Role of pannexins in vasculature

Alaa Hamed Habib (MBBS)

School of Life Sciences

University of Nottingham Medical School

U.K.

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This work is dedicated to

My father Dr Hamed habib

My mother Haifa al Habashi

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ABSTRACT FOR CONFERENCE

1. **Habib AH**, V Ralevic, WR Dunn. (2014). Pannexin Expression in Porcine Splenic Artery and Rat Aorta.7th Saudi Students Conference.Edinburgh International Conference Centre.
http://uksacb.org/ssc8/component/content/article/50-posters/172-medical-and-health-sciences-posters , 181.

2. **Habib AH**, V Ralevic, WR Dunn (2014). Pannexin inhibitors decrease responses to sympathetic nerve stimulation in porcine splenic arteries. BPS Winter Meeting 2014 Queen Elizabeth II Conference Centre London.
www.pA2online.org, 240P.

3. **Habib AH**, V Ralevic, WR Dunn. (2015). Pannexin inhibitors decrease responses to sympathetic nerve stimulation in porcine splenic arteries. 8th Saudi Students Conference. Queen Elizabeth II Conference Centre London.
http://uksacb.org/ssc8/component/content/article/50-posters/172-medical-and-health-sciences-posters, 248.

4. **Habib AH**, V Ralevic, WR Dunn. (2016). Pannexin channels do not influence endothelial function in porcine coronary arteries 9th Saudi Students. International Convention Centre (ICC) Birmingham Conference.
http://uksacb.org/ssc9/component/content/article/22-general/319-posters-medical-and-health-sciences, 832.

5. **Habib AH**, V Ralevic, WR Dunn. (2016). Pannexin channels do not influence endothelial function in porcine coronary arteries 7th European congress of pharmacology. Military Museum and Cultural Centre Istanbul.
http://readgur.com/doc/1547183/y-2016---7th-european-congress-of-pharmacology.P01.

6. **Habib AH**, V Ralevic, WR Dunn. (2016).The expression of pannexins in porcine splenic arteries. BPS Winter Meeting 2016 Queen Elizabeth II Conference Center London.
https://www.bps.ac.uk/BPSMemberPortal/media/BPSWebsite/Assets/Pharmacology-2016-programme-book.pdf,PB129

ABSTRACT

Pannexins are newly discovered proteins that were first discovered by Panchin in 2000. The pannexin family has three isomers, i.e. pannexin-1, pannexin-2, and pannexin-3. In 2011, Billaud *et al* suggested that pannexin1 channels contribute to the spread of vasoconstriction after activation of α_{1D}-adrenoceptors present on the surface of vascular smooth muscle cell (VSMCs) of thoracodorsal resistance arteries (TDA) isolated from mice. Phenylephrine acting upon the α_{1D}-adrenoceptors activated pannexin1 channels present in the cell to release ATP, which in turn activated P2Y receptors on neighbouring cells to produce a co-ordinated contractile response. This aim of this work was to further investigate the role of pannexin in the regulation of contractile responses in the vasculature. To this end, the present study examined the presence and function of pannexins in the porcine splenic artery (PSA) and the rat aorta (RA), in which α_{1A}- and α_{1D}-adrenoceptors are present respectively. The role of pannexin channels and ATP (via activation of P2 purinoceptors) in the response to exogenous NA-induced contractile responses in the PSA and the RA were investigated, as were responses to sympathetic nerve activation in the PSA. The involvement of pannexin channels was studied using several pannexin inhibitors, i.e. mefloquine (a non-selective pannexin inhibitor), probenecid (a selective pannexin1 inhibitor at low concentrations), carbenoxolone (a selective pannexin1 inhibitor) and

Brilliant Blue FCF (a selective pannexin1 inhibitor). Additionally, the involvement of ATP in NA-induced responses was examined using P2 purinoceptor antagonists (PPADS, suramin and NF449). Further experiments examined the role of pannexins in contributing to endothelium-dependent responses in a large vessel i.e. the porcine coronary artery (PCA).

The results showed that both pannexin1 and pannexin2 are present in the PSA and the RA. In the PSA, mefloquine and probenecid reduced the responses to both NA-induced contractions and the frequency-dependent response curves generated to sympathetic nerve stimulation, whereas carbenoxolone, suramin and PPADS had no effect on responses to either exogenous NA or those caused by activating the sympathetic nerves. In the RA, mefloquine and probenecid reduced the response to NA-induced contractions, whereas BB-FCF had no effect. Purinoceptor antagonists (suramin, PPADs and NF449) had no effect on responses mediated by either α_{1A}–adrenoceptors in the PSA or α_{1D}–adrenoceptors in the RA, arguing against the role of ATP (via activation of P2 receptors) in mediating NA-induced responses in either the PSA or the RA. Conflicting results were obtained, in some cases, upon the use of three different pannexin inhibitors. The most likely reason for this is that mefloquine demonstrated non-selective inhibitory actions on contractile responses since it was also shown to inhibit responses to KCl, 5-HT, U46619 (the thromboxane mimetic), and responses to re-addition of calcium to depolarised preparations, suggesting that it acts to block L-type Ca^{2+} currents. Both mefloquine and probenecid demonstrated non-selective inhibitory effects when used at relatively high concentrations. Therefore, mefloquine and probenecid should only be used in low concentrations as pannexin1 inhibitors. It has been suggested that pannexin proteins may be involved in mediating endothelium-derived hyperpolarizing factor (EDHF) responses (Gaynullina,

Shestopalov *et al* 2015). Bradykinin (BK) was shown to induce relaxation in PCA and to a lesser extent in the PSA after inhibition of NO-synthase and cyclooxygenase. The evidence for the involvement of pannexin in mediating an EDHF response was limited in both the PSA and the PCA, since neither carbenoxolone nor probenecid had any effect. While mefloquine reduced EDH-mediated responses to bradykinin in the PCA, the questions about its selectivity make this observation difficult to interpret.

The present work therefore provided some evidence for the involvement of pannexin channels in conducting responses to NA-induced α_1-adrenoceptor-mediated vasoconstriction in blood vessels in PSA and the RA, although great care must be taken in interpreting this data on the basis of a lack of selectivity of the pharmacological agents currently available as pannexin inhibitors. In addition, there was no evidence that activation of α_1-adrenoreceptors causes the release of ATP from inside cells to act as an intercellular messenger, leading to P2 receptor-mediated contractions.

ABBREVIATIONS

2-MeSATP	**2-methylthio-ATP**
5HT	**5-hydroxytryptamine**
ABC	**ATP-binding cassette**
ACh	**Acetylcholine**
Ado	**Adenosine**
ADP	**Adenosine-5'-diphosphate**
ANOVA	**Analysis of variance**
AMP	**Adenosine-5'-monophosphate**
AMP	**Adenosine-5'-monophosphate**
ANAPP3	**ATP receptor antagonist arylazido aminopropanol ATP**
APES	**3-Aminopropyltriethoxysilane**
ATP	**Adenosine tri phosphate**
BK	**Bradykinin**
BK_{Ca}	**Large conductance calcium activated potassium channels**
BSA	**ovine serum albumin**
cAMP	**Cyclic adenosine-3',5'-monophosphate**
CCRC	**Cumulative concentration curves**
CFTR	**Cystic fibrosis transmembrane conductance regulator**
cGMP	**Cyclic guanosine monophosphate**
CRC	**Concentration response curve**
CREB	**cAMP response element-binding protein**
DAG	**Diacylglycerol**
DSMO	**Dimethyl sulfoxide**

EDHF	**Endothelium-derived hyperpolarizing factor**
EDTA	**Ethylene diaminete traacetic acid**
EETs	**Epoxyeicosatrienoic acids**
EFS	**Electrical field stimulation**
eNOS	**Endothelial NOS**
ER	**Endoplasmic reticulum**
GJ	**Gap junctions**
Gly	**Glycosylation**
GPCRs	**G-protein-coupled receptors**
GTP	**Guanine triphosphate**
IEL	**Internal elastic lamina**
IK_{Ca}	**Intermediate conductance calcium activated potassium channels**
IL	**Interleukin**
iNOS	**Inducible nitric oxide synthase**
IP3	**Inositol trisphosphate**
IP3R	**1,4,5-inositol trisphosphate receptor**
KCa	**Calcium-activated potassium channels**
KIR	**Inwardly rectifying potassium channels**
L-NAME	**NG-Nitro-L-arginine Methyl Ester**
L-NMMA	**NG -Monomethyl-L-arginine**
MEGJ	**Myoendothelial gap junctions**
mRNA	**Messenger RNA**
NA	**Noradrenaline**
NAD^+	**Nicotinamide adenine dinucleotide**
NANC	**Non-adrenergic, non-cholinergic**
nNOS	**Neuronal NOS**
NO	**Nitric oxide**
NOS	**Nitric oxide synthase**
NPY	**Neuropeptide Y**
PBS	**Phosphate-buffered saline**
PEG	**Poly Ethylene Glycol**

PSA	**Porcine splenic artery**
PSS	**Physiological salt solution**
RA	**Rat aorta**
S.e.m	**Standard error of the mean**
SK_{Ca}	**Small conductance calcium-activated potassium channels**
TDA	**Thoracodorsal resistance arteries**
UDP	**Uridine-5'-diphosphate**
UTP	**Uridine-5'-triphosphate**
VRAC	**Volume-regulated anion channel**

Chapter 1

1. Introduction

1.1. Cardiovascular hemodynamics

The cardiovascular system is designed to carry out a variety of functions to maintain whole body homeostasis. These functions include the transport of gases, nutrients and waste products, the transport of hormones, the regulation of pH and osmolality and the maintenance of body temperature (Seeley *et al* 2008).

It is crucial that blood flow is able to meet the demands of different tissue under different metabolic conditions. To achieve this requirement, blood flow needs to be maintained appropriately, and the cardiovascular system has to provide continuous blood flow through the arteries and veins, driven by the pumping action of the heart.

Systemic arteries carry oxygenated blood from the heart to the capillaries where gas, nutrient and waste exchange occurs. The deoxygenated blood is then returned to the heart via the veins, which can then be delivered to the pulmonary circulation. Deoxygenated blood enters the pulmonary arteries and is circulated around the lungs to permit gas exchange. Subsequently, the pulmonary veins return the re-oxygenated blood to the heart.

Arteries and veins share a common structure in that they are comprised of three distinctive layers (Figure 1.1). The innermost layer (tunica intima) contains a single layer of endothelial cells. This layer appears flattened and smooth. Adjacent to the tunica intima is the tunica media which is a wall containing smooth muscle cells. At the edge of this layer lie the vascular sympathetic nerves (Rutishauser, 1994). The outer layer (tunica adventitia) contains collagen and elastin fibers, which provide the

arterial walls with strength and flexibility. Arteries have a much thicker tunica media and tunica adventitia than veins, allowing them to accommodate the high level of arterial blood pressure by providing extra strength and elasticity. The capillaries are the simplest among the blood vessels, and they contain just a single layer of endothelial cells surrounded by a basement membrane (Rutishauser, 1994).

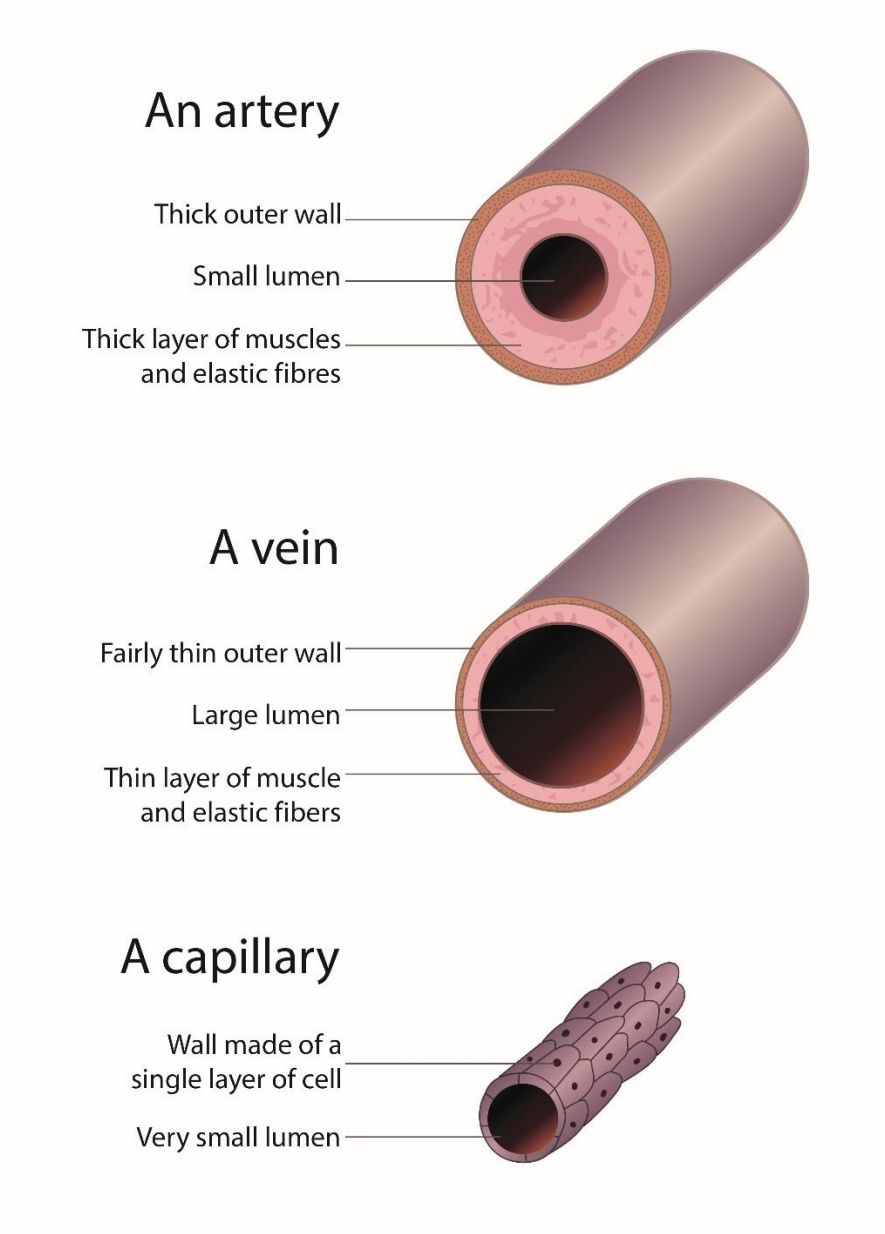


Figure 1.1 A diagram representing the comparison of blood vessels structure and functions. Adapted from Clancy & McVicar (2009).

1.2. Role of blood vessels in the regulation of systemic blood pressure

Blood pressure is the hydrostatic force that the blood exerts against the wall of the vessel. The arterial blood pressure is determined by the multiple of cardiac output and total peripheral resistance. Cardiac output is determined by heart rate and stroke volume. The latter is largely determined by the amount of blood returning to the heart, and is influenced by the force of contraction of cardiac muscle cells. The blood in veins travels at lower velocity and pressure compared to that in arteries. To facilitate the flow of the low pressure blood in veins, the contraction of the skeletal muscles squeezes the veins to increase the blood flow through them. Importantly, the extent of vasoconstriction and vasodilation of veins will influence venous return, ultimately impacting on cardiac output and blood pressure (McGeown, 2002; Seeley *et al* 2008).

