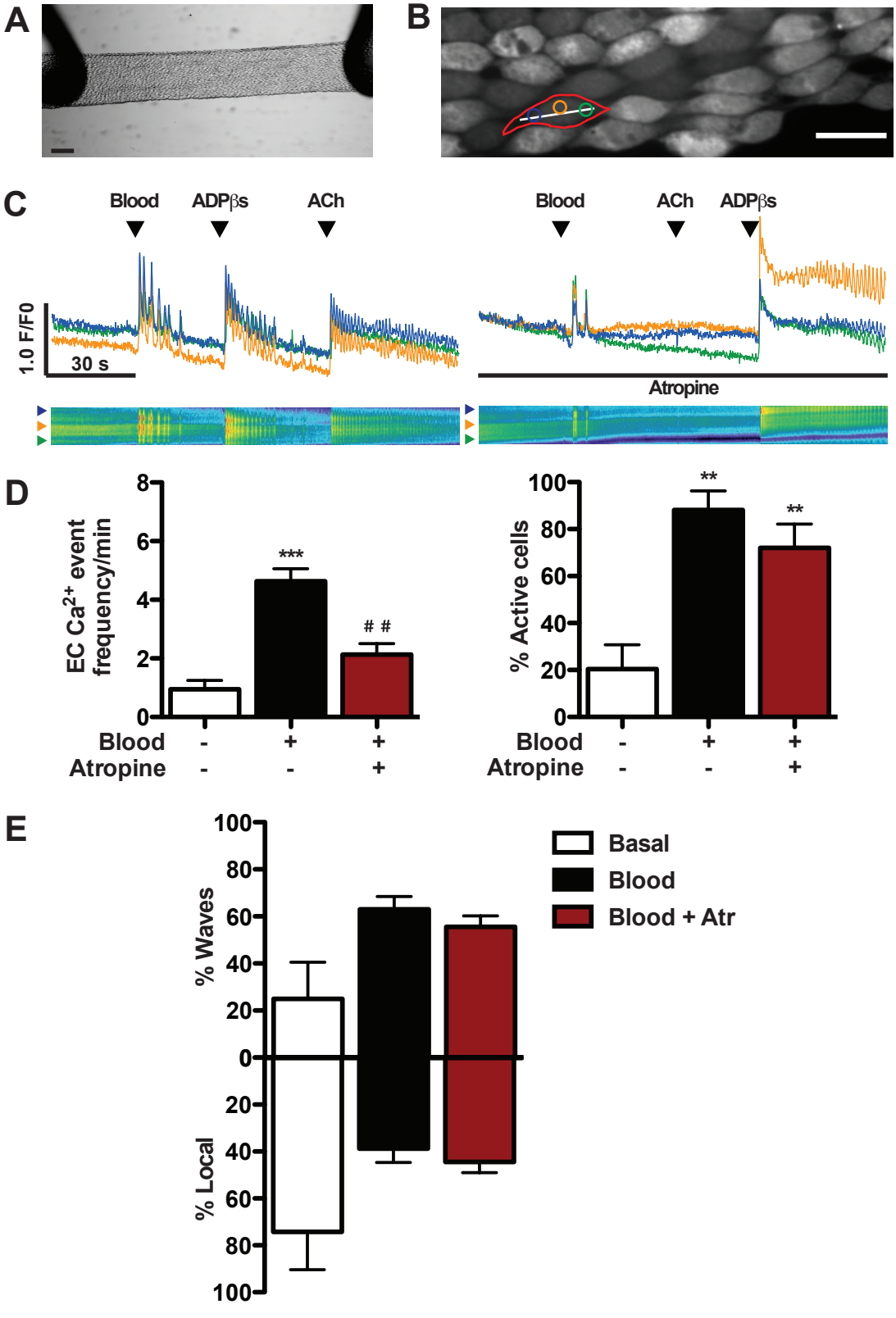


Figure 5.3.1 Atropine reduces endothelial cell tube Ca^{2+} in response to stimulation with whole blood.

Figure 5.3.1 Atropine reduces endothelial cell tube Ca^{2+} in response to stimulation with whole blood.

An endothelial cell tube (**A**) loaded with the Ca^{2+} indicator OGB-1 (**B**). Representative fluorescent intensity traces (**C**; corresponding to coloured ROIs in panel B) and linescan (**C**; corresponding to Ca^{2+} activity occurring along the white line over time in panel B) illustrating the effects of whole blood, ACh, and ADPβS in the absence and presence of atropine (**left**, **right** respectively). Summary data (**D**) showing the effects of blood on the frequency of EC Ca^{2+} activity per cell as well as the % of cells exhibiting changes in $[Ca^{2+}]_i$ in the absence and presence of atropine (1 μM, red). The shift from local Ca^{2+} events toward cell wide waves to blood is shown in panel **E**. (*n*=4; *P=<0.05, significantly different from control; $^{\#}$P<0.05, significantly different from matched data) by repeated measure ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

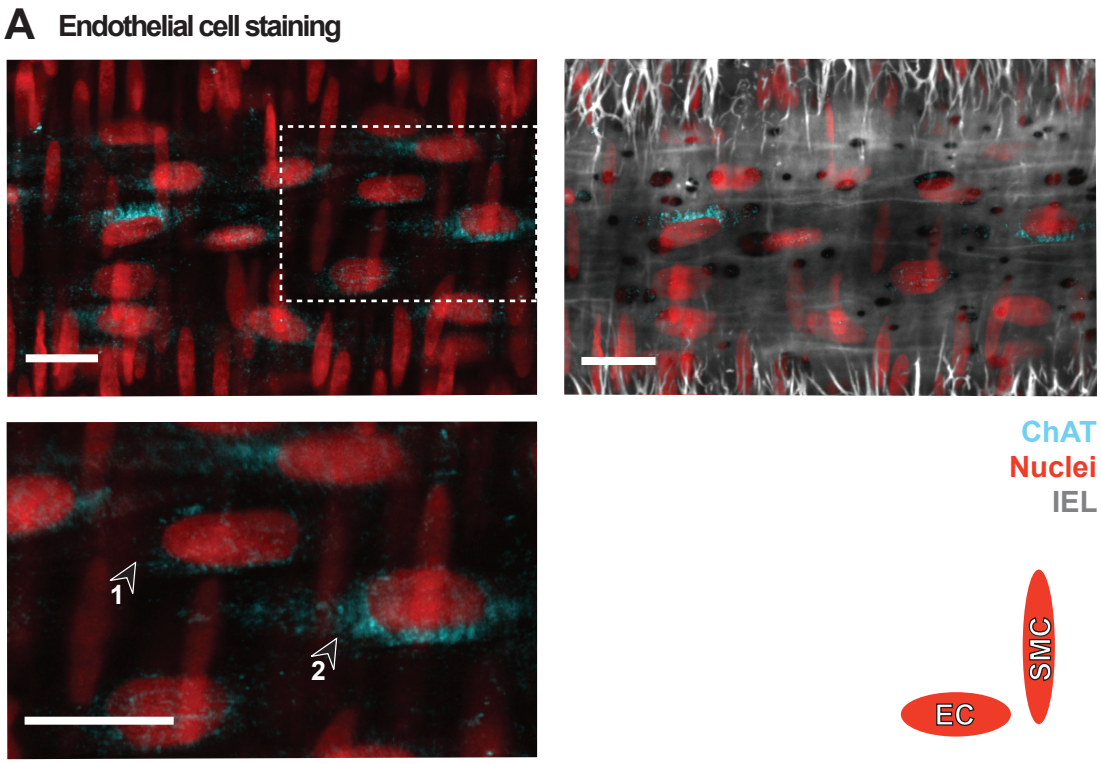


Figure 5.3.2 Expression of choline acetyltransferase (ChAT) in the endothelium of rat mesenteric arteries.

(**A**) Fluorescent immunohistochemical labelling of the acetylcholine synthesising enzyme choline acetyltransferase (ChAT; cyan) is visible in the endothelial cell layer (**top left**) of pressurised rat mesenteric arteries. Endothelial cell orientation is illustrated by nuclei staining with propidium iodide (45 μM, red) showing cells aligned horizontally and the overlay of internal elastic lamina (IEL), stained with Alexa 633 hydrazide (1 μM, grey, **top right**). An enlarged section of a few cells is provided (dotted, **below**) and demonstrates an example of the heterogeneous distribution of staining seen (cells marked **1** and **2**).

(**B**) The smooth muscle cell layer, with vertical nuclei demonstrating their perpendicular arrangement to ECs, was lacking staining for ChAT.

Scale bars = 20 μm. Images are representative of *n*=4.

As a vital part of ACh synthesis, and the rate limiting step in the neuronal cholinergic signalling pathway, we looked to focus on the uptake of choline, the pre-cursor of ACh and substrate for ACh synthesis. In order to produce ACh via ChAT, non-neuronal cells must have a reasonable supply of choline. As the concentration of choline in rat plasma is reported to be around 10 μM (Bligh, 1952), this could represent a significant source for both cells of the blood and the endothelium in the production of non-neuronal ACh.

Although all cells express low affinity choline uptake transporters for basic cell functions, we carried out immunohistochemistry to stain ECs for possible expression of the high affinity choline uptake transporter (CHT1) that is required for neuronal ACh synthesis. Again a positive control for the primary antibody was first established in rat cremaster muscle neuromuscular junction (**Figure 5.2.2C**). Staining was located all over rat mesenteric artery ECs such that the cell bodies could be identified suggesting high expression, including areas of punctate staining (**Figure 5.3.3A**). However CHT1 staining was not present in SMCs (**Figure 5.3.3B**).

Considering that choline uptake is considered the rate limiting step neuronally, we postulated that any excess available to endothelial cells from the circulation could be uptaken in response to stimulation by the blood and synthesised rapidly, resulting in autonomous ACh acting following *de novo* synthesis. This would fall in line with our observations in the previous chapter that a 'signal' (choline) may be present in the blood and plasma to drive ACh synthesis or release. By adding exogenous free choline (100 or 300 μM) to a freshly isolated endothelial cell tube (**Figure 5.3.4**) we tested the ability of available choline from the blood to drive *de novo* ACh synthesis in ECs as it were. It was found that excess free choline, as

available from the blood, was not sufficient to induce cholinergic EC Ca^{2+} activity as seen by stimulation with blood or plasma, at least on its own accord.

As it is likely that any ACh synthesised in the endothelium would originate from circulating choline, potentially after recycling from AChE hydrolysis of ACh, we investigated whether extracellular choline could represent the substrate for a source of ACh that was uptaken by ECs and stored until stimulated for release or the necessary 'signal' from the blood was present, explaining the lack of Ca^{2+} activity in **Figure 5.3.4**. Endothelial cell tubes were stimulated with whole blood both in the presence and absence of pre incubation with excess concentrations of choline (100 μM). In these experiments, choline significantly potentiated the EC Ca^{2+} response to whole blood. Subsequently this was repeated in the presence of the choline uptake transporter (CHT1) blocker, hemicholiunium-3, alongside the excess choline. Under these conditions the potentiation to choline was abolished, however a response of significant strength remained (**Figure 5.3.5**).

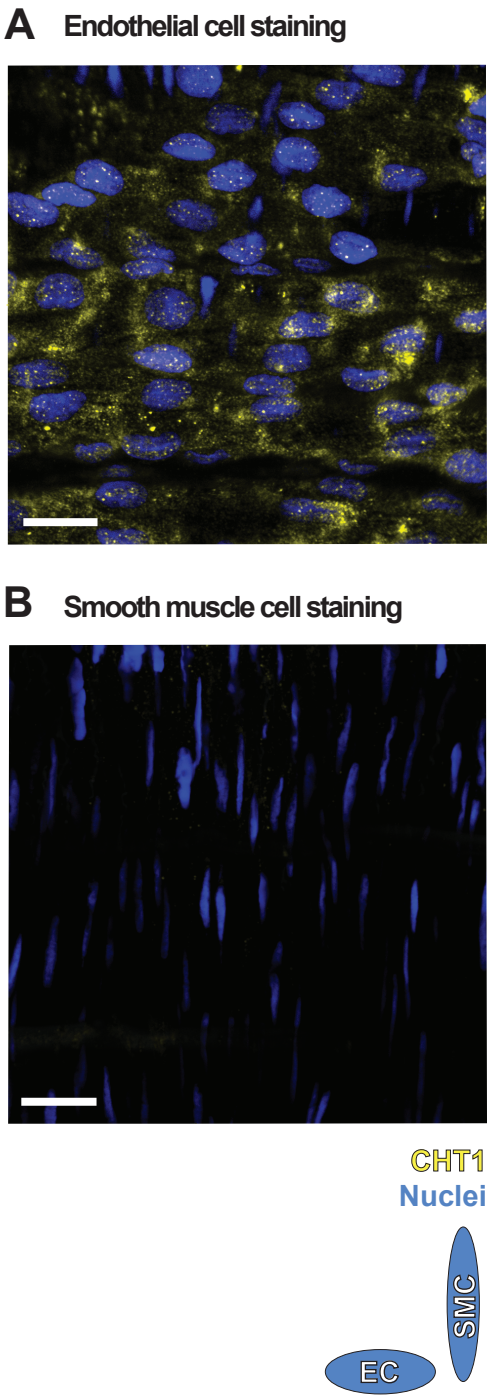


Figure 5.3.3 Expression of the high affinity choline transporter (CHT1) in the endothelium of rat mesenteric arteries.
Fluorescent immunohistochemical labelling of the high affinity choline transporter (CHT1; yellow) in the endothelial cell (**A**) and smooth muscle cell (**B**) layers of *en-face* rat mesenteric arteries. Nuclei stained with propidium iodide (45 μM, blue) showing endothelial cells aligned horizontally and smooth muscle cells oriented vertically.
Scale bars = 20 μm. Images are representative of *n*=2.

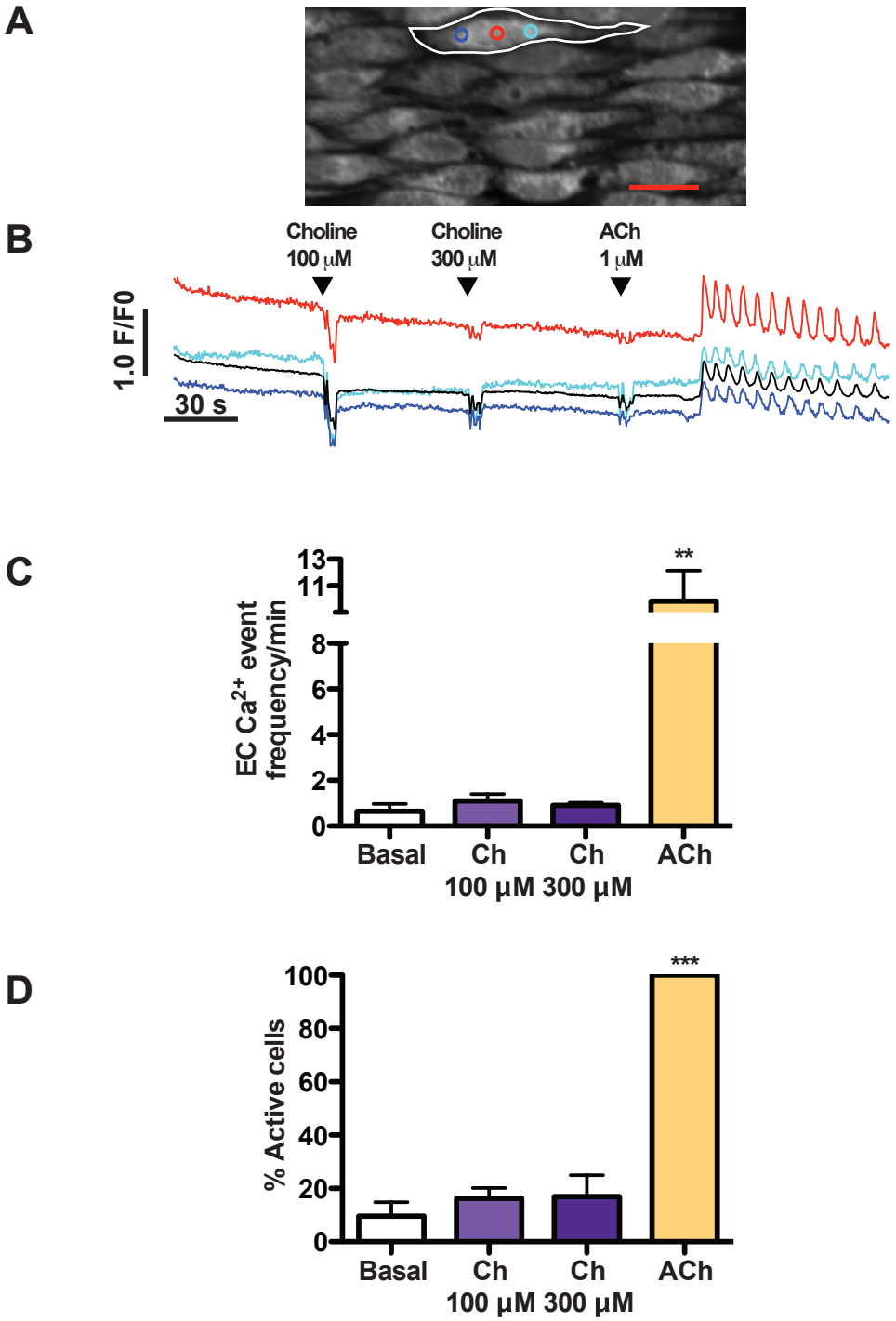


Figure 5.3.4 Extracellular choline does not increase EC Ca^{2+} events.
An endothelial cell tube loaded with the Ca^{2+} indicator OGB-1 (**A**; scale = 20 μm). Representative fluorescence intensity traces (**B**) and summary data (**C&D**) illustrate ECs do not increase $[Ca^{2+}]_i$ to choline (100, 300 μM) although cell responses to ACh (1 μM) are not lost. Paired experiments, *n*=3; *P<0.05, significantly different from control by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

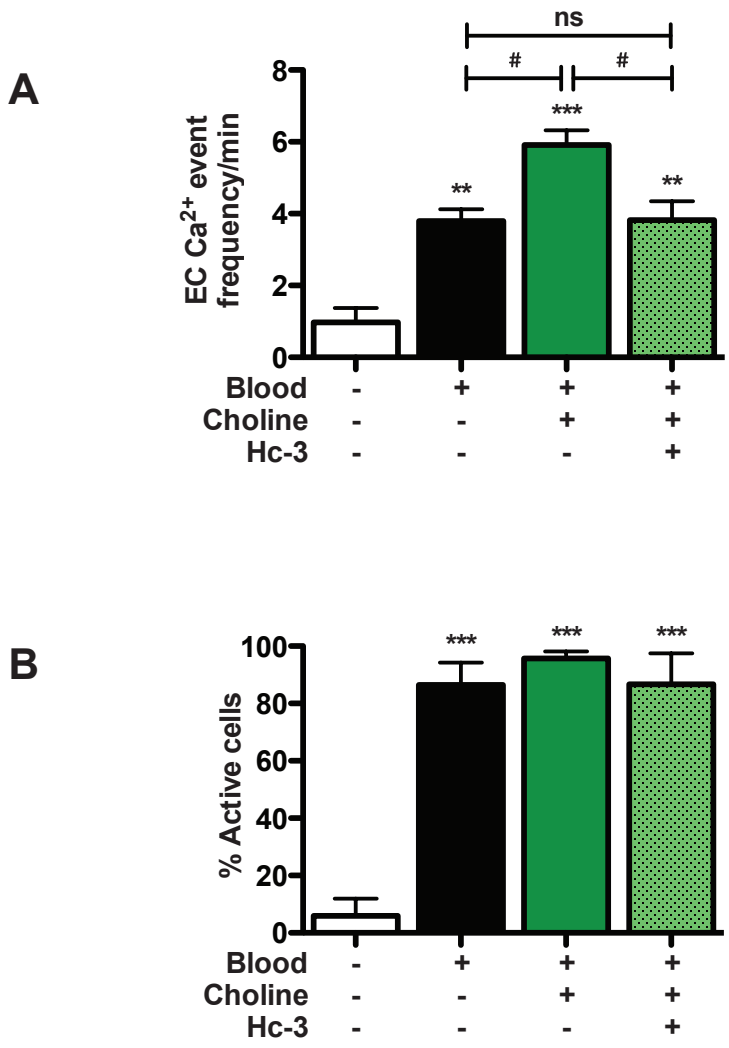


Figure 5.3.5 Choline potentiates a hemicholinium-3 sensitive increase in EC Ca^{2+} stimulated by blood.