The small arteries and arterioles are crucial in controlling peripheral resistance and hence in regulating blood pressure, thus the impedance offered by the arterioles induces the peripheral resistance and controls blood pressure. Vasoconstriction of arterioles increases peripheral resistance, whereas vasodilation reduces peripheral resistance and lowers blood pressure. This relationship has led to the primary focus of research on the cardiovascular system to principally assess arterial function. However, the blood vessel diameter of both arteries and veins is critical in regulating blood pressure. Several mechanisms are responsible for the control of vessel diameter including the sympathetic nervous system, circulating hormones such as those released by activation of the renin-angiotensin system and factors released from the vascular endothelium (Gordan *et al* 2015; Seeley *et al* 2008). A number of vasodilators are released from the endothelium as a result of physical stress or hormonal action. These

include prostacyclin, nitric oxide and endothelium-derived hyperpolarizing factor (EDHF), which cause relaxation of the vascular smooth muscle.

1.3. Sympathetic nerves

Sympathetic nerves store catecholamines in their terminal vesicles (Falck, 1962). The presence and distribution of sympathetic nerves has been demonstrated in different blood vessels in many species including terminal arterioles of the rat mesentery (Furness, 1973), monkey pulmonary arteries (El-Bermani, 1978), and rat superior mesenteric arteries and veins, where the adrenergic innervation was visualized by the Hillarp-Falck fluorescence technique, and by the immunohistochemical localization of immunoreactivity to tyrosine hydroxylase and dopamine-beta-hydroxylase. (Nilsson *et al* 1986). Furthermore, the retrograde fluorescent dye, fast blue, has also been used to identify the location of sympathetic neurons supplying mesenteric arteries of the rat (Sheppard, 1986). More recently the presence of dense sympathetic innervation in human mesenteric arteries and veins has been demonstrated using immunohistochemical staining coupled with electron microscopy (Birch *et al* 2008).

1.4. Sympathetic neurotransmission

Postganglionic sympathetic nerves activation produces biological effects via the release of neurotransmitters. Originally, it was thought that noradrenaline (NA) is the sole neurotransmitter released from sympathetic neurons, however, it was then reported that more than one neurotransmitter from the same neuron can also be released (Burnstock, 1976) (Figure 1.2). For example, adenosine triphosphate (ATP)

was implicated by Burnstock as a sympathetic neurotransmitter (Burnstock, 1972). In addition, it was shown that neuropeptide Y (NPY) is also released from the sympathetic nerves (Lundberg *et al* 1983). These discoveries prompted the development of the concept of co-transmission. Co-transmission is now a well-established concept in the central and peripheral nervous systems. However, the physiological significance of co-transmission is still not fully clear. It has been proposed that co-transmission allows a synergistic relationship between various neurotransmitters; for instance, a synergistic relationship was reported between NA as the main transmitter and ATP as a co-transmitter in rat mesenteric artery smooth muscle cells, and it has been shown that ATP can cooperate with NA to cause smooth muscle contraction in a synergistic manner (Ralevic and Burnstock, 1990).

It has also been shown that the release of cotransmitters can cause presynaptic neuromodulation and regulation of the release of the main transmitter. For instance, NPY inhibited the release of NA and ATP in the guinea-pig vas deferens (Ellis and Burnstock, 1990). In contrast, NPY enhanced the release of NA in rabbit blood vessels (Edvinsson *et al* 1984). Furthermore, it has been shown that NA release is favoured at higher impulse frequencies while NPY and ATP release is favoured at lower impulse frequencies of EFS in rat tail artery (Bradley *et al* 2003), which means that in response to different parameters of stimulation the presence of more than one neurotransmitter would allow different responses of nerves to take place.

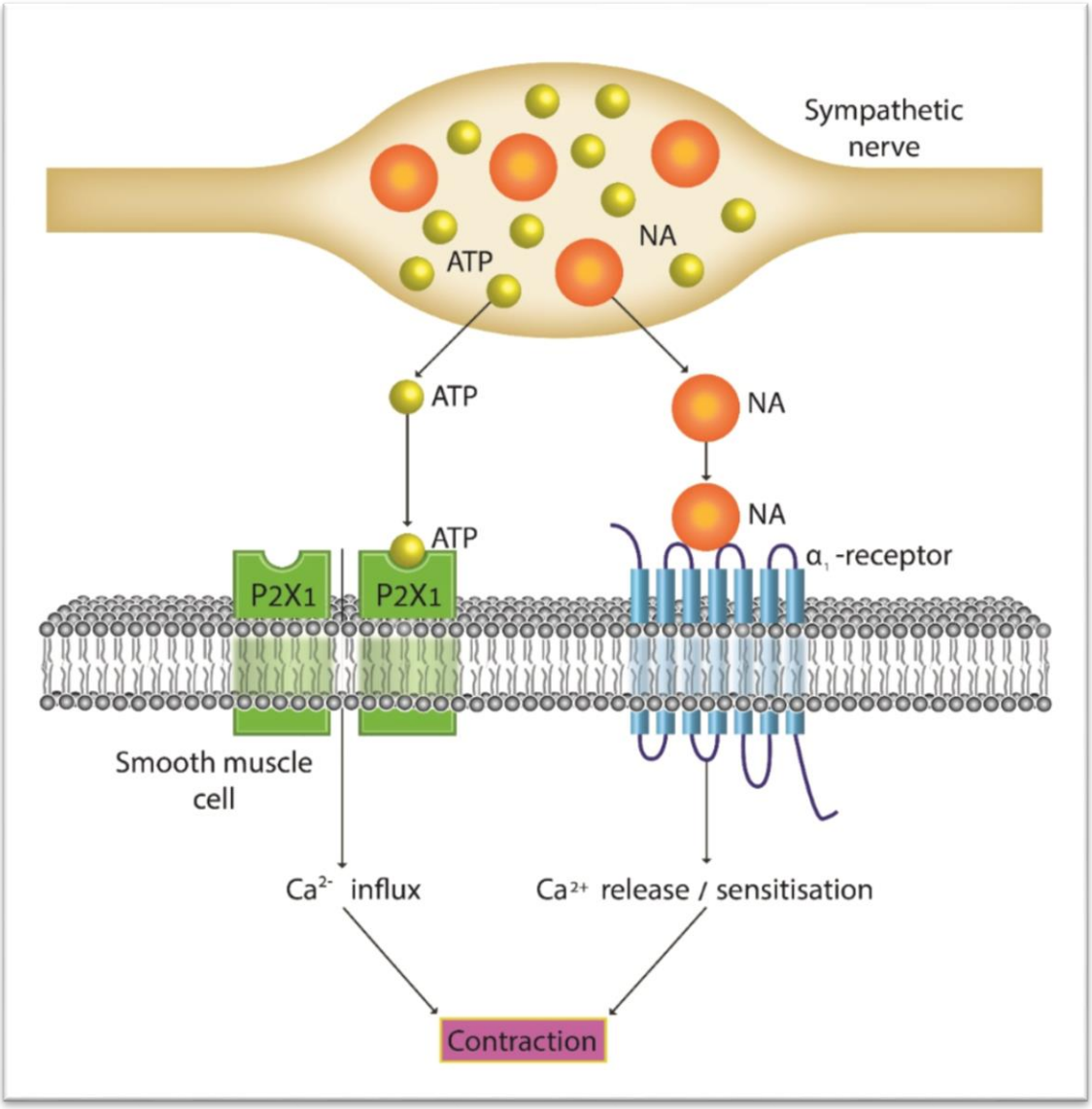


Figure 1.2 Diagram shows the release of different neurotransmitters including noradrenaline (NA) and ATP from sympathetic nerves, and their effect on different receptors; α_1- and P2X on the postsynaptic membrane to induce contraction. Adapted from (Kennedy *et al* 2013).

1.5. Noradrenaline

As mentioned above, activation of sympathetic nerves releases a number of neurotransmitters including NA. The involvement of catecholamines was first

proposed by Gaddum and Kwiatkowski, (1939) when exogenous adrenaline induced a similar response to that induced by the active substances released by adrenergic nerves in perfused rabbit ear and frog heart (Gaddum and Kwiatkowski, 1939). Subsequently, biochemical studies revealed the presence of enzymes responsible for NA synthesis in chromaffin cells and neurons, and that chromaffin cells and neurons were capable of storing and releasing catecholamines (Blaschko, 1957). Further experiments showed that NA synthesis was initiated from tyrosine in the homogenates of sympathetic nerve tissue, indicating that tyrosine acts as the main precursor of NA synthesis (Goodall and Kirshner, 1958). Tyrosine is transported by noradrenaline carriers into the cytoplasm of adrenergic neurons where it is converted to dopa by the activity of tyrosine-3-monooxygenase. Subsequently, L-amino acid decarboxylase converts dopa to dopamine, and the latter is transported into sympathetic vesicles where it undergoes hydroxylation to form NA (Blaschko, 1957). The release of NA from its storage vesicles appears in a Ca^{2+} dependent manner which occurs following activation of the terminal nerve by an action potential. Direct evidence for the release of NA was initially shown using the measurement of tritium released from tissues preloaded with [3H] NA in rabbit main pulmonary artery (Su and Bevan, 1970). Furthermore, the release of endogenous NA from sympathetic nerves has been measured using high performance liquid chromatography in rat tail artery and in the rat mesenteric artery (Buchholz and Duckles, 1992; Ralevic and Kendall, 2002). The majority of evidence which showed that NA is the main sympathetic neurotransmitter in blood vessels was based on pharmacological studies. For instance, responses to sympathetic nerve stimulation were abolished by the α_1-adrenoceptor antagonist prazosin, providing evidence that NA was the main neurotransmitter in guinea-pig, rat and rabbit small blood vessels (Angus *et al* 1988).

1.6. Adrenoceptors

Originally, adrenoceptors were described as the hypothetical structures located near the muscle or gland cells affected by adrenaline (Dale, 1906). Then researchers recognised two different groups of adrenoceptors for which the endogenous ligands were catecholamines (Ahlquist 1948). They attributed the variation in tissues response to these two groups rather than variations of neurotransmitters. These two groups were classified as alpha (α) and beta (β) based on their pharmacological responses (Ahlquist 1948); the discovery of dichloroisoprenaline, which was the first agent capable of antagonising β-adrenoceptor-mediated responses selectively, confirmed the existence of α and β subtypes (Powell and Slater, 1958).

1.6.1. α-adrenoceptors

There are two major classes of α-adrenoceptors. α_1-adrenoceptors are activated by phenylephrine, methoxamine and blocked by prazosin, while α_2-adrenoceptors are activated by UK-14,304 or BHT933 and blocked by BRL44408 (Alexander *et al* 2009). The use of cloning methods has shown three further subtypes of α_1-adrenoceptors; α_{1A}, α_{1B} and α_{1D} (Hieble *et al* 1995) and three subtypes of α_2-adrenoceptors; α_{2A}, α_{2B} and α_{2C} (Kobilka *et al* 1987; Regan *et al* 1988; Lomasney *et al* 1990).

α-adrenoceptors function

α_1-adrenoceptors couple to $G_{q/11}$ protein, activation of which results in stimulating phospholipase C enzyme which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate producing inositol triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 and DAG act as second messengers mediating intracellular Ca^{2+} release from intracellular

stores (Zhong and Minneman, 1999). Activation of α_2-adrenoceptors, which are primarily coupled to G_i-protein, results in inhibiting adenylyl cyclase which induces a reduction in intracellular adenosine 3',5'-cyclic monophosphate (cyclic AMP) production (Bylund *et al* 1994; Wise *et al* 1997).

In terms of their roles in the vasculature, postsynaptic α_1-adrenoceptors have been primarily shown to mediate contractile responses to sympathetic nerve activation, which was demonstrated in a number of blood vessels in different species. For instance, electrically-evoked contractile responses were sensitive to α_1-adrenoceptors antagonism in rabbit hindlimb (Madjar *et al* 1980), isolated pulmonary artery of the rabbit (MacLean *et al* 1993) and horse penile resistance arteries (Simonsen *et al* 1997). On the other hand, a role for α_2-adrenoceptors in mediation of postjunctional responses to the α_2-adrenoceptor agonist UK 14304 has been reported in small resistance vessels including human subcutaneous resistance vessels (Nielsen *et al* 1989). By contrast, in large arteries, activation of α_2-adrenoceptors required experimental manipulation; increasing of perfusion pressure by arginine vasopressin in the isolated vascular bed of the rat tail (Templeton *et al* 1989), or the presence of an unrelated vasoconstrictor such as angiotensin II in rabbit isolated distal saphenous artery (Dunn *et al* 1989) and the presence of U46619 and forskolin in porcine isolated ear artery (Roberts *et al* 1998).

In human blood vessels, *in vivo* studies showed the expression of postjunctional α_1- and α_2-adrenoceptors by measuring the effect on forearm blood flow induced by intra-arterial infusions of selective α_1- and α_2-adrenoceptor agonists and antagonists (Jie *et al* 1984). Presynaptic α_2-adrenoceptors were demonstrated to modulate NA release from sympathetic nerve endings via a negative feedback mechanism in humans (Jie *et*

al 1987). The involvement of both postjunctional α_1- and α_2-adrenoceptors in the mediation of the electrically-evoked vasoconstrictor responses in human gastroepiploic artery has been reported previousely (Fukui *et al* 2005).

1.6.2. β-adrenoceptors

Two subtypes of β-adrenoceptors were originally identified on the basis of different potency to agonists; the β_1-adrenoceptor (equally sensitivity to both NA and adrenaline and dominance) and the β_2-adrenoceptor (with less sensitivity to NA) (Lands *et al* 1967). Subsequently, studies on tissues expressing β-adrenoceptors showed that some of these receptors are unresponsive to β_1- and β_2- agonists. This raised the possibility of the existence of a third β-adrenoceptor subtype. The use of cloning confirmed the presence of the human β_1-adrenoceptor (Frielle *et al* 1987), mammalian β_2-adrenoceptor (Dixon *et al* 1986) and human β_3-adrenoceptor (Emorine *et al* 1989). Thus, β-adrenoceptors are classified into three types; β_1-adrenoceptors responsible for the regulation of contractility and heart rate, β_2-adrenoceptors mediating vasodilatation evoked by sympathomimetic agonists and β_3-adrenoceptors which are predominant in adipose tissue and regulate biolysis (Guimaraes and Moura, 2001; Alexander *et al* 2009).

1.7. Adenosine triphosphate (ATP) as a signalling molecule

1.7.1. ATP and purinergic receptors

Early reports showed that ATP is the source of energy for the cell and a signalling molecule (Drury, 1936). Subsequent studies observed that ATP acted as a signalling molecule on skeletal muscle (Buchthal and Folkow 1948) and the uterus (Mihich *et al* 1954). Burnstock and colleagues found stronger confirmation that reinforced the signalling action of ATP when they observed that ATP relaxed intestinal smooth muscle (Burnstock *et al* 1970) and stimulated smooth muscle of the bladder (Burnstock *et al* 1972). Following publication of these studies, Burnstock created the phrase purinergic signalling to refer to the signalling action mediated by ATP. If ATP is a ligand, it must bind to a receptor (termed a purinergic receptor) in order to produce a response. The classification of purinergic receptors is P1 receptors (adenosine-binding receptors) and P2 receptors (ATP-binding receptors) (Burnstock 1978). P2 receptors are divided into the ionotropic P2X receptors and the metabotropic P2Y receptors (Dubyak 1991). The different P2 receptors are expressed in various cell types throughout the body, including in brain stem neurons (King *et al* 2000) and in cardiovascular system (Muraki *et al*, 1998).

1.7.2. ATP release and degradation pathways

ATP release has been observed for decades in multiple systems including the nervous system (Holton 1959). ATP release was first thought to only occur in damaged cells (Burnstock, 2006b), but was later found to occur in healthy cells such as erythrocytes (Bergfeld and Forrester, 1992). ATP release is classified as lytic (from cell lysis), vesicular, or non-vesicular (Burnstock 2006a). Vesicular ATP release has been

determined to occur through exocytosis (Maroto and Hamill 2001). However, the identity of the main non-vesicular ATP release protein is still unclear. Some of the main proteins proposed to mediate the non-vesicular ATP release include: the cystic fibrosis transmembrane conductance regulator (CFTR) (Reisin *et al* 1994), ATP-binding cassette (ABC) transporters (Roman *et al* 1997), a volume-regulated anion channel (VRAC) (Hisadome *et al* 2002), a maxi-anion channel (Sabirov and Okada 2004), connexin gap junction hemichannels (connexons) (Cotrina *et al* 1998), the P2X7 receptor (Suadicani *et al* 2006), and pannexin 1 (Bao *et al* 2004).

The release of ATP following depolarizing stimuli has been demonstrated in various blood vessels including rabbit pulmonary artery and rat caudal artery (Westfall *et al* 1987, Takeuchi *et al* 1994). In addition, ATP release has been reported in cultured sympathetic nerve terminals where tetrodotoxin (neurone toxin) inhibited this release (Richardson and Brown, 1987, Von Kügelgen *et al* 1994). The release of ATP following nerve stimulation has also been shown to be a Ca^{2+}-dependent process (Brock and Cunnane, 1999).

Once ATP is released into the extracellular fluid, it is regulated by cell surface located enzymes namely ectonucleotidases. Ectonucleotidases are capable of hydrolyzing ATP to adenosine-5'-diphosphate (ADP), adenosine-5'-monophosphate (AMP) and adenosine (Burnstock, 2011, Zimmermann, 2006). However, stimulation of sympathetic nerves innervating the guinea-pig vas deferens was shown to release not only neuronal ATP, but also soluble nucleotidases which results in breaking down extracellular ATP, ADP, and AMP into adenosine (Kennedy *et al* 1997, Todorov *et al* 1997).

1.7.3. ATP as an extracellular neurotransmitter

In 1960 Burnstock and his colleagues observed inhibitory junction potentials, hyperpolarisations of smooth muscle produced by stimulation of inhibitory nerves, from intestinal smooth muscle as a result of the stimulation of noradrenergic noncholinergic (NANC) nerves. The nature of the transmitter which was involved this inhibitory effect remained unclear (Burnstock and Prosser, 1960). It was not until 1970 that Burnstock and other co-workers uncovered the nature of the transmitter which was responsible for this inhibition. They investigated the vagal noradrenergic inhibitory responses of guinea-pig and they showed the presence of ATP in the vascular secretions. Thus, they concluded that ATP was the transmitter substance released by noradrenergic inhibitory nerves (Burnstock *et al* 1970). Furthermore, it was demonstrated that ATP has postjunctional effects on the smooth muscle of vas deferens of guinea-pig (Westfall *et al* 1978). Subsequent studies showed that neurogenic responses in the vas deferens of the guinea pig were blocked by an ATP receptor antagonist arylazido aminopropanol ATP (ANAPP3) (Fedan *et al* 1981). Moreover, an ATP receptor desensitizing agent, α,β-methyleneATP, abolished the inhibitory junction potentials obtained to EFS in guinea-pig vas deferens (Sneddon and Burnstock, 1984). In blood vessels, it was demonstrated that exogenous ATP evoked vasoconstriction followed by vasorelaxation in isolated rabbit portal vein (Kennedy and Burnstock, 1985). These findings supported the concept of ATP being a cotransmitter with noradrenaline in sympathetic neurones (Lagercrantz, 1971).

1.8. Purinoceptors

In 1978, Burnstock introduced the first classification of the purinergic receptors (Burnstock, 1978). The nomenclature used by Burnstock divided these receptors into P1 and P2 purinoceptors. P1 receptors were reported to be much more responsive to the adenosine and AMP than to ATP and ADP, while P2 receptors were proposed to be much more responsive to ATP and ADP than to AMP and adenosine (Burnstock, 1978). In 1985, a report suggested a pharmacological basis for discriminating two types of P2 receptors (P2X and P2Y receptors) (Burnstock & Kennedy, 1985). According to this classification, P2X receptors were most potently activated by α,β-methyleneATP, while 2-methylthio-ATP (2-MeSATP) was at that time the most potent agonist at P2Y receptors. In 1989, it was observed that P2Y receptors responded to pyrimidine nucleotides as well as to purine nucleotides (Seifert and Schultz 1989).

The current classification was first proposed in 1994; it divides purinergic receptors on the basis of molecular structure and transduction mechanisms (Abbracchio and Burnstock 1994). Hence, purinergic receptors can be sub-divided into two major families: a P2X receptor family of ligand-gated ion channel receptors, which are activated by ATP and its analogues, in addition to a P2Y receptor family of G protein-coupled receptors (Abbracchio and Burnstock 1994). P2Y receptors are activated by ATP, ADP, uridine-5'-triphosphate (UTP), uridine-5'-diphosphate (UDP) and UDP-sugars and their analogues.

Significant developments in molecular, biochemical and pharmacological techniques have led to the further subdivision of purinoceptors. Thus at present P2 receptors are divided into two groups, P2X and P2Y. P2X receptors are divided into seven suptypes; P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7 receptors. The P2Y family is

divided into eight mammalian subtypes; $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors. P1 receptors are subdivided into A_1, A_{2A}, A_{2B}, and A_3 receptors (Ralevic and Burnstock, 1998; Alexander *et al* 2009).

1.8.1. P2X receptors

P2X receptors are ligand-gated ion channels, activation of which results in Ca^{2+} influx in addition to monovalent cations such as Na^+ and K^+, and small organic cations (Benham and Tsien, 1987; Valera *et al* 1994). The structure of P2X receptors is shown in Figure 1.3, P2X subunits have two hydrophobic, transmembrane spanning regions which cross the plasma membrane; they are connected by an extracellular loop, which contains the ATP binding site (Kennedy *et al* 2013). Using radioligand assay with [^{3}H]α,β-methyleneATP, the expression of P2X receptors on vascular smooth muscle of mesenteric, renal, and pulmonary arteries as well as in the aorta and on rat veins was confirmed (Bo and Burnstock, 1993; Hansen *et al* 1999).

In response to ATP, P2X receptors mediate vasoconstriction (Ralevic and Burnstock, 1998). In the vasculature it has been shown that P2X receptor activation mediates vasoconstriction in a number of blood vessels including rabbit basilar artery, rat pulmonary vessels and renal arteries (Lee *et al* 1976; Inscho *et al* 1994; Liu *et al* 1989). Furthermore, it was shown that the postjunctional responses of sympathetic nerve stimulation were solely mediated by the activation of P2X receptors in submucosal arterioles of the guinea-pig (Evans and Surprenant, 1992). Also, in P2X-receptor deficient mice, the P2X receptor agonist α,β-methyleneATP failed to induce any response indicating that the response-mediated by α,β-methyleneATP was due to activation of P2X receptors (Lamont *et al* 2006). In P2X1 receptor knockout mice,

vasoconstrictions to nerve stimulation were reduced by ~50% and these responses were unaffected by the P2 receptor antagonist PPADS (which is normally reduced the responses by about 50% in wild type arteries) (Vial, 2002).

In a study characterizing P2X receptor immunoreactivity, it was shown that P2X1 receptors were expressed in a number of blood vessels including femoral, pulmonary, cerebral and renal arteries of the rat (Lewis and Evans, 2001). However, the presence of other P2X receptors on endothelium has also been described such as P2X2 receptors on small cerebral arteries of the rat (Loesch and Burnstock, 2000). Furthermore, P2X4 and P2X5 receptors are expressed on human endothelial cell monolayers where ATP was shown to be released constitutively and exclusively across the membrane under basal conditions to activate the purinergic receptors and induce ATP receptor-triggered signal transduction. Hence, it has been suggested that these receptors are involved in self-regulation of endothelial function and therefore modulating vascular tone (Schwiebert *et al* 2002). P2X4 and P2X6 receptors were also found on human endothelial cells where they have been shown to play a role in cell permeability and adhesion through their colocalisation with the cell adhesion molecule VE-cadherin (Glass *et al* 2002).

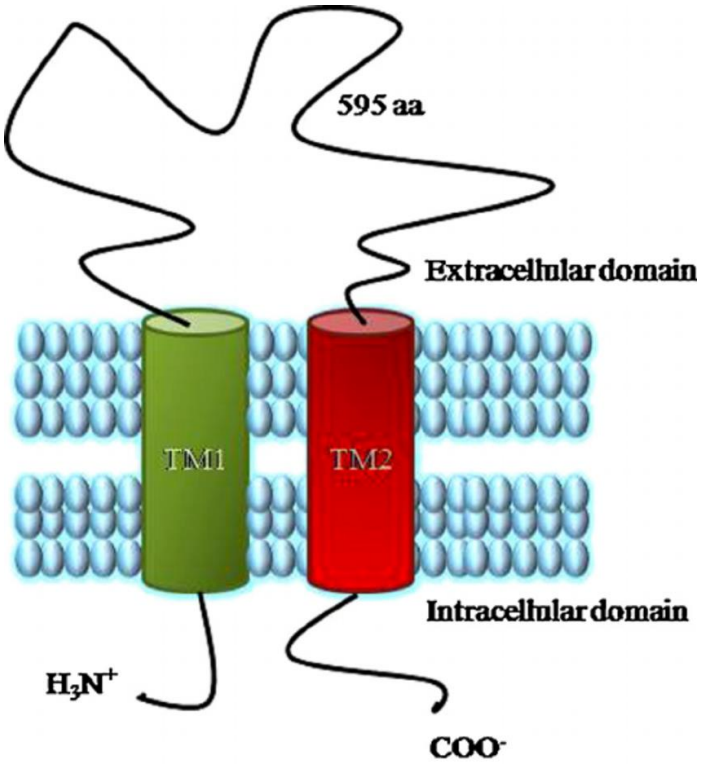


Figure 1.3 Schematic representation of a typical P2X7 receptor subunit: It consists of intracellular N-terminal and C-terminal domain and two transmembrane domains which are connected through an extracellular loop. Taken from Tewari et al. 2015.

1.8.2. P2Y receptors

P2Y receptors are G-protein-coupled receptors (GPCRs). Their structure is common to that of other G protein-coupled receptors. They contain seven α-helical transmembrane domains of hydrophobic amino acids; these domains are connected via three extracellular and three intracellular loops, with an extracellular N-terminus and an intracellular C-terminus (Jacobson *et al* 2012) (Figure 1.4). On the basis of the action of their endogenous agonists, P2Y receptors can be divided into adenine nucleotide-preferring receptors ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$) responsive to ATP or ADP, and uracil nucleotide-preferring receptors ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$)

responsive to UDP, UTP and UDP-sugars (von Kugelgen, 2006; Alexander *et al* 2009). Furthermore, according to their G protein coupling and second messenger systems, P2Y receptors can also be subdivided into two groups. One group includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors which are primarily coupled to the $G_{q/11}$ family of G proteins to activate phospholipase C, resulting in elevation of intracellular calcium levels. The other group includes $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors, which are primarily coupled $G_{i/o}$ family of G proteins to inhibit adenylyl cyclase, resulting in a reduced accumulation of cyclic AMP (Ralevic, 2009).

P2Y receptors can be expressed on the endothelial cells, for instance, in human blood vessels immunohistochemical staining showed the presence of $P2Y_2$ receptors on the endothelium of some human blood vessels including the internal mammary artery, radial artery and saphenous vein (Ray *et al* 2002). Furthermore, immunoreactivity for $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors has been shown on the vascular endothelium of human umbilical vein (Wang *et al* 2002). It seems that the most dominant functional P2Y receptors which are expressed on vascular endothelial cells are $P2Y_1$ and $P2Y_2$ receptors and they act as sensors for shear stress and hypoxia in response to locally released purines (Ralevic, 2009). However, P2Y receptors can also be found on vascular smooth muscle. For example, immunoreactivity to $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was shown on circular and longitudinal smooth muscle of human long saphenous veins (Metcalfe *et al* 2007). Functional studies have shown evidence for the involvement of $P2Y_2$ and $P2Y_6$ in regulating the blood vessels contractility. For example, $P2Y_2$ receptors have been shown to be involved in the mediation of contractile responses to uracil nucleotides in human coronary arteries (Malmsjo *et al*

2000). In addition, $P2Y_2$ and $P2Y_6$ receptors have also been shown to be involved in the mediation of contractile responses to uracil nucleotides in rat cerebral microvasculature (Lewis *et al* 2001).

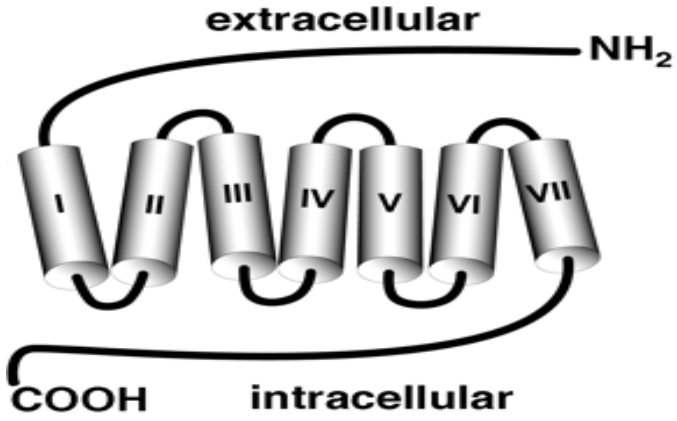


Figure 1.4 Schematic representation of the P2Y receptor subfamily. They have seven putative transmembrane domains (I-VII). These domains are linked via three extracellular and three intracellular hydrophilic loops. The arrangement of the transmembrane regions forms a pocket for the ligand binding site. Figure is taken from (Lazarowski and Boucher 2001).

1.9. Vascular endothelium

The endothelium forms an important part of the vasculature and is involved in controlling the blood flow via the complementary actions of endothelial cell-derived vasoactive factors including nitric oxide (NO) and endothelium-derived hyperpolarizing factors (EDHFs), which play an important role in endothelium-mediated vasorelaxation in many vessels by inducing a vasodilatation of the underlying smooth muscle.

1.9.1. Nitric oxide

In 1980, Furchgott and Zawadziki proposed that intact endothelial cells were required for acetylcholine to induce arterial smooth muscle cell relaxation by releasing a factor, which they termed endothelium derived relaxation factor "EDRF". They ruled out the possibility that the EDRF was bradykinin , prostacyclin, cyclic AMP or adenosine but they were unable to positively identify it (Furchgott and Zawadzki 1980).

Subsequent studies led to the identification of some factors involved in EDRF release from the vascular endothelial cells including nitric oxide (NO) (SoRelle, 1998). It is now known that NO is released from both endothelial cells and neurons and directly modulates smooth muscle tone (Förstermann *et al* 1991). It is a small hydrophobic gas which is able to diffuse across cell membranes without the aid of channels or receptors, and its action can be regulated by its concentration, proximity to the target cell and its short half-life. In blood vessels, NO diffuses from the endothelium to the smooth muscle cells and targets the haem moiety of soluble guanylate cyclase to catalyse the formation of cyclic guanine monophosphate (cGMP) from guanine triphosphate (GTP). This stimulates several protein kinases and lowers cytosolic calcium, thus initiating vascular smooth muscle relaxation (Buhimschi *et al* 1998).

NO is synthesised by nitric oxide synthases (NOS) (Hensley *et al* 1997). There are three major isoforms of NOS (neuronal NOS (nNOS or Type I), inducible NOS (iNOS or type II) and endothelial NOS (eNOS or type III). All of these isoforms have been cloned and purified, and they differ in the factors that regulate them and in their subcellular location (Alderton *et al* 2001).

Neuronal NOS (nNOS)

nNOS is a Ca^{2+}-dependent enzyme that generates NO in both the central and peripheral nervous system (Garthwaite, 1995; Bredt *et al* 1990). Following nerve stimulation, nNOS has been shown to cause vasodilation in several isolated blood vessels including canine, monkey and human cerebral arteries (Toda and Okamura, 1990).