An EC Ca^{2+} response in EC tubes stimulated with whole blood (20 μl bolus, black) was significantly increased by pre incubation of the EC tube with choline (100 μM, 10 mins, green). The potentiation of this response was inhibited by co-incubation of the CHT1 blocker hemicholinium-3 (1 μM, 20 mins, dotted) alongside the choline. Paired experiments, *n*=4; *P<0.05, significantly different from control by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

Having established the presence of ChAT and CHT1 in endothelial cells of rat mesenteric arteries and illustrated the potential importance of choline uptake in the response to blood, we investigated potential release mechanisms of any intracellular EC ACh. We used an antibody against VAChT to examine the classic, neuronal pathway utilising the involvement of vesicular exocytosis. As shown in (**Figure 5.3.6A**), dispersed staining of vesicular acetylcholine transporter is visible in the endothelial cell plane of a pressurised artery. A relatively uniform level of staining was observed throughout essentially all ECs in the field of view across the entire length of artery. Staining in most cells appeared more prominent, although not restricted to, the area surrounding the nucleus, with some cells seeming to exhibit more expression at the extremities, loosely outlining the cell shape. Although occasionally visible through holes when the IEL was overlayed, it is clear that this correlation is random as the majority of expression is spread along the cell and outside of areas beneath IEL holes. Furthermore in panels showing the z-plane, staining can be seen entirely on the endothelial side of the IEL (**Figure 5.3.6A**, **right**; **arrow**), even beneath IEL holes with no staining protruding into the planes below, indicating that VAChT is not expressed in EC projections into smooth muscle and that expression of the protein may be confined to the cytosol. Moreover no fluorescence signal was detected in any plane of the tunica media, indicating that smooth muscle cells do not express VAChT (**Figure 5.3.6B**), and intimating that regulation of protein expression is kept to specific cells in which it is functionally useful as opposed to being a product of the “near ubiquitous” non-neuronal cholinergic system.

As discussed in **section 5.1.ii**, ACh is believed to exit some cell types via specialised organic cation transporters in certain non-neuronal cells and tissues. We therefore investigated the possibility of an alternative ACh release mechanism, as

well as our evidence for VAChT expression, by performing immunohistochemistry to identify potential localisation of organic cation transporters. As OCT1 is reportedly the most common subtype of this family (and owing to a lack of available antibodies for OCT 2&3 predicted to react with rat tissue), we conducted immunohistochemistry of rat mesenteric arteries with an antibody targeting OCT1. Widespread punctate labelling demonstrated distinct expression throughout all endothelial cells in the field of view and along the length of the artery (**Figure 5.3.7A**). There was also OCT1 staining visible in the smooth muscle layer (**Figure 5.3.7B**).

Following this we studied a potential role for OCTs in mediating ACh release in response to stimulation of endothelial cell tubes with blood. As previously shown (**Figure 3.3.4**) whole blood stimulated a strong EC Ca^{2+} response in EC tubes. This was found to be significantly sensitive to the known OCT blockers corticosterone and quinine (100, 300 μM respectively). Subsequently pre incubation with the muscarinic receptor antagonist atropine (1 μM) had no further effect on the Ca^{2+} response (**Figure 5.3.8**).

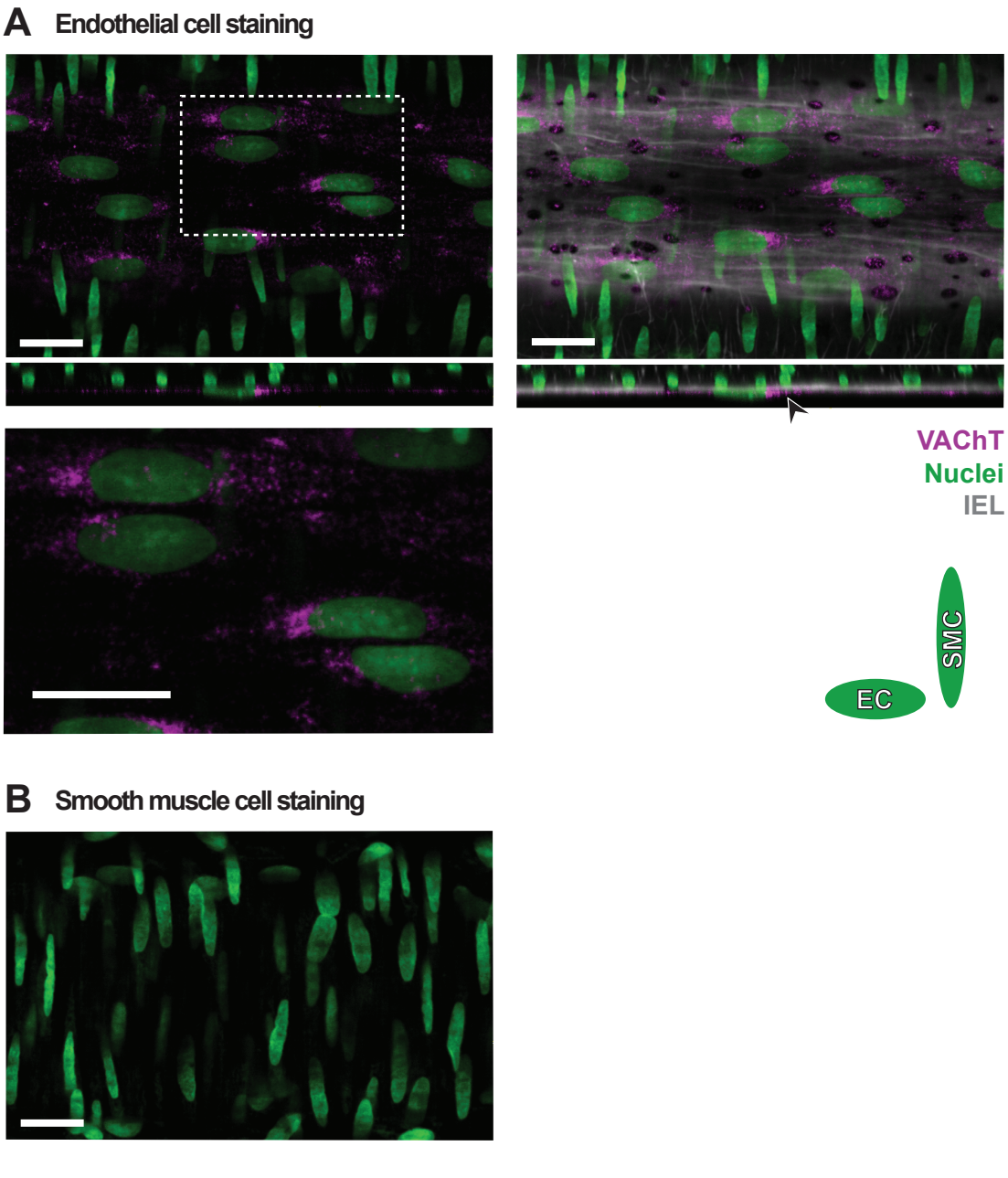


Figure 5.3.6 Expression of vesicular acetylcholine transporter (VAChT) in the endothelium of rat mesenteric arteries.

(**A**) VAChT fluorescence (purple) is visible in the endothelial cell layer (**top left**) of pressurised rat mesenteric arteries. Endothelial cell orientation is illustrated by nuclei staining with propidium iodide (45 μM, green) showing cells aligned horizontally. The overlay of internal elastic lamina (IEL), stained with Alexa 633 hydrazide (1 μM, grey, **top right**) demonstrates that in the reconstructed Z-plane image underneath, VAChT staining appears solely on the endothelial side of the IEL (arrow). An enlarged section of a few cells is provided (dotted, **below**).

(**B**) The smooth muscle cell layer, with vertical nuclei demonstrating their perpendicular arrangement to ECs, was devoid of any purple VAChT staining.

Scale bar = 20 μm, applicable to all images. Images are representative of *n*=3.

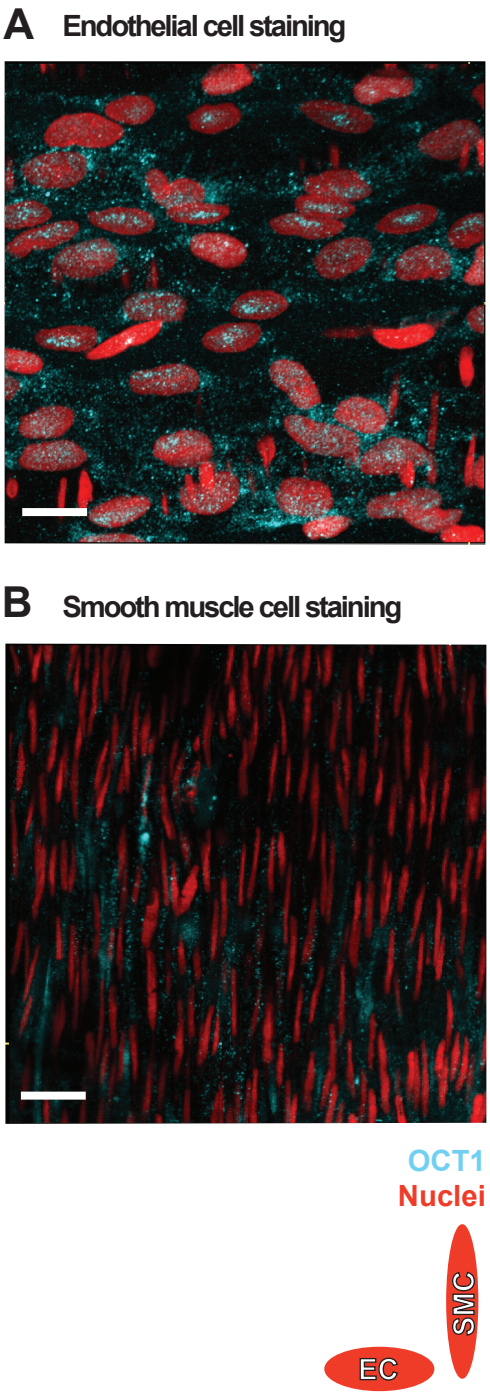


Figure 5.3.7 Expression of organic cation transporter 1 (OCT1) in endothelial and smooth muscle cells of rat mesenteric arteries.
Fluorescent immunohistochemical labelling of the membrane bound transporter OCT1 (cyan) in the endothelial cell (**A**) and smooth muscle cell (**B**) layers of *en-face* rat mesenteric arteries. Nuclei stained with propidium iodide (45 μM, red) showing endothelial cells aligned horizontally and smooth muscle cells oriented vertically.
Scale bars = 20 μm. Images are representative of *n*=2.