Inducible NOS (iNOS)

iNOS is not usually expressed in healthy vascular cells and therefore does not contribute to the regulation of basal NO levels and vascular tone. However, in conditions of infection or inflammation, bacterial lipopolysaccharides or pro-inflammatory cytokines induce the transcription of iNOS (Vallance, 2003). During these conditions, increased activity of iNOS in arteries induces decreased systemic vascular resistance and hypotension (Annane *et al* 2000).

Endothelial NOS (eNOS)

eNOS was described in endothelial cells and it is associated with endothelial cell membranes (Buhimschi *et al* 1998). eNOS is stimulated by a variety of stimuli including acetylcholine, bradykinin , serotonin, ATP and shear stress to catalyse the reaction of L-arginine, NADPH and oxygen to citrulline, NADP and NO in a Ca^{2+}/calmodulin dependent manner (Alderton *et al* 2001; Arnal *et al* 1999).

The synthesis of NO synthase inhibitors such as N^G -Monomethyl-L-arginine (L-NMMA) and N^G-Nitro-L-arginine Methyl Ester (L-NAME) has proved crucial in investigating the physiological importance of NO and its contribution to vascular dysfunction: NOS inhibitors compete with L-arginine for the eNOS binding site. It has been demonstrated that intravenous administration of L-NAME caused a dose–

dependent pressor response in rats (Gardiner *et al* 1990). Vasoconstrictor effects were most profound in the mesenteric vascular bed. The effect of L-NAME could be reduced or reversed by L-arginine addition. Gardiner *et al* (1990) also showed that oral administration of L-NAME caused a more prolonged hypertension (Gardiner at al., 1990).

Vallance *et al* (1989) demonstrated that endothelium-derived NO was continuously released in the forearm arterial bed and therefore contributed to basal blood flow. They also showed that L-NMMA inhibited acetycholine-induced relaxation in single dorsal hand veins; this effect was reversed by treatment with L-arginine (Vallance *et al* 1989).

Subsequently, NO release has been shown to occur in many other blood vessels including large arteries such as the rat aorta (Shimokawa *et al* 1996) and brachial arteries (Urakami-Harasawa *et al* 1997), as well as in smaller arteries including distal arteries in the rat mesenteric bed (Shimokawa *et al* 1996) and small branch arteries in the rabbit ear (Berman *et al* 2002). The majority of experiments have found that NO has a predominant effect on larger arteries than smaller arteries. NO release has also been shown to occur in veins including rat skeletal muscle venules (Dornyei *et al* 1997), and the dorsal hand vein (Vallance *et al* 1989). Alterations in NO levels have been associated with cardiovascular diseases. Reduction in endothelial NO production can lead to abnormal vascular regulation and consequently increased peripheral resistance which could lead to hypertension. In hypertensive individuals, the response of forearm blood flow to an infusion of L-NMMA is significantly less than that of normal individuals: potentially indicating reduced basal NO-mediated vasodilation (Calver *et al* 1992). It is not known whether this occurs due to a reduction in NO

release, a decrease of smooth muscle sensitivity to NO, or due to inactivation of NO (Vallance and Chan, 2001).

During early investigations examining endothelial function in blood vessels, it became apparent that a vasodilator response remained even after inhibition of prostacyclin and NO production. This unidentified mechanism was termed endothelium-derived hyperpolarizing factor (EDHF) since it was associated with smooth muscle hyperpolarization (Chen *et al* 1988).

1.9.2. Endothelium-Derived Hyperpolarizing Factor (EDHF)

EDHF mediated responses are defined as vasodilator modulators which require an intact endothelium. They are distinct from endothelium-derived NO and they involve hyperpolarizing the vascular smooth muscle and activation of calcium-activated potassium channels (KCa). The KCa are activated by calcium released from internal calcium stores via phospholipase C and inositol triphosphate-gated Ca^{2+} release channels (Bryan *et al* 2005). Evidence of the role of K^+ channels in the action of EDHF comes from the fact that EDHF can be blocked by the intermediate conductance KCa channel inhibitors (Vanhoutte, 2004).

Unlike NO, EDHF has been shown to be more predominant in smaller rather than larger arteries, with its impact increasing as vessel size decreases. Shimokawa *et al* (1996) examined the relaxation responses to acetylcholine in rat aorta and proximal and distal arteries from the rat mesentery. They observed a greater role for NO in the aorta, whereas EDHF had a greater role in the vasodilation of the distal microvessels. The hyperpolarization induced by bradykinin was more significant in the distal

microvessels than the larger gastroepiploic arteries, which suggests a greater EDHF response in the smaller vessels (Urakami-Harasawa *et al* 1997). Berman *et al* (2002) examined vasodilator responses in different sized arteries from the rabbit ears, and found responses to be mediated predominantly by NO in the central ear arteries, while EDHF was found to be the predominant vasodilator in the smaller branch arteries. Since smaller vessels are often involved in controlling vascular resistance, EDHF may play an important role in determining blood flow.

Parkington *et al* (2002) provided evidence for a role for EDHF *in vivo*. In the presence of L-NAME and indomethacin, acetylcholine caused a vasodilator response in rat mesenteric and hindlimb arteries which was sensitive to the addition of charybdotoxin and apamin (EDHF blockers). Furthermore, an important role for EDHF has been implicated in humans since L-NMMA reduced basal blood flow of human forearm arteries, but did not completely abolish the responses to bradykinin, with the residual vasodilator response being attributed to EDHF (Halcox *et al* 2001). Katz and Krum (2001) investigated EDHF responses in healthy human subjects by measuring forearm blood flow in response to acetylcholine and comparing the responses with those obtained in individuals suffering from chronic heart failure suggesting a more prominent role for EDHF during vascular dysfunction. In isolated vessels EDHF responses have been observed in rat mesenteric arteries (Cheung *et al* 1999), bovine coronary arteries (Gebremedhin *et al* 1998) and coronary arteries (He, 2002). As with NO, much less information is known about EDHF within the venous system. While the evidence above suggests that EDHF has an important role to play in vascular control, EDHF has not yet been chemically identified. Several factors have been proposed to be EDHF.

1.9.3. Epoxyeicosatrienoic Acids

Epoxyeicosatrienoic acids (EETs) are arachidonic acid–derived products of cytochrome P450 epoxygenases. EDHF responses in bovine coronary arteries can be blocked by cytochrome P450 inhibitors, SKF 525A and miconazole, TEA (K_{Ca} channel inhibitor) and high extracellular potassium (Campbell *et al* 1996). It was concluded that EETs activated KCa channels and were responsible for the EDHF response. In addition, in bovine coronary arteries, bradykinin caused the release of 14,15-EET, which in turn caused a concentration-dependent vasodilatation which was sensitive to 14,15-EEZE (an EET agonist) (Gauthier *et al* 2005). However, in human mesenteric arteries, blockage of the cytochrome P450 epoxygenase with specific and non-specific inhibitors, sulfaphenazole and 17-ODYA, respectively had no effect on the EDHF response (Matoba *et al* 2002). Hence, the involvement of EETs in EDHF responses is still controversial.

1.9.4. Potassium ions

Activation of the endothelial Calcium-sensitive potassium (KCa) channels and intermediate conductance calcium activated potassium channels induces efflux of K^+ ions into the extracellular space (Edwards *et al* 1998). In rat hepatic arteries, the concentration of K^+ detected in the myoendothelial space was raised to ~6 mM during ACh-induced hyperpolarization (Edwards *et al* 1998). In these vessels, the increase in K^+ concentration in the myoendothelial space was abolished in the presence of apamin and charybdotoxin, inhibitors of the Calcium-sensitive potassium (KCa) channels, intermediate conductance calcium activated potassium channels (IK_{Ca}) and large

conductance calcium activated potassium channels (Edwards *et al* 1998). Likewise, in human coronary arterioles, flow-induced vasodilatation and hyperpolarization were abolished in the presence of charybdotoxin which indicates that K_{Ca} channels play a role in the shear stress-induced endothelium-dependent vasorelaxation (Miura *et al* 2003).

The efflux of K^+ ions from the endothelium can activate the Na^+/K^+-ATPase pump and Kir channels on vascular smooth muscle leading to hyperpolarization and relaxation (Edwards *et al* 1998). In rat hepatic and mesenteric arteries, it was reported that extracellular K^+ mimics the effect of EDHF response (Edwards *et al* 1998). These EDH-type responses were abolished by the presence of ouabain (Na^+/K^+-ATPase inhibitor) and barium (Kir channel blocker), hence they concluded that K^+ is likely to be a factor for EDH-type response via activation of the Na^+/K^+-ATPase and Kir channels (Edwards *et al* 1998).

However, studies from other laboratories using different vessels; rat hepatic arteries, human subcutaneous arteries, porcine coronary arteries, guinea-pig carotid and coronary arteries showed that K^+ is unlikely to be a factor for EDH-type responses and that neither ouabain nor barium had an effect on the bradykinin-induced hyperpolarization (Quignard *et al* 1999). In addition, Lacy *et al* (2000) demonstrated that the response seen to raising extracellular potassium in rat arteries was different from the response seen after increasing acetylcholine concentration. Therefore, K^+ is not the EDHF released by acetylcholine. Furthermore, blocking Na^+/ K^+ ATPase and inward rectifying K^+ channels did not block the EDHF response in myogenically active mesenteric resistance arteries, thus under physiological conditions K^+ is not considered as an EDHF (Mathewson and Dunn 2014).

1.9.5. Hydrogen peroxide

Several studies have demonstrated a role for hydrogen peroxide (H_2O_2) as an EDHF (Matoba, *et al* 2002). Bradykinin-induced EDHF responses in human mesenteric arteries were inhibited by addition of catalase (an endogenous peroxidase which breaks down H_2O_2), and by raised extracellular potassium suggesting that H_2O_2 induced hyperpolarization (Matoba, *et al* 2002). K_{Ca} channels have been shown to be involved in the H_2O_2 EDHF-mediated response. Using patch clamp experiments with porcine coronary arteries, Barlow and White (1998) demonstrated that H_2O_2 activates large conductance calcium activated potassium channels (BK_{Ca}), increasing their open probability. H_2O_2 is a reactive oxygen species, a product of superoxide breakdown by either superoxide dismutase or spontaneous breakdown. Superoxides are produced from a variety of enzyme including eNOS, cyclooxygenase, lipoxygenase, cytochrome P450 epoxygenases and NAD(P)H oxidases. It has been proposed that H_2O_2 can also stimulate large conductance calcium activated potassium channels (BK_{Ca}) indirectly by increasing arachidonic acid metabolism, and lipoxygenase derivatives of arachidonic acid promote vasorelaxation by stimulating large conductance calcium activated potassium channels (BK_{Ca}) channel activity.

1.9.6. Gap junctions

Although no universal EDHF has emerged, a large body of evidence has shown a role for myoendothelial gap junctions (MEGJ). MEGJ are channels formed between endothelial and smooth muscle cells by the docking of two connexons (one from each cell). Each connexon is composed of six connexins, which in vascular preparations are

predominantly connexins 37, 40 and 43 (Griffith, 2004). Gap junctions allow passage of inorganic ions and water soluble molecules smaller than 1kDa. Matoba *et al* (2002) blocked gap junctions with 18α-glycyrrhetinic acid in human mesenteric arteries and found that the EDHF response was slightly but significantly reduced. Sandow *et al* (2002) compared EDHF and MEGJ presence in the rat mesenteric and femoral arteries. In femoral arteries, acetylcholine did not induce EDHF hyperpolarizations in smooth muscle cells, whereas hyperpolarizations were induced in smooth muscle cells in the mesenteric arteries. This correlated with the presence of more MEGJ in mesenteric arteries than femoral arteries. This may explain why EDHF responses are more prominent in small resistance arteries than in conduit arteries. Finally, they stimulated the smooth muscle cell and measured hyperpolarizations in the endothelial cells. Hyperpolarization was only recorded in the endothelial cells of mesenteric arteries, suggesting electrical coupling between the endothelial and smooth muscle cells in mesenteric but not in femoral arteries, thus indicating a role for MEGJs. It has been proposed that the candidates for EDHF may act by activating MEGJ, with the EDHF factors that activate MEGJ varying in different tissues and species (Griffith, 2004).

1.10. Discovery of pannexins

Pannexins are a relatively recently discovered family of proteins that were discovered in the year 2000 by Panchin *et al* They were described as a second family of gap junction proteins in the human genome, in addition to the connexins. Four years before Panchin discovered the sequence of pannexin1, the protein was cloned and deposited in GenBank by Graeme Bolger (1996). He titled the sequence as MRS1. In 2004,

Baranova *et al* worked on the MRS1 clone and recognised the importance of pannexin1 as an ATP release channel (Dahl and Keane, 2012).

Before the discovery of pannexins, there were only two families of gap junction proteins namely, connexins and innexins. Connexins are the gap junction proteins found in vertebrates (figure 1.5) (Dahl and Keane, 2012). The connexin family of gap junction proteins in vertebrates is very well characterised. On the other hand, gap junction molecules with no sequence homology to connexins were identified in insects and nematodes. These proteins were specific to invertebrates and were termed innexins. Panchin was searching for sequences homologous to innexins in the human genomes when he discovered the pannexins. The mRNA encoding pannexin 1 displayed statistically significant similarities to innexins including certain cysteine residues and the location of four putative transmembrane domains. Pannexins follow a ubiquitous distribution in the animal kingdom and hence the name as suggested by Baranova *et al* (2004) (figure 1.5).

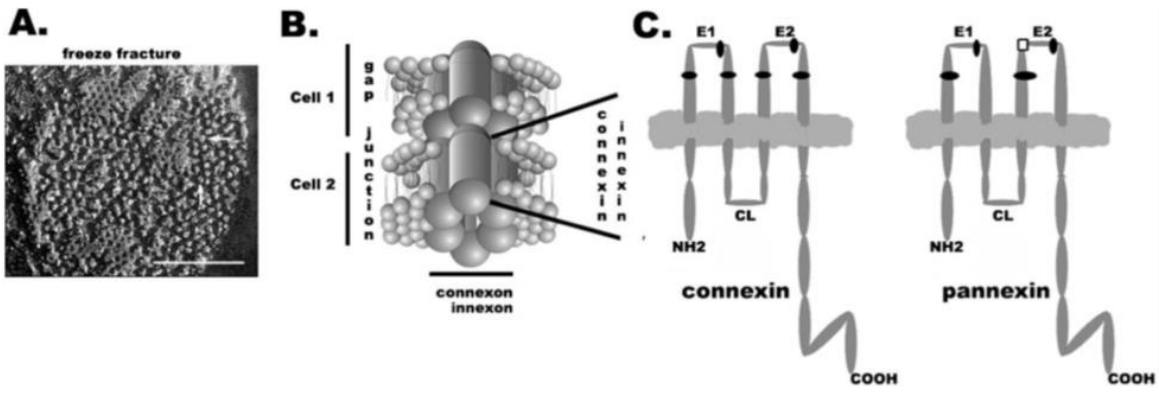


Figure 1.5 Gap junction structure and protein membrane topology (Scemes *et al* 2007).

1.10.1. Structure of pannexins

The pannexins are a class of integral membrane glycoproteins and are composed of three members - pannexin1, pannexin2 and pannexin3 (~ 50-60% sequence similarity between them). Both human and mouse genomes contain all three forms of pannexins. Pannexin1 and pannexin3 are more homologous to each other, while pannexin2 has a larger C terminal domain which conveys unique functions to it, regulation, targeting or macromolecular interactions (Figure 1.6) (Yen and Saier, 2007).

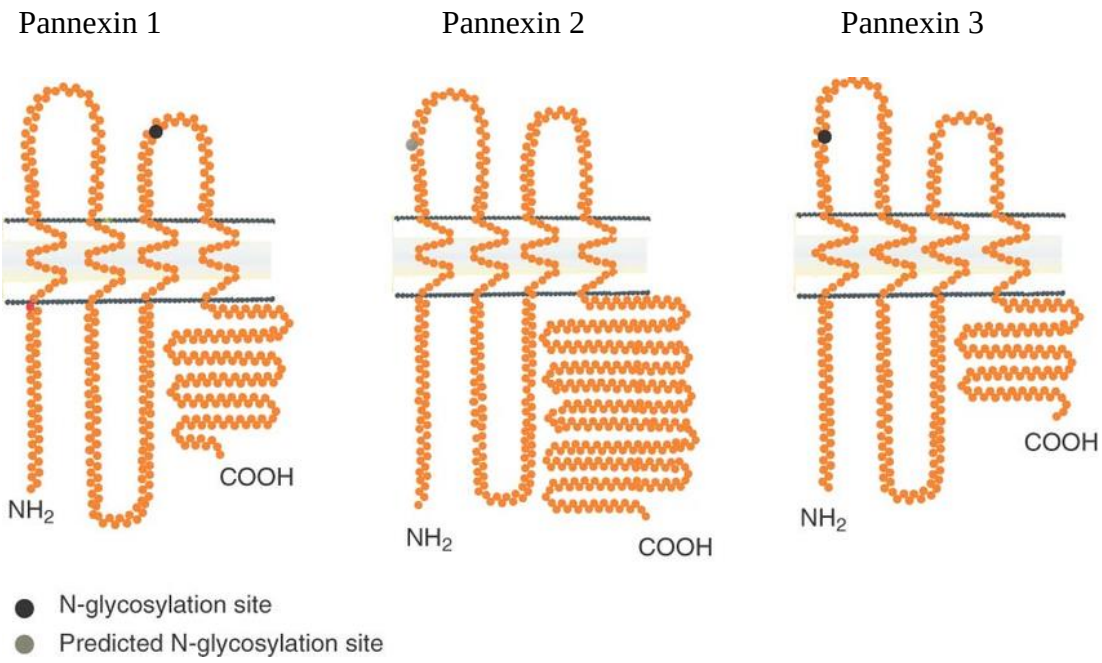


Figure 1.6 The structure of pannexins (Penuela and Laird, 2012).

Pannexin proteins span the lipid bilayer of the membrane four times to give rise to two extracellular loops and a cytoplasmic loop with the amino and carboxy terminals exposed to the cytoplasm. In topology therefore, pannexins are similar to connexins and innexins. They differ in the number of cysteine residues in the extracellular loops.

There are two cysteine residues in each extracellular loop in pannexins (Scemes *et al* 2009). The extracellular loops in pannexins are glycosylated to a high degree and this glycosylation is thought to play an important role in preventing pannexins from forming gap junctions (Bossa *et al* 2007). Both the N and C terminals of pannexins are least conserved with the C terminal having the highest sequence variability of the two (Panchin *et al* 2000).

Pannexin membrane channels are formed by six monomers (hexameric oligomers), embedded in a single plasma membrane in the case of pannexin1 (Ambros *et al* 2010; Penuela *et al* 2012) and eight monomers for pannexin2. However, the oligomeric number of a pannexin3 membrane channel is not clear yet although it is expected to be closer to pannexin1 than pannexin2 due to their similarity in size and sequence (Sosinsky *et al* 2011). The oligomeric form of pannexins is referred to as a pannexon.

1.10.2. The pannexin genes

The genes for human pannexin1 and pannexin3 are found in the same arm of chromosome 11. The gene for pannexin2 is located on chromosome 22. The genes for murine pannexin1 and pannexin3 are located in chromosome 9 whereas the pannexin2 gene is located on chromosome 15 (Baranova *et al* 2004). It has been reported that pannexin1 and pannexin3 share a high degree of sequence homology with each other when compared with pannexin2 in both species (Penuela *el al* 2009).

Pannexins have a complex gene structure that includes exons and introns. The pannexin gene structure with intervening introns throughout their coding sequences led to the generation of many different expression products (Penuela *et al* 2012). In addition to the diversity of pannexins being brought about by alternative splicing, post-

translational modifications also contribute to their diversity. It is thought that pannexins do not undergo post-translational phosphorylation, even though they contain several phosphorylation sites in their C-terminus, intracellular loop and N-terminus (Penuela *et al* 2007 and Barbe *et al* 2006). Moreover, pannexin1 and pannexin3 contain N-glycosylation sites in their extracellular loop and this has been confirmed by several mutagenesis studies. Glycosylation of pannexin1 and pannexin3 occurs at two levels and this leads to the formation of 3 distinct species – the non-glycosylated species (Gly0), the predominant endoplasmic reticulum resident high mannose species (Gly1) and the complex glycosylated species (Gly2). Pannexin2 also contains an N-linked glycosylation site in its first extracellular loop and is sensitive to deglycosylating enzymes. There are studies that suggest that the N-glycosylation of pannexins plays an important role in regulating their distribution since the Gly2 complex is abundant in the plasma membrane and the Gly0 and Gly1 prefer to reside in the endoplasmic reticulum (Penuela *et al* 2009).

Pannexin2 seems to undergo an additional post-translational modification - palmitoylation. However further studies have to be carried out to confirm this report and also to determine if pannexin1 and pannexin3 also undergo palmitolyation (Swayne *et al* 2010). Cysteine residues could play an important role in controlling pannexin channels (Bunse *et al* 2011). It has been observed that when any of the four extracellular cysteines of pannexin1 are mutated, the protein loses its channel function (Bunse *et al* 2011 and Wang and Dahl, 2010), hence, it has been suggested that cysteines intrinsically control pannexin channel activity by post-translational modification processes such as glutathionation or S-nitrosylation. However, more studies have to be carried out to confirm if cysteine residues do indeed undergo S-nitrosylation in pannexin1, as in the case of connexins (Billaud *et al* 2011). Pannexin1

interacts with pannexin2 and pannexin3 in a glycosylation dependent manner. Pannexin2 interacts with the Gly0 and Gly1 species of pannexin1 but not with pannexin3 (Penuela *et al* 2009). Studies showed that the C-terminal end of pannexin1 binds to actin directly. There are further suggestions that the interaction of pannexin1 and pannexin3 with actin microfilaments as well as cytoskeleton microfilaments is mandatory for the proper delivery of pannexin1 and dynamics at the cell plasma membrane surface. Moreover, these interactions with the cytoskeleton microfilaments help to stabilise the cell surface populations of pannexin1 and pannexin3 (Bhalla-Gehi *et al* 2010).

1.10.3. Pannexin expression and genomics

The expression of all three pannexins has now been identified in at least seven species – humans, mouse, rat, dog, cow, zebrafish and puffer fish (Penuela *et al* 2013; Yen *et al* 2007). However, they are most well studied in human and murine tissues. It is important to note that the expression levels of pannexins are dynamically controlled by physiological and pathological processes. For example, pannexin2 is expressed in astrocytes only during ischemia or reperfusion (Zappala *et al* 2007). Also, the expression of pannexin1 and pannexin2 increases upon epileptic and seizure activity (Zappala *et al* 2007; Samoilova *et al* 2003; Zappala *et al* 2006)

Pannexin1

Pannexin1 is expressed in several human tissues including the brain, heart, skeletal muscle, prostate, lung, liver, small intestine, pancreas, spleen, colon, vascular endothelium and erythrocytes (Baranova *et al* 2004). In the central nervous system,

pannexin1 was detected in the cerebellum, cortex, lens, retina, pyramidal cells, interneurons, hippocampus, amygdala, substantia nigra, olfactory bulb, neurons (Baranova *et al* 2004; Bruzzone *et al* 2003; Dvoriantchikova *et al* 2006, Dvoriantchikova *et al* 2006; Ray *et al* 2006; Shestopalov *et al* 2008). The expression of pannexin1 was found to be substantial in the brain; however, expression of pannexin in lung, kidney, spleen, heart ventricle, skin and cartilage in 3 weeks old mice was found to be variable (Penuela *et al* 2007). Pannexin1 was also found in rodent cochlea and in the vascular smooth muscle of TDA (Penuela *et al* 2007; Wang *et al* 2009; Billaud *et al* 2011).

Pannexin2

The expression of pannexin2 is limited to various area of the human adult brain including the cerebellum, cerebral cortex, medulla, occipital pole frontal lobe, temporal lobe and putamen (Baranova *et al* 2004). High levels of pannexin2 were detected in rodent brain, spinal cord and eye but on the other hand, rodent thyroid, kidney and liver showed low levels of pannexin2 expression (Bruzzone *et al* 2003; Dvoriantchikova *et al* 2006; Dvoriantchikova *et al* 2006). There are reports that pannexin1 and pannexin2 are co-expressed in several areas of adult rat brain (hippocampus), interneuron cells and the Purkinje cells of the cerebellum (Vogt *et al* 2005).

Pannexin3

Pannexin3 has been found in osteoblasts, synovial fibroblasts, skin; mouse paws joint and the inner ear cartilage. Additionally, pannexin3 expression was also detected in the human hippocampus but at very low levels (Baranova *et al* 2004). There are also

reports that pannexin3 is expressed in the mouse cochlear bone as well as in the prehypertrophic chondrocytes, perichondrium and osteoblasts in embryonic mice (Wang *et al* 2009; Iwamoto, 2010). Pannexin3 is also endogenously expressed in osteoblasts, both in murine calvaria and reference cell lines (Bond, 2011 and Penuela, 2008).

1.10.4. Localisation profiles of pannexins

Ectopically expressed pannexin1 and pannexin3 predominantly accumulate at the cell surface. However, these pannexins are also present within intracellular compartments (Penuela *et al* 2009). Similar distribution profiles have been reported for endogenously expressed pannexin1 in a variety of cells and tissues (Bhalla-Gehi *et al* 2010; Boassa *et al* 2007; Wang *et al* 2009; Kienitz *et al* 2011). Though pannexin3 is usually found in the intracellular compartments in rat cochlear bone, it has also been found at the cell surface and intracellular compartments in murine chondrocytes (Iwamoto *et al* 2010). Pannexin2 localises mainly in intracellular compartments but can also be found in limited amounts at the cell surface. The cell surface expression of pannexin2 is increased when co-expressed with pannexin1 (Penuela *et al* 2009). Pannexin1 and pannexin3 are found to be especially enriched in dynamic cell surface protrusions at areas deficient in contacting cells (Penuela *et al* 2009).

1.10.5. Pannexin hemichannel modulation

The pannexin channels are mainly implicated in ATP release and intercellular calcium wave propagation. Pannexin1 is also known as a channel for ions, metabolites and

signalling molecules with a molecular weight less than 1kDa (D'hondt *et al* 2011). Previous studies report that pannexin1 channels open in response to stimuli like mechanical stress or membrane depolarisation and release ATP to the extracellular media. It is well documented that various physiological stimuli, including extracellular ATP and mechanical stress initiate Ca^{2+} waves. This calcium wave can be propagated by two pathways: either by direct intercellular flux of inositol triphosphate (IP_3) through gap junction channels or an extracellular pathway involving ATP release and purinergic receptors (Hassinger *et al* 1996).

The physiological ATP concentration ranges between 1 and 10 mM (Yaginuma *et al* 2014). A large gradient between intracellular and extracellular ATP concentration drives ATP efflux through the open pannexin1 channel. The amount of ATP released has been quantified by bioluminescent luciferin-luciferase based assay which shows that the cellular ATP loss is limited. When released into the extracellular spaces, ATP and other metabolites like ADP and AMP can bind to purinergic receptors on neighbouring cells to produce metabotropic and ionotropic P2X downstream responses (Burnstock 2012, Abbracchio *et al* 2009). Pannexin hemichannels are also involved with entry and exit of other metabolites and fairly large molecules such as nicotinamide adenine dinucleotide (NAD^+) (Bruzzone *et al* 2001; Verderio *et al* 2001), prostaglandins (Cherian *et al* 2005), glutamate, a major excitatory neurotransmitter between neurons as well as the neuron-glial signaling network (Bezzi and Volterra 2001; Ye *et al* 2003; Stridh *et al* 2008), glutathione (GSH) (Stridh *et al* 2008) and inositol 1,4,5-trisphosphate (IP_3) (Gossman and Zhao., 2008), which can serve as transmitters between cells (Bennett *et al* 2012).

1.11. Functional roles of pannexins

1.11.1. Paracrine signalling by pannexin1

a. Calcium waves

Changes in intracellular calcium ion concentration drives many cellular responses. Initiation of calcium ion waves can be brought about by the activation of purinergic receptors such as P2Y and P2X in human mesenteric arteries (Matoba *et al* 2002). When ATP binds to P2Y receptors, there is an increase in inositol 1,4,5-triphosphate. This in turn, releases calcium ions from the ER. The calcium ions then activate the calcium channel and this leads to further release of ATP and proliferation of signals to neighbouring cells (Figure 1.7) (Barbe *et al* 2006; Scemes *et al* 2009). Pannexin1 is known to be a part of the P2X7 receptor complex that is necessary for ATP release (Pelegrin and Surprenant, 2006). Similarly, ATP-induced ATP release was also observed when pannexin1 channels were activated through P2Y receptors and cytoplasmic calcium ions. Thus, pannexin1 plays a role in the initiation and propagation of regenerative calcium signalling. In the brain, one hypothesis is that the interaction of pannexin1 channels with NMDA receptors releases calcium ions in the hippocampal pyramidal neurons, which can promote epilepsy (Thompson *et al* 2008). This role has been confirmed *in vivo* using mice devoid of pannexin1, as they showed that activation of pannexin1 in juvenile mouse hippocampi contributes to neuronal hyperactivity in seizures (Santiago *et al* 2011).

b. Taste sensation

There are two distinct types of cells that constitute taste buds – the taste receptor (receptor cells) and the synapses (presynaptic cells). Pannexin1 is expressed in the

receptor cell and on taste stimulation can release ATP (Huang *et al* 2007). Once ATP is released, it can stimulate P2 receptors of the presynaptic cells which in turn release serotonin (5-HT). Hence pannexin1 helps in cell to cell signalling and information processing within taste buds (Ransford *et al* 2009).

c. Airway defence

The primary role of airway epithelial cells is to provide a defence mechanism by clearing mucus from the airway. When pannexin1 channels are activated (e.g. under hypotonic stress) they can release ATP via pannexin1 channels which then regulates ciliary activity which is important for mucus clearance (Ransford *et al* 2009).

d. Pannexin1 and HIV

ATP release through pannexin1 channels is thought to play a significant role in HIV viral infection of $CD4^+$ cells. It has been suggested that the envelope proteins of the virus interact with special receptors on $CD4^+$ cells. This interaction triggers ATP release though pannexin1 channels and the ATP then interacts with $P2Y_2$ purinergic receptors to activate Pyk2 kinases. As a result, membrane depolarisation occurs, allowing membrane to membrane fusion and facilitates viral entry into the cell as well as cell to cell transmission. Pannexin1 forms a part of the infection synapse that is organised by the virus to facilitate infection. In addition to pannexin1, $P2Y_2$ and Pyk2 are also recruited to the contact site to form a part of this infection synapse (Seror *et al* 2011). This observation is important because by inhibiting any of the components mentioned above, HIV infection could be reduced and viral replication could be disrupted; hence leading to their use as a potential retroviral therapy (Penuela *et al* 2012).