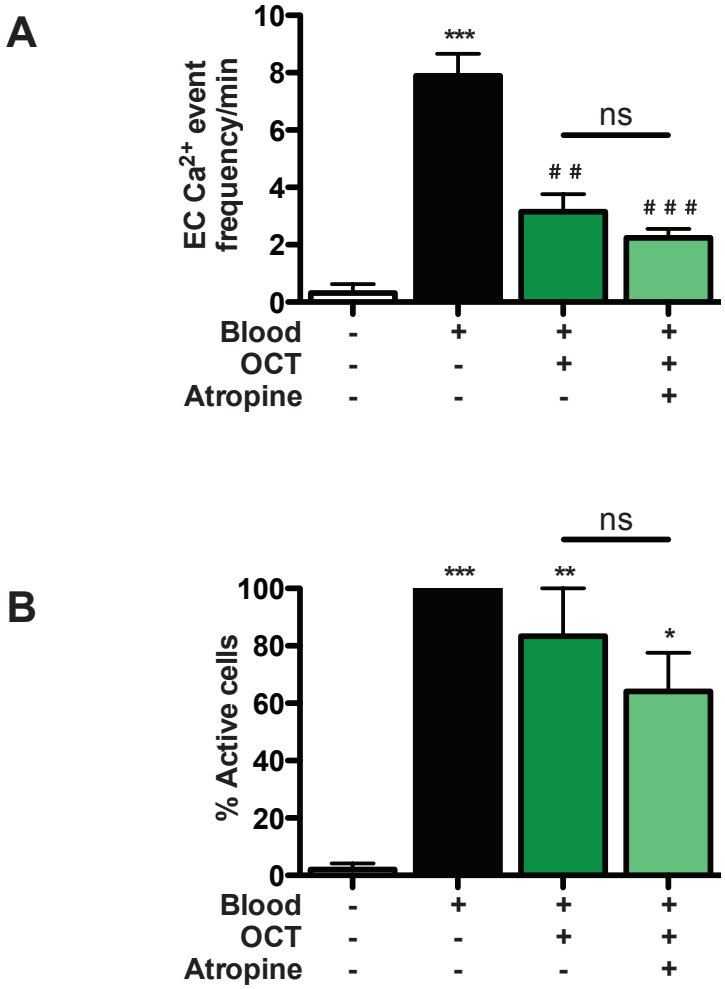


Figure 5.3.8 Organic cation transporter blockade in endothelial cell tubes reduces EC Ca^{2+} activity in response to stimulation with whole blood.

Summary data to show the frequency of EC Ca^{2+} events (**A**) and % of cells exhibiting Ca^{2+} activity (**B**) in isolated endothelial cell tubes in response to organic cation transporter (OCT) blockade. Application of blood in all conditions raised EC Ca^{2+} activity above basal levels, however pre-incubation of EC tubes with OCT blockers corticosterone (100 μM) and quinine (300 μM) caused a significant reduction. No further difference was observed in the presence of the muscarinic receptor antagonist atropine (1 μM). Paired experiments, *n*=4, *P<0.05 against basal (white), #P<0.05 against blood (black), by repeated measures ANOVA with Bonferroni post-tests to compare selected data sets as indicated.

5.4 Discussion

The data presented in this chapter demonstrate that:

1) Essential components of the cholinergic system (ChAT, CHT1 and VAChT) were localised in endothelial cells by immunohistochemistry indicating that endothelial cells may synthesize and release ACh.
2) Increased uptake of choline increased the EC Ca^{2+} response to blood in EC tubes, which could be inhibited by block of CHT1.
3) Pharmacological blockade of organic cation transporters, previously suggested to mediate ACh release in non-neuronal cells, reduced the EC Ca^{2+} response to blood in EC tubes.

The EC Ca^{2+} response generated when blood was applied over ECs was in part determined to be ACh mediated as the degree of Ca^{2+} activity could be reproducibly attenuated by muscarinic antagonism. Our demonstration of this response in endothelial cell tubes confirms that the response is EC specific (**Figure 5.3.1**). Results from **chapter 4** determined that ECs were likely to be the source of ACh implicated in the response to blood over originating from blood cells.

Previous studies have been limited in their study of non-neuronal cholinergic systems in endothelial cells. Many demonstrate the presence of particular enzymes in isolation and therefore do not establish a complete set of cholinergic proteins necessary to constitute an entire cholinergic system in the same vascular bed. The present work has provided evidence for the presence of a collection of essential cholinergic system machinery necessary to facilitate for uptake of substrate, synthesis, potential storage and release of ACh in rat mesenteric arteries, a commonly studied artery of the resistance vasculature.

5.4.i The ACh synthesising enzyme ChAT is expressed in rat mesenteric artery ECs.

Previous efforts to demonstrate immunohistochemical staining of ChAT in ECs have also largely remained constrained by their use of cultured cells. Endothelial cells in culture may result in the loss of specific functions or introduce new metabolic characteristics which are not present in ECs obtained from isolated vessels. The current body of work demonstrates the presence of ChAT within ECs from isolated rat mesenteric artery for the first time (**Figure 5.3.2**). Smooth muscle cells did not exhibit any ChAT staining, suggesting that the protein is not found ubiquitously and its expression is regulated to necessary cell types. The expression of ChAT would infer the endothelium may produce ACh and therefore is potentially a source of ACh that affects EC muscarinic receptors in an auto or paracrine fashion.

5.4.ii The high affinity choline transporter is expressed and is sensitive to hemicholinium-3 in rat mesenteric artery ECs.

The high affinity choline uptake mechanism is more targeted in its expression as unlike the low affinity uptake system, which is associated with choline supply for membrane phospholipid synthesis, it classically provides choline for ACh synthesis (ileum; Girardin *et al.*, 2012). The high affinity re-uptake of choline is essential to meet the demands for production of a new cycle of cellular ACh synthesis in cholinergic signalling. Our immunohistochemical localisation of CHT in ECs is therefore a significant advance in our understanding of non-neuronal cholinergic signalling in rat mesenteric arteries. The high affinity choline transporter (CHT1) has previously been demonstrated in endothelial cells. The endothelium of mouse brain capillaries (Sawada *et al.*, 1999), rat thoracic and human cerebral arteries (Estrada *et al.*, 1990) contain both the mRNA for and protein of CHT1, the authors

demonstrating hemicholinium-3 sensitivity of choline uptake, a key feature of the neuronal transporter. These findings are in accordance with ours, that ECs of rat mesenteric arteries express CHT1 and display sensitivity to hemicholinium-3 and taken together indicates that the endothelium may share a similar mechanism of choline uptake with neurons. However these previous reports, like those of ChAT, were all undertaken in cell culture and our experiments therefore demonstrate their presence in isolated rat arteries potentially for the first time.

In response to confirmation of CHT1 expression in ECs, choline alone was tested for its ability to initiate an EC Ca^{2+} response. Due to its distribution at high concentration throughout blood and plasma (Lips *et al.*, 2003), *de novo* synthesis of ACh upon exposure of ECs to choline offered a unifying factor to explain the results seen in **chapter 4**, where whole blood, erythrocytes, and plasma all raised an atropine sensitive cholinergic response. However choline alone was insufficient to generate a response (**Figure 5.3.4**), arguing against ACh involvement as a result of *de novo* synthesis.

The presence of CHT1 is corroborated by the ability of extracellular choline to potentiate the blood response in a hemicholinium-3 sensitive manner (**Figure 5.3.5**). This not only establishes active involvement of CHT1 in our model, but this uptake of choline is indirectly contributing to the EC Ca^{2+}, indicative of a cholinergic contribution to the response. This implicates the ChAT seen is perhaps also functional. Supplementary experiments utilising bromoacetylcholine, an inhibitor of ChAT, will be telling in this regard should its use also diminish a choline-enhanced response to EC stimulation with blood.

5.4.iii The vesicular acetylcholine transporter is present in rat mesenteric artery ECs.

We illustrate that endothelial cells of rat mesenteric arteries express immunolabelling for the enzyme vesicular acetylcholine transporter (VAChT; **Figure 5.3.6**). We noted that the profile of staining in ECs was comparable to that of ChAT (**Figure 5.3.2**) in that both were largely concentrated around the nucleus of each cell. The cholinergic locus is a phylogenetically conserved area of the genome responsible for encoding both ChAT and VAChT (Bligh, 1952). Correspondingly, mRNA and protein expression of VAChT and ChAT are often colocalised in cholinergic cells in order to facilitate packaging of ACh into vesicles rapidly following its synthesis (Eiden, 1998). Our chosen primary antibodies against VAChT and ChAT were both raised in rabbit, preventing double labelling studies. An alternative ChAT antibody was tested but was dismissed when it failed to express signal in our positive control tissue. Future double labelling would prove interesting, especially if a functional vesicular release mechanism can be revealed.

Vesicular release neuronally is evoked following Ca^{2+} influx through voltage gated calcium channels that open upon receipt of an action potential. Endothelial cell Ca^{2+} is mobilised in response to many stimuli. It is possible some modest local increase from intracellular stores (but not calcium influx, **Figure 3.3.6**) can raise Ca^{2+} sufficiently to evoke exocytosis, but no evidence has been shown for ACh release in response to EC activation by exogenous agonist stimulation. Although the exact pathway mediating exocytosis is unclear, evidence exists for endothelial vesicular signalling (Arvidsson *et al.*, 1997; Weihe *et al.*, 1998). Several agents have been implicated in disruption of vesicular trafficking. Vesamicol inhibits uptake of acetylcholine into vesicles by inhibiting VAChT (Bodin & Burnstock, 2001). We

initially chose not to test vesamicol in our EC response to blood stimulation as prevention of vesicular filling would also require depletion of the stored ACh before block was observed. It was suggested that prior initiation of the response may deplete pre exisiting stores, however this could not be confirmed. Instead we chose to target general vesicular trafficking pathways. Previous studies have shown that N-ethylmaleimide (NEM) targets NEM-sensitive factor (NSF), an essential ATPase involved in membrane fusion, thereby preventing vesicular exocytosis (Prior *et al.*, 1992). NEM has been shown to inhibit exocytosis of ATP containing vesicles in osteoblasts (Block *et al.*, 1988; Kuner *et al.*, 2008) and endothelial cells (Genetos *et al.*, 2005; Orriss *et al.*, 2009). However in pilot experiments, NEM appeared to have off target effects that severely damaged or inhibited cell function in our rat mesenteric arteries, as they were unable to properly constrict and dilate following NEM incubation (data not shown). Ideally botulinum toxin could be obtained in order to cleave soluble NSF attachment protein receptor (SNARE) complexes that also mediate vesicle fusion (Bodin & Burnstock, 2001) in order to test the involvement of ACh containing vesicles in ECs.

5.4.iv Organic cation transporter blockade inhibited cholinergic EC Ca^{2+} increases.

As discussed in **section 5.1.ii**, ACh is believed to exit some cell types via specialised organic cation transporters (OCT) in certain non-neuronal cells and tissues (Nigam & Nigam, 2010) and these OCTs have been previously shown in ECs, although not for their ability to transport ACh (Bader *et al.*, 2014; Kummer *et al.*, 2008; Wessler *et al.*, 2001). In this study we observed immunohistochemical labelling of OCT1 in rat mesenteric endothelial cells as well as smooth muscle cells. The pattern of endothelial cell expression appeared to be homogeneous, with

widespread staining throughout cell bodies and present throughout all cells viewed along the length of the vessel (**Figure 5.3.7**). Reliable primary antibodies targeting rat OCT subtypes 2-3 are not commercially available, therefore we were unable to also to utilise immunohistochemistry to probe the endothelial expression of these subtypes. However, it has been suggested that all subtypes (OCT1, 2 and 3) are pharmacologically sensitive to corticosterone and quinine (Lin *et al.*, 2010), and that OCT1 and possibly OCT3 are the most likely candidates for ACh transport (Alexander, 2013; Wessler *et al.*, 2001). It would however be interesting to further probe the importance of different OCT subtypes in ECs, both in terms of whether all, or only OCT1 are expressed on ECs, and which of these are involved in mediating passage of ACh.

Use of the known OCT inhibitors corticosterone and quinine significantly reduced the EC Ca^{2+} response induced by whole blood in isolated endothelial cell tubes (**Figure 5.3.8**), but still left a substantial response that remained above basal levels. When repeated in the presence of atropine as well as the OCT blockers, there was no further drop in EC Ca^{2+} activity, indicating overlapping pathways. Corticosterone and quinine are not thought to act as muscarinic receptor antagonists, indicating their reduction of EC Ca^{2+} in our preparations may be due to removal of ACh involvement altogether, and that organic cation transporters may be a vital mechanism for ACh release from ECs.

These data are suggestive of an ACh release mechanism via OCTs, however substrates may pass bi-directionally through OCTs, and their purpose in the passage of other organic cations should also be considered. OCTs may provide an alternate mechanism for choline uptake alongside CHT1 (Bader *et al.*, 2014; Wessler *et al.*, 2001) and it is possible that by inhibiting OCTs with corticosterone

and quinine, we are interfering with acetylcholine production within ECs. Further investigation will be necessary to determine whether this represents a primary or alternate pathway for ACh release, with immunohistochemical localisation of VAChT (**Figure 5.3.6**) suggestive of the capability to release ACh via vesicular exocytosis.

5.4.v Additional implications for an unknown ACh mobilising or inhibitory signal.

Given that EC Ca^{2+} activity was not observed during incubation with choline (up to 10 min; **Figure 5.3.4**) and Ca^{2+} activity was augmented only upon stimulation with blood (**Figure 5.3.5**), it seems that the blood probably regulates EC ACh release. This unknown ACh mobilising signal must also be present in plasma (**Figure 4.3.3**) or is a condition of the blood that is detectable by the endothelium (hypoxia, pH, hypothermia etc.) that acts through an as yet, undetermined mechanism. Physiologically, circulating choline is ever present (~10 μM; Bligh, 1952), providing a constant substrate for on-going ACh production. Depending on conditions required to invoke release, ACh turn over could feasibly be very high. This might mean that ACh needn't be confined solely to storage options; cytosolic ACh may be released via the opening of an ACh conduit channel (such as OCTs) as well as, or in place of, classic vesicular exocytosis, either of which could be stimulated by the unknown blood signal.

As blood flow, and as such, stimulus for ACh release is constantly present physiologically it remains to be investigated whether ACh is released constitutively or not, as suggested in several other non-neuronal cholinergic systems (Lin *et al.*, 2010). In this case, signals could have the opposite effect, instead regulating the pathway by feedback inhibition, for example the uptake of choline by CHT1 is

regulated by cytoplasmic ACh concentrations (Lips *et al.*, 2005; Wessler *et al.*, 2001). If OCTs prove to be the predominant release mechanism over vesicular exocytosis, noradrenaline has also been shown to inhibit OCTs via substrate inhibition (Jenden *et al.*, 1976), so perhaps ACh release is regulated by circulating catecholamine levels physiologically. In addition, a number of clinically prescribed drugs are substrates and potential inhibitors of OCTs and therefore may interfere with the release of non-neuronal acetylcholine (Wessler *et al.*, 2001).

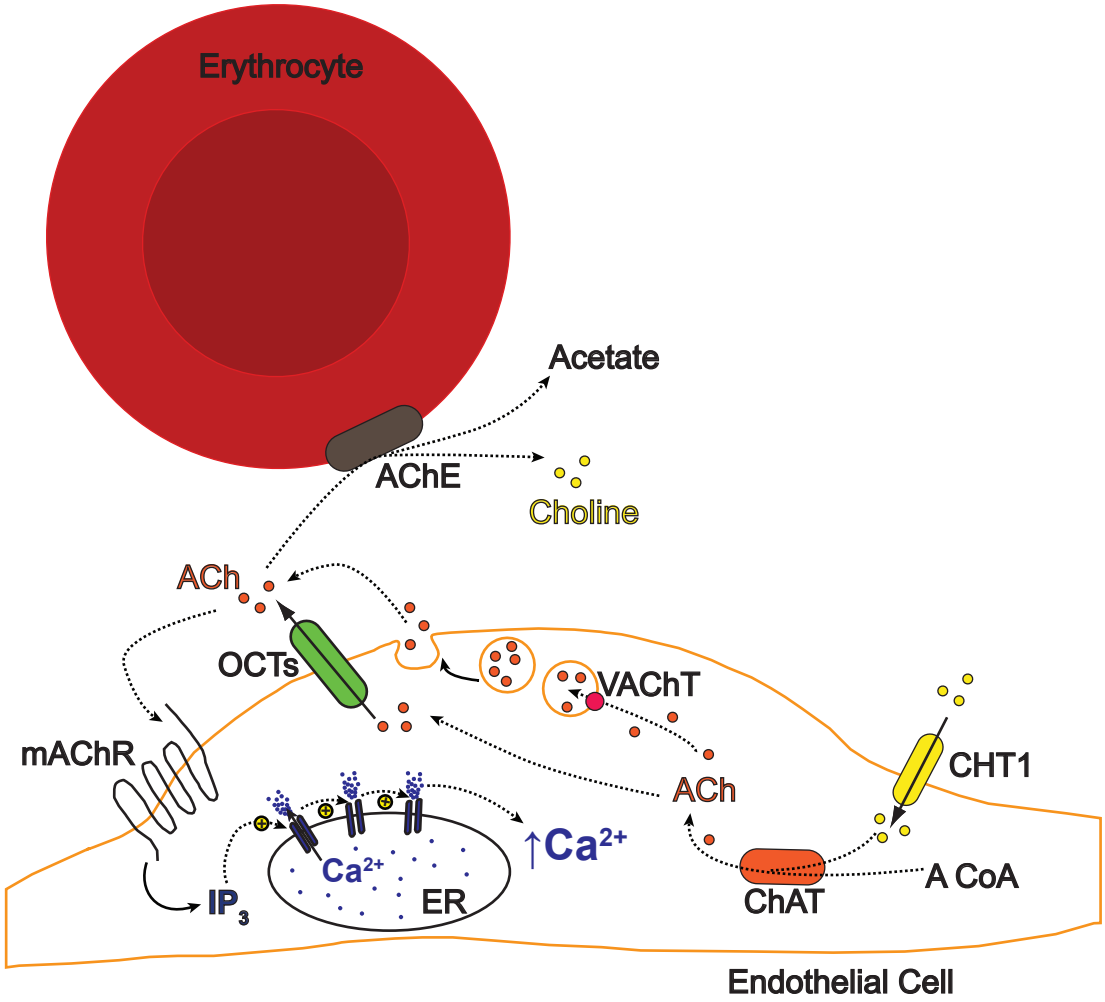


Figure 5.4.1 Proposed endothelial non-neuronal cholinergic signalling pathway. Schematic illustrating the hypothesised non-neuronal cholinergic signalling pathway in endothelial cells (ECs). Freely circulating choline (yellow) is uptaken into the cytosol of ECs by the high affinity choline transporter (CHT1). Choline and acetyl coenzyme A (A CoA) are substrates for the synthesis of acetylcholine (ACh; orange) by the enzyme choline acetyltransferase (ChAT). Cytosolic ACh may either leave cells via organic cation transporters (OCTs) or be packaged into storage vesicles by the enzyme vesicular acetylcholine transporter (VAChT). ACh release via exocytosis or OCTs is potentially stimulated in response to an acetylcholine mobilising signal received from the blood, the identity and mechanism of which are as yet unclear (not shown). Released ACh may act upon nearby ECs via action at muscarinic receptors, to raise IP_3 and mobilise Ca^{2+} from intracellular endoplasmic reticulum (ER) stores. This EC Ca^{2+} may then presumably initiate endothelium dependent hyperpolarisation and relaxation pathways to induce vasodilation (not shown).

5.5 Conclusion

Our major investigations in this chapter demonstrate the presence by immunolabelling of essential enzymes and transporters required to outline a complete non-neuronal cholinergic system in rat mesenteric arteries. This is the first time ChAT, VAChT and CHT1 have been located together in this tissue. Extracellular choline was demonstrated to indirectly increase EC Ca^{2+} in a hemicholinium-3 sensitive manner, suggestive of the uptake of choline via CHT1 and synthesis of ACh within isolated EC tubes. The share of EC Ca^{2+} activity shown to be atropine sensitive in response to blood stimulation was removed by blockade of organic cation transporters in EC tubes, implicating these as potential release pathways for endothelial ACh. Taken together the data are indicative of the ability for ECs to synthesise and release ACh that acts upon endothelial mAChRs thereby raising $[Ca^{2+}]_i$ in response to stimulation with blood. Although the signal that triggers release of ACh is as yet unknown, it is of great interest to present evidence for the action of this endogenous ACh and the physiological importance of endothelial mAChRs.