1.12. Cellular responses mediated by pannexin1

a. Immune response

Pannexin1 interacts with P2X7 receptors to elicit an immune response by releasing the cytokine interleukin-1B (IL-1B) in response to the stimulation of the receptors by ATP followed by the activation of caspase-1 (Pelegri and Surprenant, 2006). High extracellular potassium ion levels in neuronal/astrocytic inflammasomes facilitate the P2X7 mediated activation of pannexin1 channels (Silverman *et al* 2009). On the other hand, a more recent study stated that pannexin1 channels are not required for the assembly of caspase-1-activating inflammasome complex (Qu *et al* 2011).

b. Tumorigenesis

The exogenous expression of pannexin1 has been thought to help with tumour suppressive properties in C6 gliomas (Lai *et al* 2007). When pannexin1 is released, the C6 gliomas exhibit a flattened morphology and also show a reduction in proliferation, motility and anchorage-independent growth. Experiments carried out in nude mice determined that cells overexpressing pannexin1 displayed reduced *in vivo* tumour growth when compared to control mice. Thus, they concluded that pannexin1 has an important role as a tumour suppressor. A similar study also reported a role for pannexin2 (Qu *et al* 2011; Lai *et al* 2007).

c. Ischemic cell death and epilepsy

When oxygen and glucose decrease in the mouse hippocampus, it has been suggested that pannexin1 channels open and conduct large currents, leading to neuronal death

due to significant ionic dysregulation (Thompson *et al* 2008). Additionally, the opening of the pannexin1 channels associated with N-methyl-D-aspartate receptors enhances epileptic seizure activity in pyramidal neurons. Studies carried out using pannexin1 knockout mice have confirmed that pannexin1 does contribute to severe epileptic seizures *in vivo* (Santiago *et al* 2011).

d. Apoptosis

When pannexin1 is co-expressed with P2X7 receptors, ATP induced zeiosis (blebbing) in Xenopus oocytes was observed which was not the case was when pannexin1 was expressed alone or together with PY_2 receptors. Hence, it has been suggested that pannexin1 forms a part of the P2X7 death complex (Locovei *et al* 2006). Pannexin1 mediates the release of nucleotide signals from apoptotic cells that helps in the recruitment of activated monocytes. Therefore, specific cell death signalling happens due to the interaction of pannexin1 with P2X7 receptors. Caspases activate pannexin1 channels and open the channel for ATP and UTP release which help to attract phagocytes (Chekeni *et al* 2010).

e. Keratinocyte differentiation

It has been observed that when pannexin1 is ectopically expressed, it reduces the proliferation rate of rat epidermal keratinocytes. The overexpression of pannexin1 in organotypic cultures is known to have a disruptive effect on the overall architecture of the epidermis. Pannexin1 reduces the thickness of the living cell layer and also re-localises cytokeratin 14 (the basal cell marker) throughout the living cell layer. Hence, this research argues that pannexin1 has a significant role to play in the regulation of keratinocyte differentiation (Celetti *et al* 2010).

1.13. Cellular responses mediated by pannexin2 and pannexin3

a. Neuronal differentiation

It has been observed that pannexin 2 is usually expressed by both progenitor neuronal cells as well as mature neurons. The expression of pannexin2 in these cells seems to be regulated by the presence of the pannexin2 species that has been post-transitionally modified by S-palmitoylation. These pannexin2 species are localised in the intracellular compartments of the progenitor neuronal cell but not in mature neurons. Pannexin2 is re-expressed in the plasma membrane of mature neurons. In addition, researchers have observed that when pannexin2 is knocked out in neuro2a cells, it significantly enhances the rate of neuronal differentiation (Swayne *et al* 2010).

b. Differentiation and development

The channel forming properties of pannexin3 have not been studied in detail. However, pannexin3 can reduce the rate of proliferation of epidermis keratinocytes without altering their migration properties like pannexin1. Overexpressed pannexin3 however maintains the integrity of the epidermis and keratinocyte differentiation in organotypic cell lines (Celetti *et al* 2010). Additionally, it has been reported that pannexin3 expressed in cartilage, is associated with the differentiation of chondrocytes. Pannexin3 specifically promotes ATP release into the extracellular media and thereby reduces the proliferation of chondrocytes by decreasing calcium levels and also by inhibiting the phosphorylation of CREB in parathyroid hormone-mediated chondrocyte proliferation (lwamoto *et al* 2010). From this study, it can be elucidated that pannexin3 has a role in switching the properties of chondrocytes from one of proliferation to one of differentiation. Recent reports also suggest that pannexin3 has

an important role in chondrocyte and osteoblast differentiation because the pannexin3 promoter is responsive to Runx2 activation (Bond *et al* 2011). Pannexin3 can also function as an ER calcium channel, plasma membrane channel or even as a gap junction protein (Ishikawa *et al* 2011). Pannexin3 expression has also been associated with the simultaneous increase in the expression of connexin Cx43 and therefore it is not clear if the gap junction function is due to pannexin3 or due to the increase in Cx34. Pannexin3 has also been found to be expressed in rat leydig cells, the apical region of the epididymis and in the efferent ducts; hence pannexin 3 could possibly play a role in the transport and maturation of sperm (Turmel *et al* 2011).

1.14. Role of pannexins in vasculature

Previous studies established the location of pannexin1 on erythrocytes which on activation either by stress or hypoxia release ATP. It is also documented that pannexin1 channels initiate and propagate Ca^{2+} waves. Thus, they control blood flow by releasing ATP from red blood cells and stimulation of purinergic receptors on endothelial cells which causes calcium wave initiation and propagation (Locovei *et al* 2006). It has been shown recently by Billaud *et al* (2011) that pannexin1 has a significant role in forming a complex with α1D-adrenergic receptors in vascular smooth muscle cells which in turn contributes to vasoconstriction of resistance arteries. Thus, pannexin1 could be important in the coordination of vascular smooth muscle cell constriction and also the regulation of blood pressure (Penuela *et al* 2013). Billaud *et al* have shown that after α_1-adrenoceptor activation (by phenylephrine) pannexin channels open with the subsequent release of ATP. ATP then acts as an

intercellular signalling molecule to propagate vasoconstriction by activating P2Y receptors on neighbouring cells (Figure 1.7).

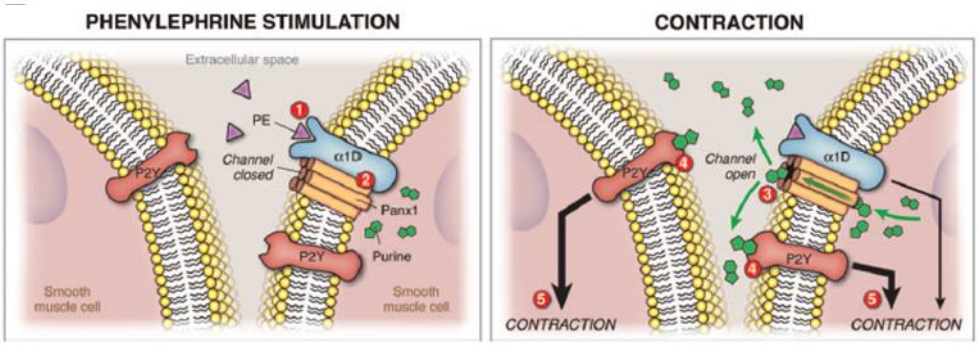


Figure 1.7 Pannexin1 contributes to α-1-Adrenergic Receptor–Mediated Vasoconstriction (Billaud *et al* 2011).

Burns *et al* have shown the presence of pannexin1 in vascular smooth muscle and pannexin2 in both smooth muscle and the endothelium (Burn *et al* 2012; Chen, Burns *et al* 2008; Li, He *et al* 2015). In the smooth muscle of small arteries, pannexin1-mediated ATP release was shown to participate in vascular tone regulation by contributing to arterial contractile response to α_1-adrenoceptor activation (Billaud *et al* 2011). By contrast, Dina *et al* (2014) studied the functional role of pannexin1 in facilitating vasodilator and anticontractile effects mediated by the endothelium and suggested a major role of pannexin1 in endothelium-dependent responses, mediated by EDHF (Figure 1.8). They found that endothelial function was impaired in conduit arteries of pannexin1 knockout mice. The mechanism by which pannexins provoke their vasorelaxation effects is summarised in Figure 1.8.

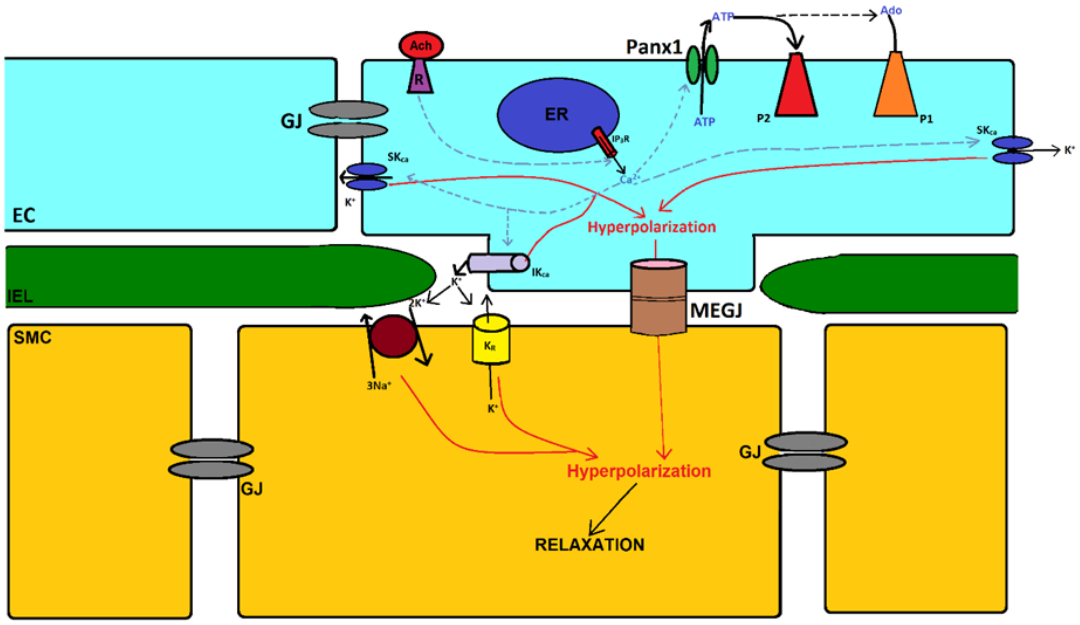


Figure 1.8 Diagram showing mechanisms of pannexin1 involvement in the functioning of EDHF-like mechanism during agonist-induced stimulation followed by smooth muscle relaxation. Intermediate conductance calcium activated potassium channels (IK_{Ca}) – endothelial intermediate conductance calcium-activated potassium channels; SK_{Ca} – small conductance calcium-activated potassium channels; R – receptor; ACh – acetylcholine; Ado – adenosine; ER – endoplasmic reticulum; IP3R – 1,4,5-inositol trisphosphate receptor; EC – endothelial cell; IEL – internal elastic lamina; SMC – smooth muscle cell; MEGJ – myoendothelial gap junctions; GJ – gap junctions; KIR – inwardly rectifying potassium channels. Adapted from Gaynullina *et al* 2015.

Aim and objectives

The key aim of this dissertation was to investigate whether pannexin protein was present in the porcine splenic and coronary arteries and in the rat thoracic aorta. Subsequent functional studies aimed to determine whether pannexins were involved in transducing responses to NA and, more importantly, whether they provide a mechanism for producing a generalised vasoconstriction in arteries after the discrete release of focal neurotransmitters after activation of the sympathetic nerves. Later studies investigated whether pannexins are involved in endothelium-dependent response in porcine splenic and coronary arteries.

Chapter 2

2. Materials and methods

2.1. Tissue preparation for Western blotting

- Porcine tissues

Tissues were collected from Wood and Sons Abattoir (Clipston, Nottingham, UK) and transported to the laboratory in cold Krebs–Henseleit solution. In the dissection room, on a clean surface, the porcine splenic and coronary arteries were dissected and placed in a petri dish containing sufficient buffer to keep the tissue from drying out where they were finely dissected to remove the connective tissue.

Segments of porcine arteries were extracted and stored at -80 0C. Samples were homogenized in lysis buffer [Tris (20mM), ethylene glycol tetraacetic acid (EGTA, 1 mM), Triton X-100 0.1% (v/v), sodium fluoride (NaF, 1 mM), β-glycerophosphate (10 mM), pH7.6], containing 1 %(v/v) protease inhibitor cocktail (Sigma-Aldrich,Dorset, UK) EDTA free.The samples were then centrifuged at 6000x g for 5 minutes at 4 0C. The supernatant was used as total soluble protein sample which was developed using PAGE assay.

- Rat aorta

Male Wistar rats weighting between 180-220g were stunned by a blow to the cranium and killed by bleeding. The rat thoracic aorta was dissected and placed in a petri dish containing sufficient buffer to keep the tissue from drying out. The rat thoracic aorta was finely dissected to remove the connective tissue.

Segments of rat aorta were extracted and stored at -80 0C. Samples were homogenized in lysis buffer [Tris (20mM), ethylene glycol tetraacetic acid (EGTA, 1 mM), Triton

X-100 0.1% (v/v), sodium fluoride (NaF, 1 mM), β-glycerophosphate (10 mM), pH7.6], containing 1 %(v/v) protease inhibitor cocktail (Sigma-Aldrich,Dorset, UK) EDTA free.The samples were then centrifuged at 6000x g for 5 minutes at 4 0C. The supernatant was used as total soluble protein sample which was developed using PAGE assay.

2.2. Lowry test

A Lowry test was used to determine the protein concentration of the supernatants (Lowry *et al.,* 1951). A stock bovine serum albumin (BSA, 1mg/ml) solution was prepared in distilled water; a serial dilution (from 0.05-0.45 mg/ml) was prepared from the stock to produce a standard curve. Supernatants and standards were mixed with Lowry A (0.4 % (w/v) NaOH, 0.2 % (w/v) sodium dodecyl sulphate (SDS), 2 % (w/v) Na2CO3) and Lowry B (1 % (w/v) CuSO4, 2 % (w/v) NaK Tartrate), and incubated for 10 minutes at room temperature. Subsequently, folin phenol reagent, diluted 1:1 in distilled water, was added to the supernatants and standards and incubated for 45 minutes at room temperature. The samples were then transferred to a 96 well plate and the absorbance was measured using SpectraMAX 340 PC plate reader at 750 nm wavelength. The protein concentrations of the samples were extrapolated from the BSA standard curve.

The supernatant from the remaining samples (not used in the Lowry test) was diluted 1:6 in solubilisation buffer (6x SB, 24 % (w/v) SDS, 30 % (v/v) glycerol, 5 % (v/v) beta mercaptoethanol, 2.5 % (v/v) bromophenol blue, 1.5M Tris-hydrochloride, pH 6.8) and heated at 95 oC for 5 minutes, followed by centrifugation for 1 minute at 13000x g.

2.3. Western blotting

Samples were run on on 4-20 % Tri Glycine (PAGE) Gold Precast Gels (Bio Rad, Hercules, CA, USA) in electrophoresis buffer (0.19 M Tris hydrochloride, 1.9 M glycine and 35 mM SDS). The lysis samples were placed in the heat block for 5 minutes at 95°C. Following this they were vortexed and centrifuged for 1 minute at 6000x g.

One µl of the molecular marker (Bio-Rad, precision plus protein) was loaded in the first well and the rest of the wells were loaded with 5µg of the porcine tissue and 10µg of the rat aorta sample. Electrophloresis was performed at 175V for 35 minutes using Bio-rad mini protean 3 electrophloresis tank. The gel was blotted onto nitrocellulose membranes which was performed at 100V for 60 minutes at 4°C. To check for transfer of protein, ponceau s solution (Sigma, UK) was added to the nitrocellulose membrane, which was then washed in tris buffered saline solution with the detergent Tween® 20 (TBST) (25mM Tris, 12mM NaCL, 10l dH_2O, 10ml Tween 20, pH 7.6). Following this, the nitrocellulose membrane was blocked in 5% (w/v) milk solution in TBST for 1 hour at room temperature on a rocking platform. After that the nitrocellulose membrane was incubated overnight at 4^oC with the primary antibodies Rabbit anti-pannexin1 (Abcam cat No. ab124969), Rabbit anti- pannexin2 (Abcam cat No. ab55917) and Rabbit anti-pannexin3 (Abcam cat No. ab98093, added at 1:1000 concentration in 5% (w/v) milk solution Mouse monoclonal anti-GAPDH antibody (G8795 Sigma, UK) (1:40 000) was simutaniously used as house keeping and loading control. All antibodies where sealed in a plastic bag with the nitrocelluose and incubated over night at 4^oC.

The following day the primary antibodies were removed by putting the nitrocellulose membrane in a glass container and washing with TBST (3 rinses, 3x5 minute washes and 3x15 minute washes). In the final step the nitrocellulose membranes were incubated again for 1 hour at room temperature with with to the corresponding secondary antibodies: IRDye® 680LT Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) and IRDye® 800CW Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK).After that the secondary antibodies were removed and the nitrocellulose was washed as before (3rinses, 3x5 minute washes and 3x15 minute) and analysed using Li-Cor Odyssey Imager Scanner and densities of the bands were determined using Odyssey (Application Software Version 3.0LI-COR Biosciences, Cambridge, UK).

2.4. Immunohistochemistry

2.4.1. Paraffin embedding tissue and sectioning

Porcine splenic and coronary arteries and rat thoracic aorta were dissected and placed in a sponged petri dish full of Krebs–Henseleit solution, where they were finely dissected to remove the connective tissue, then cut to 1 cm in length, immediately placed in 4% formaldehyde and incubated overnight at 4º C. The next day, tissues were processed using a Leica bio-system tissue processor 1020 for the tissue to dehydrate. A gentle exchange was achieved by passing the tissue through increasing concentrations of ethyl alcohol (50, 70, 90, 96 & 100%). Once the water had been replaced by alcohol, the alcohol was then replaced with xylene, which is a transitional solvent which is miscible with both the alcohol and molten wax. The final step in processing was to infiltrate the tissue with molten paraffin wax. The following day,

the tissue is arranged in a steel mould in the correct orientation for example the porcine arteries were arranged transversely and embedded in molten wax. They were then left to cool and harden prior to sectioning. 5µm sections were cut using a Leica RM2125 microtome (Leica), floated in a water bath set to 42˚ C and transferred to 3-Aminopropyltriethoxysilane (APES) coated slides, which is a chemical solution used to add a charge to the slides, thus helping sections to adhere to the slides better. The slides were then left to dry.

2.4.2. Histology

APES coated slides containing 5µm sections were dewaxed using xylene over 5 minutes. The sections were then rehydrated by immersing the slides in Industrial Methylated Spirits (IMS), moving through 2x100%, 90% 70%, 50% then down to water each step was done for for 30 seconds.

For the antigen retrieval, slides were placed onto a plastic rack and positioned in a microwavable plastic container containing citrate saline buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0). The container was then moved into the microwave with the lid on. The slides were then irradiated at mid-high power for 5 minutes followed by 15 minutes in low power then left under running cold tap water for 15 minutes. They were then permeabilized using 0.1% triton in phosphate-buffered saline (PBS) for 10 minutes, blocked with 10% goat serum for 30 minutes to prevent non – specific staining and then drained off. The primary antibodies were diluted in (PBS 0.1%, 0.1% Tween-20, 1% bovine serum albumin (BSA)) to a dilution of 1:500. This dilution was chosen following optimisation and successful use in immunostaining experiments using same antibody concentration in different tissue. The slides were

then placed in a humidity chamber and the primary antibodies were applied to the sections and left to bind overnight (24 h). No primary antibody was applied to the negative control; instead blocking solution only was applied overnight. The excess primary antibody was removed the following day by washing with PBS. Then the secondary antibody, The goat anti-rabbit antibody (secondary antibody) FITC (F0382) was diluted to 1:500 and goat anti-rabbit IgG (H+L) secondary antibody, Rhodamine Red conjugate was diluted to 1:1000 in (PBS 0.1%, 0.1% Tween-20, 1%BSA) and applied to the sections for 1 h. The excess secondary antibody was then removed by washing with PBS. The slides were placed in a humidity chamber which was then covered in aluminium foil to prevent bleaching of the Rhodamine red and FITC marker. After1 hour, excess antibody was removed by washing with PBS. Following this they were incubated with DAPI (1in 500 dilution) for 5 min in the dark. Incubation with DAPI was followed by 2 washes with TBS. Coverslips were placed on microscope slides applied using mounting medium (1% (v/v) 1, 4-diazobicyclo-[2, 2, 2,]-octane (DABCO) fluorescence mounting medium-SS04. Slides were then stored and covered with aluminium foil.

2.4.3. Fluorescence microscopy

The immunostaining sections were viewed using a light microscope and a wide field fluorescence microscope (Leica DMRB). Sections were viewed at 20x and 40X (plan, air objective with a 0.5 NA) magnifications to assess pannexin location in the arteries. Images were captured using Micro Manager. The following filters were used to visualise the immunostains. DAPI - Excitation 350/50nm, emission 460/50 nm, FITC - Excitation 480/40nm, emission 535/50 nm to visualise the Goat anti-rabbit Antibody

and Rhodamine Red - Excitation 545/20nm, emission 610/75 nm for the Goat anti-rabbit IgG (H+L) secondary antibody.

2.5. Isometric tension recording

Porcine tissue was obtained from Wood and Sons Abattoir (Clipston, Nottingham, UK) from breeds of modern hybrid pig, but the sex and exact age of each animal was unknown. Porcine spleens and hearts were isolated at the abattoir and stored in Krebs–Henseleit solution at 4˚C during transport to the laboratory. On arrival, gross dissection of splenic arteries and coronary arteries were carried out leaving the adipose and connective tissue directly surrounding the vessels intact. Vessels were stored overnight in Krebs–Henseleit solution at 4˚C pre-gassed with a 95% O_2: 5% CO_2 gas mixture. The next day vessels were pinned in a dissection dish and the remaining adipose and connective tissue was removed whilst ensuring vessels were not stretched or damaged. The length of the vessel segments was approximately 5 mm. Tissue was kept in Krebs–Henseleit solution and allowed to normalize to room temperature before being setup in the isometric force transducer .

Vessels were attached to two metal hooks placed through the lumen, ensuring that the hooks were not overlapping. One hook was attached to a glass rod and the other was attached to a non-stretchable silk thread. The vessel was then transferred to an organ bath where the glass rod was anchored into the system (Figure 2.1). The thread attached to one of the hooks was attached to a force transducer (AD Instruments, UK) on a rack and pinion, enabling adjustment of tension. The organ bath contained 20ml Krebs–Henseleit solution and was kept at a constant temperature of 37 ˚C using a

thermal circulator pump. The Krebs–Henseleit solution was constantly gassed with the required gas mixture for each experiment.

The force transducer was attached to a Bridge Amplifier (ADInstruments, UK) to measure alterations in voltage recorded by the transducer due to changes in the tension applied to the transducer. The Bridge Amplifier was in turn connected to a PowerLab unit (AD Instruments, UK), which connected the transducer to a computer where data was recorded using Lab Chart software (AD Instruments, UK). Transducers were calibrated with a 10g weight daily to ensure consistency of measurement.

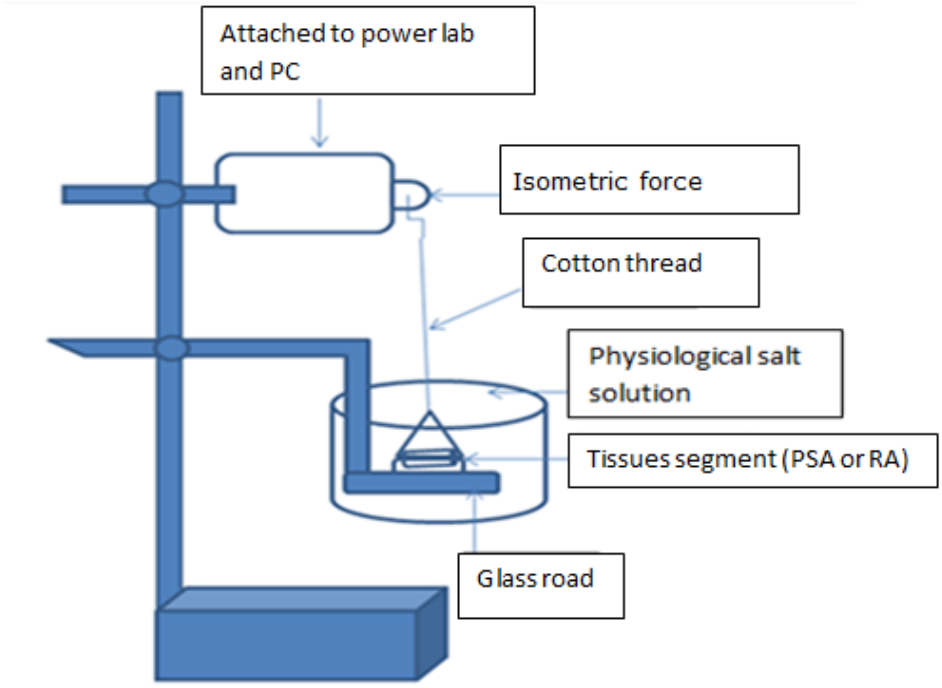


Figure 2.1 Pictorial representation of the apparatus used in the isometric tension recording experiments

2.6. Statistics analysis

Data analysis was carried out using Prism (Graph Pad Software).

Data was expressed as mean ± standard error of the mean (s.e.m). Where the N represents the number of animals used in every experiemnt. The difference between means was considered statistically significant at a value of $P < 0.05$. Statistical significance was determined using two-tailed unpaired Students t-test between two data sets or one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc test carried out as appropriate (Prism 6.0 software (San Diego, CA)).

Contractions to all agonists were measured in grams and expressed as a percentage of the KCl-induced contraction in porcine tissue and expressed as grams in rat tissue. In Electrical field stimulation experiment the responses were measured in grams and expressed as a percentage of NA-induced contractions. The contractions to 5HT and U46619 were expressed as a percentage of their own maximum response in porcine tissue and as grams in rat tissue. All data are presented as mean +/- standard error of the mean (s.e.m). In some experiments, the log concentration-response curves were used to determine potency (log EC_{50} values) and maximum response (R_{max}) values. EC_{50} is the concentration of a drug that produces a response equal to 50% of the maximum response (GraphPad Prism software).

To assess the affinity of antagonists at α_1-adrenoceptors in the porcine splenic artery, log EC_{50} values were obtained in the absence and presence of different concentrations of the antagonist and further used for Schild analysis, using the following equations:

$$DR = \frac{EC_{50}`}{EC_{50}}$$

$$KB = \frac{(DR - 1)}{A}$$

Where (DR) is concentration ratio, ($EC_{50}`$) is the concentration of the agonist that causes 50% of the observed action in presence of the antagonist, (EC_{50}) is the concentration of the agonist that causes 50% of the observed action in absence of the antagonist and (A) is the concentration of the antagonist. Subsequently a Schild plot (Arunlakshana *et al* 1959) was performed by plotting log (DR-1) against the logarithm of the concentration of the antagonist. From the equation, the slope of the plot was obtained and the intersection of the log (DR-1) with the x-axis was used to determine the -logK_B (pA_2) value of the antagonist.

In the endothelium dependent vasorelaxation study the data are presented as mean percentage relaxation of U46619-induced tone with standard error of the mean (s.e.m.). The maximum percentage relaxation (R_{max}) and the negative log of concentration required to produce half the maximal relaxation of the induced tone (EC_{50}) were calculated by fitting the data to the logistic equation:

$$R = \frac{R_{max} \, X \, A^{nH}}{EC_{50}{}^{nH} + A^{nH}}$$

Where R is the reduction in tone, R_{max} is the maximum vasorelaxation of the established tone; A is the concentration of the vasorelaxant. nH is the slope function and EC_{50} is the concentration of the vasorelaxant required to produce half the maximal relaxation (McCulloch & Randall, 1998). Data were analysed using one-way ANOVA was used for multiple groups, P-values of less than 0.05 were considered statistically significant.

2.7. Drugs and solutions

The following solutions used were:

Krebs–Henseleit solution contains (in mM/L): NaCl 118; KCl 4.8; $NaHCO_3$ 25; KH_2PO_4 1.25; $MgSO4.7H_2O$ 1.2; Glucose 11.1; and $CaCl_2$ 1.25.

Calcium-free Krebs–Henseleit solution

The composition of the calcium-free Krebs–Henseleit solution, in mM, was as follows: (118 mM NaCl, 4.8mM KCl, 1.1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11.6 mM D-glucose)

The following compounds were used

Ethylene diamine tetra-acetic acid disodium salt (EDTA) (BDH Laboratory Supplies, England), noradrenaline, carbenoxolone, 2-(2,6-Dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride (WB 4101) hydrochloride, 5HT 5-Hydroxytryptamine hydrochloride , pannexin 1 blockers – mefloquine and probenecid, Brilliant Blue FCF, Pyridoxalphosphate-6-azophenyl-2',4'- disulfonic acid (PPADS), thromboxane A_2 mimetic, 9,11-dideoxy-11 alpha, 9 alpha-epoxy-methano-prostaglandin F_2 alpha (U46619), 4,4',4'',4'''-[Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3 benzenedisulfonic acid, octasodium salt (NF449)**,** N-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, bradykinin and Guanethidine (Sigma, UK) and prazosin hydrochloride (Sigma, UK).

The drugs used in this study was made up in distilled water except for NA which was made up in 23µM EDTA, and probenecid, U46619 and mefloquine and indomethacin which were dissolved in ethanol with further dilutions made up from the original stock.

Antibodies

Anti- pannexin1, Anti- pannexin2 and Anti-pannexin3 antibodies used for western blotting were purchased from Abcam (UK) and LI-COR Biosciences. All antibodies were made up to a final concentration of 1:1 000 using semi-skimmed milk.

The goat anti-rabbit IR Dye antibody (secondary antibody) was purchased from LI-COR (UK) and was made to a final concentration of 1:1 000 using skimmed milk. An anti- pannexin1 (AV42783-50UG) antibody used for immunohistochemistry was purchased from Sigma, UK and was made up to a final concentration of 1:500 using semi- skimmed milk. The goat anti-rabbit IRDyeantibody (secondary antibody) FITC (F0382) was purchased also from Sigma, UK and was made up to a final concentrateion of 1:1000 in (PBS 0.1%, 0.1% Tween-20, 1%BSA). Goat anti-rabbit IgG (H+L) Secondary antibody Rodamine Red conjugate (R-6394) was purchased from (Thermo Scientific) and was made up to a final concentration of 1:1000 (PBS 0.1%, 0.1% Tween-20, 1%BSA).