5.6 Future Directions

Taken together the data presented in this thesis provide evidence for a non-neuronal cholinergic system in the endothelium of rat mesenteric arteries. Although we provide immunohistochemical evidence for the presence of essential cholinergic proteins and demonstrable activity of endothelial CHT1, it would be advantageous to demonstrate functional activity of endothelial ChAT in particular, in future experiments. As an inhibitor of ChAT, and therefore ACh synthesis, bromoacetylcholine would inhibit increases in ACh production and potentially magnitude of cholinergic response, but likely not exhibit complete block owing to potential stored ACh. However this may be useful in informing whether ACh is indeed stored as part of the system. As our data indicate that the ACh response is potentiated by the presence of extracellular choline (**Figure 5.3.5**), it would expand our model by demonstrating that this could be inhibited by bromoacetylcholine (Brailoiu & der Kloot, 1996).

We also provide evidence for two forms of ACh release; vesicular exocytosis mediated by VAChT and through organic cation transporters (OCTs). As previously discussed (**section 5.4.iii**), it would be beneficial to inhibit the mechanism of vesicular exocytosis over vesicle filling through VAChT inhibition with vesamicol. This could reliably be achieved through use of botulinum toxin to cleave soluble NSF attachment protein receptor (SNARE) complexes that also mediate vesicle fusion (Bodin & Burnstock, 2001) in order to test the involvement of ACh containing vesicles in ECs. As well as being released from the endothelium via pannexin channels (Billaud *et al.*, 2014), ATP has been suggested to be released from the endothelium by vesicular exocytosis and inhibited by use of the agents monensin and brefeldin A (Bodin & Burnstock, 2001; Lim To *et al.*, 2015). Monensin is an

inhibitor of vesicular formation from the Golgi apparatus (Cecchelli *et al.*, 1986) and brefeldin A inhibits secretory protein transport to the Golgi apparatus in order to disrupt vesicular signalling (Misumi *et al.*, 1986). It would therefore be useful to revisit the disruption of vesicular trafficking in our models in order to confirm whether vesicular exocytosis has a role in the release of ACh from the endothelium. Furthermore, if successful, this could be used conjointly with OCT blockers to determine if both release pathways are applicable for ACh in ECs and which may be predominant.

The detection of ACh from endothelial cells in previous studies by high performance liquid chromatography (HPLC) (Kirkpatrick *et al.*, 2003; Kirkpatrick *et al.*, 2001) or radioimmunoassay (Kawashima *et al.*, 1990) could be adapted to measure ACh release from endothelial cell tubes in place of intact arteries to confirm endothelial cell specificity and has the advantage of coming from native ECs and not cultured cells. The ability to measure ACh in samples would also confirm that free ACh is not present in our plasma or washed erythrocyte samples to further validate our proposal that ACh may be released in response to an unknown blood signal.

The identity of a signal that provokes release of ACh from ECs remains elusive. Our investigation suggests this may be dependent on erythrocytes (**Figure 4.3.4**) but also transmissible to the plasma (**Figure 4.3.1**). Aside from some messenger produced by the blood, it is possible ECs are responding to detection of some other non-ligand based signal emanating from the blood. It may prove that ECs detect changes in arterial pH, temperature or pO_2 amongst other states and respond by releasing vasodilator agents including ACh.

Chapter 6

References

Abbracchio MP, & Burnstock G (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64**:** 445-475.

Alexander SPHB, H.E.; Faccenda, E.; Pawson, A.J.; Sharman, J.L.; Spedding, M.; Peters, J.A.; and Harmar, A.J. (2013). The Concise Guide to PHARMACOLOGY 2013/14: Transporters. Br J Pharmacol 170**:** 1706–1796.

Ando J, Komatsuda T, & Kamiya A (1988). Cytoplasmic calcium response to fluid shear stress in cultured vascular endothelial cells. In Vitro Cell Dev Biol 24**:** 871-877.

Anfossi G, & Trovati M (1996). Role of catecholamines in platelet function: pathophysiological and clinical significance. Eur J Clin Invest 26**:** 353-370.

Apparsundaram S, Ferguson SM, George AL, Jr., & Blakely RD (2000). Molecular cloning of a human, hemicholinium-3-sensitive choline transporter. Biochem Biophys Res Commun 276**:** 862-867.

Arvidsson U, Riedl M, Elde R, & Meister B (1997). Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J Comp Neurol 378**:** 454-467.

Bader S, Klein J, & Diener M (2014). Choline acetyltransferase and organic cation transporters are responsible for synthesis and propionate-induced release of acetylcholine in colon epithelium. European journal of pharmacology 733**:** 23-33.

Bagher P, Beleznai T, Kansui Y, Mitchell R, Garland CJ, & Dora KA (2012). Low intravascular pressure activates endothelial cell TRPV4 channels, local Ca^{2+} events, and IK_{Ca} channels, reducing arteriolar tone. Proc Natl Acad Sci U S A 109**:** 18174-18179.

Berridge MJ (1993). Inositol trisphosphate and calcium signalling. Nature 361**:** 315-325.

Berridge MJ (1997). Elementary and global aspects of calcium signalling. The Journal of experimental biology 200**:** 315-319.

Berridge MJ, Lipp P, & Bootman MD (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1**:** 11-21.

Billaud M, Lohman AW, Johnstone SR, Biwer LA, Mutchler S, & Isakson BE (2014). Regulation of cellular communication by signaling microdomains in the blood vessel wall. Pharmacol Rev 66**:** 513-569.

Bligh J (1952). The level of free choline in plasma. J Physiol 117**:** 234-240.

Block MR, Glick BS, Wilcox CA, Wieland FT, & Rothman JE (1988). Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. Proc Natl Acad Sci U S A 85**:** 7852-7856.

Bodin P, & Burnstock G (2001). Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 38**:** 900-908.

Boittin FX, Macrez N, Halet G, & Mironneau J (1999). Norepinephrine-induced Ca^{2+} waves depend on $InsP_3$ and ryanodine receptor activation in vascular myocytes. Am J Physiol 277**:** C139-151.

Boullin DJ, & Green AR (1972). Mechanisms by which human blood platelets accumulate glycine, GABA and amino acid precursors of putative neurotransmitters. Br J Pharmacol 45**:** 83-94.

Brailoiu E, & der Kloot WV (1996). Bromoacetylcholine and acetylcholinesterase introduced via liposomes into motor nerve endings block increases in quantal size. Pflugers Archiv : European journal of physiology 432**:** 413-418.

Brayden JE, & Bevan JA (1985). Neurogenic muscarinic vasodilation in the cat. An example of endothelial cell-independent cholinergic relaxation. Circ Res 56**:** 205-211.

Brini M, & Carafoli E (2000). Calcium signalling: a historical account, recent developments and future perspectives. Cell Mol Life Sci 57**:** 354-370.

Brunner F, & Kukovetz WR (1991). Radioligand binding to muscarinic receptors of bovine aortic endothelial cells. Br J Pharmacol 102**:** 373-380.

Bruzzone R, Hormuzdi SG, Barbe MT, Herb A, & Monyer H (2003). Pannexins, a family of gap junction proteins expressed in brain. Proc Natl Acad Sci U S A 100**:** 13644-13649.

Burnstock G (1978). A basis for distinguishing two types of purinergic receptors. In Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. eds Straub R.W., & Bolis L. Raven Press: New York, pp 107-118.

Burnstock G (1990). Local mechanisms of blood flow control by perivascular nerves and endothelium. J Hypertens Suppl 8**:** S95-106.

Burnstock G (2006). Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 58**:** 58-86.

Burnstock G, & Kennedy C (1985). Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 16**:** 433-440.

Carvalho FA, de Almeida JP, Freitas-Santos T, & Saldanha C (2009). Modulation of erythrocyte acetylcholinesterase activity and its association with G protein-band 3 interactions. J Membr Biol 228**:** 89-97.

Cavalli A, Eder-Colli L, Dunant Y, Loctin F, & Morel N (1991). Release of acetylcholine by Xenopus oocytes injected with mRNAs from cholinergic neurons. EMBO J 10**:** 1671-1675.

Cecchelli R, Cacan R, Porchet-Hennere E, & Verbert A (1986). Dilatation of Golgi vesicles by monensin leads to enhanced accumulation of sugar nucleotides. Biosci Rep 6**:** 227-234.

Chen G, Suzuki H, & Weston AH (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. Br J Pharmacol 95**:** 1165-1174.

Cohen KD, & Jackson WF (2005). Membrane hyperpolarization is not required for sustained muscarinic agonist-induced increases in intracellular Ca^{2+} in arteriolar endothelial cells. Microcirculation 12**:** 169-182.

Colden-Stanfield M, Schilling WP, Ritchie AK, Eskin SG, Navarro LT, & Kunze DL (1987). Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. Circ Res 61**:** 632-640.

Crane GJ, Gallagher N, Dora KA, & Garland CJ (2003). Small- and intermediate-conductance calcium-activated K^+ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. J Physiol 553**:** 183-189.

de Almeida JP, & Saldanha C (2010). Nonneuronal cholinergic system in human erythrocytes: biological role and clinical relevance. J Membr Biol 234**:** 227-234.

Dietrich HH, Ellsworth ML, Sprague RS, & Dacey RG, Jr. (2000). Red blood cell regulation of microvascular tone through adenosine triphosphate. Am J Physiol Heart Circ Physiol 278**:** H1294-1298.

Dora KA, Doyle MP, & Duling BR (1997). Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. Proc Natl Acad Sci U S A 94**:** 6529-6534.

Dresser MJ, Zhang L, & Giacomini KM (1999). Molecular and functional characteristics of cloned human organic cation transporters. Pharm Biotechnol 12**:** 441-469.

Duling BR, & Berne RM (1970). Propagated vasodilation in the microcirculation of the hamster cheek pouch. Circ Res 26**:** 163-170.

Duza T, & Sarelius IH (2004). Localized transient increases in endothelial cell Ca^{2+} in arterioles in situ: implications for coordination of vascular function. Am J Physiol Heart Circ Physiol 286**:** H2322-2331.

Edwards G, Dora KA, Gardener MJ, Garland CJ, & Weston AH (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 396**:** 269-272.

Eiden LE (1998). The cholinergic gene locus. J Neurochem 70**:** 2227-2240.

Emerson GG, & Segal SS (2000). Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. Circ Res 87**:** 474-479.

Estrada C, Bready J, Berliner J, & Cancilla PA (1990). Choline uptake by cerebral capillary endothelial cells in culture. J Neurochem 54**:** 1467-1473.

Falcone JC, Kuo L, & Meininger GA (1993). Endothelial cell calcium increases during flow-induced dilation in isolated arterioles. Am J Physiol 264**:** H653-659.

Fleming I, & Busse R (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 284**:** R1-12.

Fujii T, Takada-Takatori Y, Horiguchi K, & Kawashima K (2012). Mediatophore regulates acetylcholine release from T cells. J Neuroimmunol 244**:** 16-22.

Fujii T, Yamada S, Watanabe Y, Misawa H, Tajima S, Fujimoto K*, et al.* (1998). Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. J Neuroimmunol 82**:** 101-107.

Fujii T, Yamada S, Yamaguchi N, Fujimoto K, Suzuki T, & Kawashima K (1995). Species differences in the concentration of acetylcholine, a neurotransmitter, in whole blood and plasma. Neurosci Lett 201**:** 207-210.

Furchgott RF, Cherry PD, Zawadzki JV, & Jothianandan D (1984). Endothelial cells as mediators of vasodilation of arteries. J Cardiovasc Pharmacol 6 Suppl 2**:** S336-343.

Furchgott RF, & Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288**:** 373-376.

Furuichi T, Yoshikawa S, Miyawaki A, Wada K, Maeda N, & Mikoshiba K (1989). Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature 342**:** 32-38.

Galea E, & Estrada C (1991). Periendothelial acetylcholine synthesis and release in bovine cerebral cortex capillaries. J Cereb Blood Flow Metab 11**:** 868-874.

Garland CJ, Hiley CR, & Dora KA (2011). EDHF: spreading the influence of the endothelium. Br J Pharmacol 164**:** 839-852.

Genetos DC, Geist DJ, Liu D, Donahue HJ, & Duncan RL (2005). Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. J Bone Miner Res 20**:** 41-49.

Girardin M, Dionne S, Goyette P, Rioux J, Bitton A, Elimrani I*, et al.* (2012). Expression and functional analysis of intestinal organic cation/L-carnitine transporter (OCTN) in Crohn's disease. J Crohns Colitis 6**:** 189-197.

Gitterman DP, & Evans RJ (2001). Nerve evoked P2X receptor contractions of rat mesenteric arteries; dependence on vessel size and lack of role of L-type calcium channels and calcium induced calcium release. Br J Pharmacol 132**:** 1201-1208.

Gonzalez JL, & Santo-Benito FF (1987). Synthesis of acetylcholine by endothelial cells isolated from rat brain cortex capillaries. Brain Res 412**:** 148-150.

Gordon JL (1986). Extracellular ATP: effects, sources and fate. Biochem J 233**:** 309-319.

Grundemann D, Gorboulev V, Gambaryan S, Veyhl M, & Koepsell H (1994). Drug excretion mediated by a new prototype of polyspecific transporter. Nature 372**:** 549-552.

Haas TL, & Duling BR (1997). Morphology favors an endothelial cell pathway for longitudinal conduction within arterioles. Microvasc Res 53**:** 113-120.

Haberberger RV, Bodenbenner M, & Kummer W (2000). Expression of the cholinergic gene locus in pulmonary arterial endothelial cells. Histochem Cell Biol 113**:** 379-387.

Haberberger RV, Pfeil U, Lips KS, & Kummer W (2002). Expression of the high-affinity choline transporter, CHT1, in the neuronal and non-neuronal cholinergic system of human and rat skin. J Invest Dermatol 119**:** 943-948.

Hanna-Mitchell AT, Beckel JM, Barbadora S, Kanai AJ, de Groat WC, & Birder LA (2007). Non-neuronal acetylcholine and urinary bladder urothelium. Life Sci 80**:** 2298-2302.

Hanson MS, Stephenson AH, Bowles EA, Sridharan M, Adderley S, & Sprague RS (2008). Phosphodiesterase 3 is present in rabbit and human erythrocytes and its inhibition potentiates iloprost-induced increases in cAMP. Am J Physiol Heart Circ Physiol 295**:** H786-793.

Hao C, Huang ZH, Song SW, Shi YQ, Cheng XW, Murohara T*, et al.* (2014). Arterial baroreflex dysfunction impairs ischemia-induced angiogenesis. J Am Heart Assoc 3**:** e000804.

Hasegawa Y, Kunihara M, & Maruyama Y (1982). Determination of picomole amounts of choline and acetylcholine in blood by gas chromatography-mass spectrometry equipped with a newly improved pyrolyzer. J Chromatogr 239**:** 335-342.

Heemskerk JW, Farndale RW, & Sage SO (1997). Effects of U73122 and U73343 on human platelet calcium signalling and protein tyrosine phosphorylation. Biochim Biophys Acta 1355**:** 81-88.

Holland WC, & Greig ME (1952). The synthesis of acetylcholine by human erythrocytes. Arch Biochem Biophys 39**:** 77-79.

Horiuchi Y, Kimura R, Kato N, Fujii T, Seki M, Endo T*, et al.* (2003). Evolutional study on acetylcholine expression. Life Sci 72**:** 1745-1756.

Huestis WH, & McConnell HM (1974). A functional acetylcholine receptor in the human erythrocyte. Biochem Biophys Res Commun 57**:** 726-732.

Ignarro LJ, Byrns RE, Buga GM, & Wood KS (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 61**:** 866-879.

Israel M, Lesbats B, Morel N, Manaranche R, Gulik-Krzywicki T, & Dedieu JC (1984). Reconstitution of a functional synaptosomal membrane possessing the protein constituents involved in acetylcholine translocation. Proc Natl Acad Sci U S A 81**:** 277-281.

Jenden DJ, Jope RS, & Weiler MH (1976). Regulation of acetylcholine synthesis: does cytoplasmic acetylcholine control high affinity choline uptake? Science 194**:** 635-637.

Johns A, Lategan TW, Lodge NJ, Ryan US, Van Breemen C, & Adams DJ (1987). Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. Tissue Cell 19**:** 733-745.

Kansui Y, Garland CJ, & Dora KA (2008). Enhanced spontaneous Ca^{2+} events in endothelial cells reflect signalling through myoendothelial gap junctions in pressurized mesenteric arteries. Cell Calcium 44**:** 135-146.

Karnovsky MJ, & Roots L (1964). A "Direct-Coloring" Thiocholine Method for Cholinesterases. J Histochem Cytochem 12**:** 219-221.

Kawashima K, & Fujii T (2000). Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 86**:** 29-48.

Kawashima K, & Fujii T (2008). Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. J Pharmacol Sci 106**:** 167-173.

Kawashima K, Fujii T, Moriwaki Y, & Misawa H (2012). Critical roles of acetylcholine and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune function. Life Sci 91**:** 1027-1032.

Kawashima K, Oohata H, Fujimoto K, & Suzuki T (1989). Extraneuronal localization of acetylcholine and its release upon nicotinic stimulation in rabbits. Neurosci Lett 104**:** 336-339.

Kawashima K, Watanabe N, Oohata H, Fujimoto K, Suzuki T, Ishizaki Y*, et al.* (1990). Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett 119**:** 156-158.

Kirkpatrick CJ, Bittinger F, Nozadze K, & Wessler I (2003). Expression and function of the non-neuronal cholinergic system in endothelial cells. Life Sci 72**:** 2111-2116.

Kirkpatrick CJ, Bittinger F, Unger RE, Kriegsmann J, Kilbinger H, & Wessler I (2001). The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. Jpn J Pharmacol 85**:** 24-28.

Kohler R, Heyken WT, Heinau P, Schubert R, Si H, Kacik M*, et al.* (2006). Evidence for a functional role of endothelial transient receptor potential V4 in shear stress-induced vasodilatation. Arterioscler Thromb Vasc Biol 26**:** 1495-1502.

Kruse HJ, Grunberg B, Siess W, & Weber PC (1994). Formation of biologically active autacoids is regulated by calcium influx in endothelial cells. Arterioscler Thromb 14**:** 1821-1828.

Kummer W, Lips KS, & Pfeil U (2008). The epithelial cholinergic system of the airways. Histochem Cell Biol 130**:** 219-234.

Kuner T, Li Y, Gee KR, Bonewald LF, & Augustine GJ (2008). Photolysis of a caged peptide reveals rapid action of N-ethylmaleimide sensitive factor before neurotransmitter release. Proc Natl Acad Sci U S A 105**:** 347-352.

Lawson AA, & Barr RD (1987). Acetylcholinesterase in red blood cells. Am J Hematol 26**:** 101-112.

Ledoux J, Bonev AD, & Nelson MT (2008a). Ca^{2+}-activated K^{+} channels in murine endothelial cells: block by intracellular calcium and magnesium. J Gen Physiol 131**:** 125-135.

Ledoux J, Taylor MS, Bonev AD, Hannah RM, Solodushko V, Shui B*, et al.* (2008b). Functional architecture of inositol 1,4,5-trisphosphate signaling in restricted spaces of myoendothelial projections. Proc Natl Acad Sci U S A 105**:** 9627-9632.

Lew MJ, Rivers RJ, & Duling BR (1989). Arteriolar smooth muscle responses are modulated by an intramural diffusion barrier. Am J Physiol 257**:** H10-16.

Lim To WK, Kumar P, & Marshall JM (2015). Hypoxia is an effective stimulus for vesicular release of ATP from human umbilical vein endothelial cells. Placenta 36**:** 759-766.

Lin CJ, Tai Y, Huang MT, Tsai YF, Hsu HJ, Tzen KY*, et al.* (2010). Cellular localization of the organic cation transporters, OCT1 and OCT2, in brain microvessel endothelial cells and its implication for MPTP transport across the blood-brain barrier and MPTP-induced dopaminergic toxicity in rodents. J Neurochem 114**:** 717-727.

Lips KS, Pfeil U, Reiners K, Rimasch C, Kuchelmeister K, Braun-Dullaeus RC*, et al.* (2003). Expression of the high-affinity choline transporter CHT1 in rat and human arteries. J Histochem Cytochem 51**:** 1645-1654.

Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D*, et al.* (2005). Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol 33**:** 79-88.

Lipton MA, & Barron ES (1946). On the mechanism of the anaerobic synthesis of acetylcholine. J Biol Chem 166**:** 367-380.

Locovei S, Bao L, & Dahl G (2006). Pannexin 1 in erythrocytes: function without a gap. Proc Natl Acad Sci U S A 103**:** 7655-7659.

Lohman AW, Weaver JL, Billaud M, Sandilos JK, Griffiths R, Straub AC*, et al.* (2012). S-nitrosylation inhibits pannexin 1 channel function. J Biol Chem 287**:** 39602-39612.

Mallet J, Houhou L, Pajak F, Oda Y, Cervini R, Bejanin S*, et al.* (1998). The cholinergic locus: ChAT and VAChT genes. J Physiol Paris 92**:** 145-147.

McSherry IN, Spitaler MM, Takano H, & Dora KA (2005). Endothelial cell Ca^{2+} increases are independent of membrane potential in pressurized rat mesenteric arteries. Cell calcium 38**:** 23-33.

Mehta D, & Malik AB (2006). Signaling mechanisms regulating endothelial permeability. Physiol Rev 86**:** 279-367.

Mendoza SA, Fang J, Gutterman DD, Wilcox DA, Bubolz AH, Li R*, et al.* (2010). TRPV4-mediated endothelial Ca^{2+} influx and vasodilation in response to shear stress. Am J Physiol Heart Circ Physiol 298**:** H466-476.

Meyer EM, Jr., Engel DA, & Cooper JR (1982). Acetylation and phosphorylation of choline following high or low affinity uptake by rat cortical synaptosomes. Neurochem Res 7**:** 749-759.

Michiels C, Arnould T, & Remacle J (2000). Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions. Biochim Biophys Acta 1497**:** 1-10.

Mikoshiba K, Huchet M, & Changeux JP (1979). Biochemical and immunological studies on the P400 protein, a protein characteristic of the Purkinje cell from mouse and rat cerebellum. Dev Neurosci 2**:** 254-275.

Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, & Burnstock G (1990). Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. Proc Biol Sci 241**:** 245-248.

Miseta A, Bogner P, Berenyi E, Kellermayer M, Galambos C, Wheatley DN*, et al.* (1993). Relationship between cellular ATP, potassium, sodium and magnesium concentrations in mammalian and avian erythrocytes. Biochim Biophys Acta 1175**:** 133-139.

Misumi Y, Misumi Y, Miki K, Takatsuki A, Tamura G, & Ikehara Y (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J Biol Chem 261**:** 11398-11403.

Mulvany MJ, & Aalkjaer C (1990). Structure and function of small arteries. Physiol Rev 70**:** 921-961.

Nagao T, Illiano S, & Vanhoutte PM (1992). Heterogeneous distribution of endothelium-dependent relaxations resistant to NG-nitro-L-arginine in rats. Am J Physiol 263**:** H1090-1094.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ*, et al.* (1995). Relaxation of arterial smooth muscle by calcium sparks. Science 270**:** 633-637.

Nigam PK, & Nigam A (2010). Botulinum toxin. Indian J Dermatol 55**:** 8-14.

O'Neil RG, & Heller S (2005). The mechanosensitive nature of TRPV channels. Pflugers Arch 451**:** 193-203.

Orriss IR, Knight GE, Utting JC, Taylor SE, Burnstock G, & Arnett TR (2009). Hypoxia stimulates vesicular ATP release from rat osteoblasts. J Cell Physiol 220**:** 155-162.

Paleus S (1947). On the Localization of the Specific Cholinesterase in Human Blood. Archives of Biochemistry 12**:** 153-154.

Panchin Y, Kelmanson I, Matz M, Lukyanov K, Usman N, & Lukyanov S (2000). A ubiquitous family of putative gap junction molecules. Curr Biol 10**:** R473-474.

Pfeil U, Lips KS, Eberling L, Grau V, Haberberger RV, & Kummer W (2003). Expression of the high-affinity choline transporter, CHT1, in the rat trachea. Am J Respir Cell Mol Biol 28**:** 473-477.

Pierzga JM, & Segal SS (1994). Spatial relationships between neuromuscular junctions and microvessels in hamster cremaster muscle. Microvasc Res 48**:** 50-67.

Prior C, Marshall IG, & Parsons SM (1992). The pharmacology of vesamicol: an inhibitor of the vesicular acetylcholine transporter. Gen Pharmacol 23**:** 1017-1022.

Ray B, Bailey JA, Simon JR, & Lahiri DK (2012). High-affinity choline uptake (HACU) and choline acetyltransferase (ChAT) activity in neuronal cultures for mechanistic and drug discovery studies. Curr Protoc Neurosci Chapter 7**:** Unit 7 23.

Ringer S (1883). A further Contribution regarding the influence of the different Constituents of the Blood on the Contraction of the Heart. J Physiol 4**:** 29-42 23.

Rivers RJ, & Duling BR (1992). Arteriolar endothelial cell barrier separates two populations of muscarinic receptors. Am J Physiol 262**:** H1311-1315.

Rodriguez-Rodriguez R, Yarova P, Winter P, & Dora KA (2009). Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries. Br J Pharmacol 158**:** 1609-1620.

Rosenmeier JB, Hansen J, & Gonzalez-Alonso J (2004). Circulating ATP-induced vasodilatation overrides sympathetic vasoconstrictor activity in human skeletal muscle. J Physiol 558**:** 351-365.

Sawada N, Takanaga H, Matsuo H, Naito M, Tsuruo T, & Sawada Y (1999). Choline uptake by mouse brain capillary endothelial cells in culture. J Pharm Pharmacol 51**:** 847-852.

Schedel A, Thornton S, Schloss P, Kluter H, & Bugert P (2011). Human platelets express functional alpha7-nicotinic acetylcholine receptors. Arterioscler Thromb Vasc Biol 31**:** 928-934.

Siegel-Axel DI, & Gawaz M (2007). Platelets and endothelial cells. Semin Thromb Hemost 33**:** 128-135.

Socha MJ, Hakim CH, Jackson WF, & Segal SS (2011). Temperature effects on morphological integrity and Ca^{2+} signaling in freshly isolated murine feed artery endothelial cell tubes. Am J Physiol Heart Circ Physiol 301**:** H773-783.

Sonkusare SK, Bonev AD, Ledoux J, Liedtke W, Kotlikoff MI, Heppner TJ*, et al.* (2012). Elementary Ca^{2+} signals through endothelial TRPV4 channels regulate vascular function. Science 336**:** 597-601.

Sprague RS, Ellsworth ML, Stephenson AH, & Lonigro AJ (1996). ATP: the red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol 271**:** H2717-2722.

Sridharan M, Adderley SP, Bowles EA, Egan TM, Stephenson AH, Ellsworth ML*, et al.* (2010). Pannexin 1 is the conduit for low oxygen tension-induced ATP release from human erythrocytes. Am J Physiol Heart Circ Physiol 299**:** H1146-1152.

Sterling GH, & O'Neill JJ (1978). Citrate as the precursor of the acetyl moiety of acetylcholine. J Neurochem 31**:** 525-530.

Straub AC, Lohman AW, Billaud M, Johnstone SR, Dwyer ST, Lee MY*, et al.* (2012). Endothelial cell expression of haemoglobin α regulates nitric oxide signalling. Nature 491**:** 473-477.

Straub AC, Zeigler AC, & Isakson BE (2014). The myoendothelial junction: connections that deliver the message. Physiology (Bethesda) 29**:** 242-249.

Streb H, Irvine RF, Berridge MJ, & Schulz I (1983). Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306**:** 67-69.

Supattapone S, Worley PF, Baraban JM, & Snyder SH (1988). Solubilization, purification, and characterization of an inositol trisphosphate receptor. J Biol Chem 263**:** 1530-1534.

Tangsucharit P, Takatori S, Sun P, Zamami Y, Goda M, Pakdeechote P*, et al.* (2012). Do cholinergic nerves innervating rat mesenteric arteries regulate vascular tone? Am J Physiol Regul Integr Comp Physiol 303**:** R1147-1156.

Thompson RH, & Tickner A (1953). Cholinesterase activity of arteries. J Physiol 121**:** 623-628.

Tracey WR, & Peach MJ (1992). Differential muscarinic receptor mRNA expression by freshly isolated and cultured bovine aortic endothelial cells. Circulation research 70**:** 234-240.

Tsukahara T, Kassell NF, Hongo K, Vollmer DG, & Ogawa H (1989). Muscarinic cholinergic receptors on the endothelium of human cerebral arteries. J Cereb Blood Flow Metab 9**:** 748-753.

Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A*, et al.* (1996). Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. Proc Natl Acad Sci U S A 93**:** 8063-8067.

Walch L, Taisne C, Gascard JP, Nashashibi N, Brink C, & Norel X (1997). Cholinesterase activity in human pulmonary arteries and veins. Br J Pharmacol 121**:** 986-990.

Waldron GJ, & Garland CJ (1994). Effect of potassium channel blockers on L-NAME insensitive relaxations in rat small mesenteric artery. Can J Physiol Pharmacol 72 (Suppl. 1).

Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C*, et al.* (2002). P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol 40**:** 841-853.

Watanabe M, Kimura A, Akasaka K, & Hayashi S (1986). Determination of acetylcholine in human blood. Biochem Med Metab Biol 36**:** 355-362.

Weihe E, Schafer MK, Schutz B, Anlauf M, Depboylu C, Brett C*, et al.* (1998). From the cholinergic gene locus to the cholinergic neuron. J Physiol Paris 92**:** 385-388.

Wessler I, Kirkpatrick CJ, & Racke K (1998). Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 77**:** 59-79.

Wessler I, Kirkpatrick CJ, & Racke K (1999). The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 26**:** 198-205.

Wessler I, Roth E, Deutsch C, Brockerhoff P, Bittinger F, Kirkpatrick CJ*, et al.* (2001). Release of non-neuronal acetylcholine from the isolated human placenta is mediated by organic cation transporters. Br J Pharmacol 134**:** 951-956.

Whiteheart SW (2011). Platelet granules: surprise packages. Blood 118**:** 1190-1191.

Winter P, & Dora KA (2007). Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. J Physiol 582**:** 335-347.

Wright DL, & Plummer DT (1973). Multiple forms of acetylcholinesterase from human erythrocytes. Biochem J 133**:** 521-527.

Yajima T, Inoue R, Matsumoto M, & Yajima M (2011). Non-neuronal release of ACh plays a key role in secretory response to luminal propionate in rat colon. J Physiol 589**:** 953-962.

Yamamoto K, Korenaga R, Kamiya A, & Ando J (2000). Fluid shear stress activates Ca^{2+} influx into human endothelial cells via $P2X_4$ purinoceptors. Circ Res 87**:** 385-391.

Yamamura HI, & Snyder SH (1973). High affinity transport of choline into synaptosomes of rat brain. J Neurochem 21**:** 1355-1374.