Chapter 3

3. Pannexin function in porcine splenic artery

3.1. Introduction

Pannexin channels were first discovered by Panchin (2000) as integral membrane proteins in mammalian genomes. The pannexin family comprises three members, i.e. pannexin-1, pannexin-2, and pannexin-3 (Bond and Naus, 2014). These proteins have some resemblance in their membrane topology to connexons (Ambrosi *et al* 2010; Kar *et al* 2012). However, the role for pannexin-1 oligomers (that forms a channel between the cytosol and extracellular space) is different from that of connexon oligomers (that form intercellular gap junctions between adjacent cells). The key dissimilarity is possibly due to the mechanism whereby pannexin-1 is exceedingly glycosylated on its extracellular loops, which might inhibit docking with pannexin-1 channels on the adjacent cells (Ohbuchi *et al* 2011; Poornima *et al* 2012; Sorge *et al* 2012; Sumi *et al* 2010). Pannexin-1 is present in many human tissues (Cea *et al* 2014; Abeele *et al* 2006; Baranova *et al* 2004; Shestopalov and Panchin, 2008), whereas the expression of pannexin-2 is more limited to the central nervous system (Ray *et al* 2006; Dvoriantchikova *et al* 2006), and pannexin-3 is expressed in many cultured cell lines (Ishikawa *et al* 2011; Bond *et al* 2011; Turmel *et al* 2011). Recently pannexins have been shown to be expressed in the vasculature (Billaud *et al* 2011), and while their function has yet to be fully determined, Billaud *et al* (2011) proposed a mechanism by which the vasoconstriction induced by α_1-adrenoceptors in thoracodorsal resistance arteries involved pannexin1 channels**.** They found that phenylephrine acting upon the α_{1D}-adrenoceptors caused release of ATP through pannexin channels in mouse thoracodorsal small arteries. ATP then activated P2 receptors on neighbouring smooth

muscle cells to enhance vasoconstriction. They concluded that pannexin1 could contribute to the coordination of vascular smooth muscle cell constriction and the regulation of blood pressure as an intermediary for catecholamines released by sympathetic nerves (Billaud *et al* 2011). Although Billaud *et al* (2011) have presented evidence for the involvement of pannexins in controlling the constriction of thoracodorsal resistance arteries. The role of pannexins in mediating responses to NA in other arteries has not been studied. Hence, the aim of this chapter was to build on the research carried out by Billaud *et al* (2011) to provide an overall understanding of the role of pannexin in the regulation of vascular tone. The objectives were to determine (i) the presence of pannexins in PSA by (using Western blot and immunohistochemistry), (ii) the α_1-subtype population responsible for mediating NA-induced vasoconstriction in PSA, and (iii) whether pannexins are involved in mediating contractile responses produced by exogenous NA or activation of the sympathetic nerves in PSA.

3.2.Material and methods

3.2.1. Westren blotting

Porcine spleens were collected from the abattoir and processed as described in section 2.3. Samples were run on 7% SDS-PAGE gels using a Biorad Mini-protein 3 system as described in section 2.3 Subsequently, the nitrocellulose membrane was incubated overnight with the primary antibody Rabbit anti-pannexin1 (Abcam cat No. ab124969), Rabbit anti-pannexin2 (Abcam cat No. ab55917) and Rabbit anti-pannexin3 (Abcam cat No. ab98093 which were added at 1:1 000 concentration in 5%(w/v) milk solution, and mouse monoclonal anti-GAPDH antibody (G8795 Sigma,

UK) (1:40 000), followed by incubation with corresponding secondary antibodies IRDye®680LT Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) and IRDye® 800CW Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) for 1 hour at room temperature. After that the secondary antibodies were removed and the blots where washed the images where processed on a Licor Odyessy scanner using Image studio v3.0 software.

3.2.2. Immunohistochemistry

3.2.2.1. Paraffin embedding tissue and sectioning

Porcine splenic arteries were dissected from the spleen and processed as described in section 2.4.1. Immunohistochemistry experiment was performed as described in section 2.4.1. The primary antibodies were diluted in (PBS 0.1%, 0.1% Tween-20, 1%BSA) and incubated overnight, while the secondary antibody, goat anti-rabbit FITC, was diluted in (PBS 0.1%, 0.1% Tween-20, 1%BSA) to a dilution of 1:500. And applied to the sections for 1 h. Following this they were incubated with DAPI (1in 500 dilution) for 5 min in the dark. Coverslips were placed on microscope slides using mounting medium (1% (v/v) 1, 4-diazobicyclo-[2, 2, 2,]-octane (DABCO) DABCO fluorescence mounting medium-SS04. The slides and sections were examined at X20 and X40 using a light microscope and a wide field Fluorescence microscope to assess pannexin location. The following filters were used to visualise the immunostains. DAPI - Excitation 350/50nm, emission 460/50 nm, FITC - Excitation 480/40nm, emission 535/50 nm to visualise the Goat anti-rabbit Antibody.

3.2.3. Isometric tension recording

3.2.3.1. Tissue collection and sample preparation

Porcine splenic arteries were dissected cut into 4 equal strips approximately 5mm in length as described in section 2.5.k Four sets of experiments were carried out to investigate the contractile response of porcine splenic arteries to NA in the absence and presence of pannexin inhibitors (mefloquine or probenecid or carbenoxolone) or P2 receptor antagonist (PPADS or suramin).

3.2.3.2. The general protocol for recording isometric tension

As described in section 2.5, segments were exposed to increasing concentrations of NA (10^{-8} -10^{-4}M) raised in 3–fold increments to generate cumulative concentration curves (CCRC) to NA. The concentration of NA was changed by adding appropriate amounts of NA to the bath as soon as the previous concentration had produced a plateau. After the CCRC was completed, tissues were washed and the whole procedure was repeated again to check the reproducibility of the CCRC curves after a further 30-minute equilibration period. This was compared to CCRC curves to NA generated in the presence of either suramin (10^{-4}M), PPADS (10^{-5}M), mefloquine ($2x10^{-5}$M) or probenecid ($5x10^{-4}$M).

Experiments were also carried out on porcine splenic artery using 5HT and U46619 as contractile agents. 5HT was applied at concentrations between $3x10^{-10}$ and 10^{-8}M and U46619 at concentrations from $3x10^{-8}$ to 10^{-6}M to produce cumulative concentration curves. The effect of mefloquine ($2x10^{-5}$) M on 5HT and U46619 responses were then determined.

3.2.3.3. Electrical field stimulation experiment in porcine splenic artery

Porcine splenic artery ring segments were set up as described previously. Tissues were exposed to NA (10^{-5} M), followed by a washout using Krebs'-Henseleit solution. Exposure to NA was repeated at least twice before tissues were exposed to electrical field stimulation (EFS). EFS was applied to the artery segments at increasing frequencies (1, 2, 5, 10, 20Hz, at a voltage of 20 V, pulse width 0.5ms, for 10s) at 5 minutes intervals using an SRI electrical stimulator. Frequency- response curves were repeated in the presence and absence of mefloquine ($2x10^{-7}$, $2x10^{-6}$, $2x10^{-5}$), probenecid ($5x10^{-4}$M) carbenoxolone (10^{-5}, $2x10^{-5}$ and10^{-4} M), suramin (10^{-4}M), and PPADS (10^{-5}M).

3.2.4. Statistical analysis

As described in section 2.6.

3.3.Results

3.3.1. Western Blotting analysis

Western blot analysis was carried out to determine pannexin1 and pannexin2 expression in PSA using anti-pannexin1 and 2 specific antibodies.

1. Pannexin1 expression in porcine splenic artery

The expression of pannexin1 was shown as a 50 kDa band in all samples. The lanes show splenic artery samples from 11 different pigs. The 50 kDa bands observed during

western blotting correspond to pannexin1 protein (http://www.abcam.com/pannexin-1-antibody-EPR5556-ab124969-references.html) (Figure 3.1).

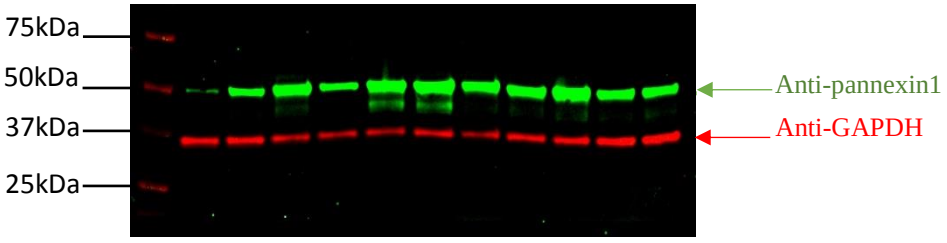


Figure 3.1 Western blot analysis to determine pannexin1 expression in porcine splenic artery. N =11.

2. Pannexin2 and pannexin3 expression in porcine splenic artery

The expression of pannexin2 was shown as a 100kDa band in all lanes. The lanes show splenic artery samples from 4 different pigs. The 100kDa bands observed during western blotting correspond to the pannexin2 protein (http://www.abcam.com/pannexin-2-antibody-ab55917-references.html). In addition, the expression of pannexin3 (http://www.abcam.com/pannexin-3-antibody-ab98093.html). Was examined using anti-pannexin3 antibody, but the expression of pannexin3 was not evident in PSA as we did not detect a band corresponding to pannexin3 (Figure 3.2).

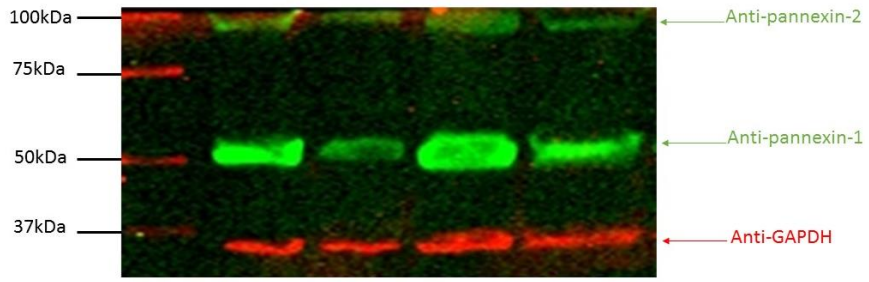


Figure 3.2. Western blot analysis to determine pannexin1 and pannexin2 expression in porcine splenic artery. N = 4.

3.3.2. Pannexin1 expression assessed by immunohistochemistry in porcine splenic artery

Immunolabelling of pannexin1 using anti-pannexin1 specific antibody was carried out in cross sections and showed endothelium and smooth muscle cells of porcine splenic artery labelled for presence of pannexin1 under light and fluorescence microscope at 20X and 40X. The image shows expression of pannexin1 in the endothelium and the smooth muscle cells of porcine splenic artery (Figure 3.3).

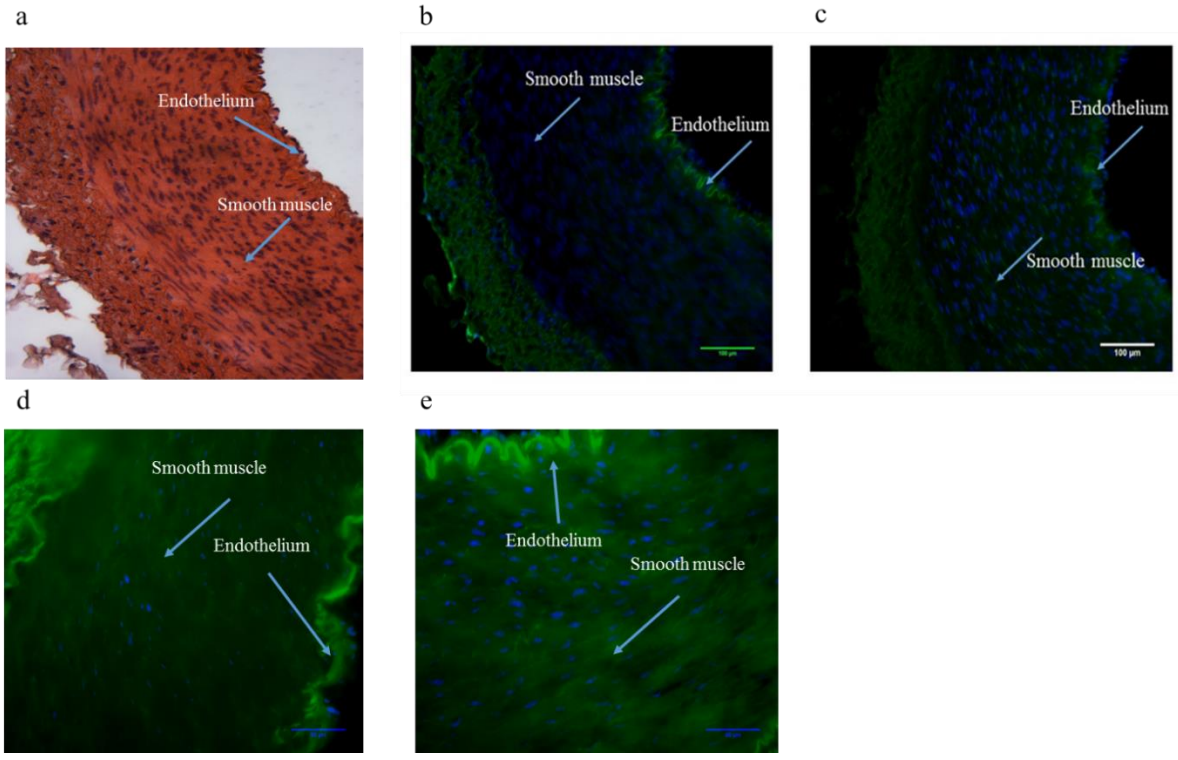


Figure 3.3. Light microscopic images of porcine splenic artery at X20 (a) stained without primary antibody. Expression of pannexin1 shown by immunohistochemistry in the absence (b) and presence (c) of anti-pannexin1 specific antibody at X20. Expression of pannexin1 shown by immunohistochemistry in the absence (d) and presence (e) of anti-pannexin1 specific antibody at X40. Expression was evident in the endothelium and the smooth muscle cells of porcine splenic artery. N =6.

3.3.3. Isometric tension recording

3.3.3.1. Eeffect of noradrenaline on tension of porcine splenic artery

To test whether the response to NA was reproducible in PSA, consecutive concentration-response curves were obtained. The maximum responses did not significantly alter between the two curves; R_{max} values were 230.3 ± 27.58% and 221.4 ± 37.8% of the KCl response for curves one and two, respectively (Figure 3.4). Similarly, the sensitivity to NA did not change between the curves; Log EC_{50} values were -6.04 ± 0.27 and -6.33 ± 0.28 for curves one and two, respectively ($p > 0.05$, Student's *t-test*, N = 8).

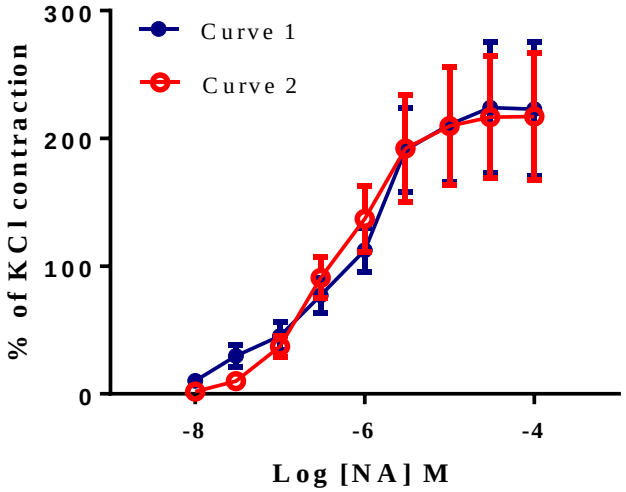


Figure 3.4 Responses to noradrenaline in the porcine splenic artery. The contractions caused by noradrenaline were expressed as a percentage of the contraction produced by KCl. Data are presented as the mean value ± s.e.m. There was no significant difference between the concentration-response curves ($P > 0.05$, Student's *t-test*, N = 8).

3.3.3.2. Effect of mefloquine on noradrenaline induced contraction in porcine splenic artery

Mefloquine at $2x10^{-5}$M reduced the magnitude of NA-induced contraction in PSA. The maximum responses to NA were 231.2 ± 8.87% in the absence and 118.3 ± 9.44% in the presence of mefloquine ($P < 0.0001$, Student's *t-test*, N=7) (Figure 3.5). However, mefloquine did not alter the sensitivity of responses to NA in PSA; log EC_{50} values were -5.69 ± 0.08 in the absence and -5.62 ± 0.16 in the presence of mefloquine ($P > 0.05$, Student's *t-test*) (Figure 3.5).

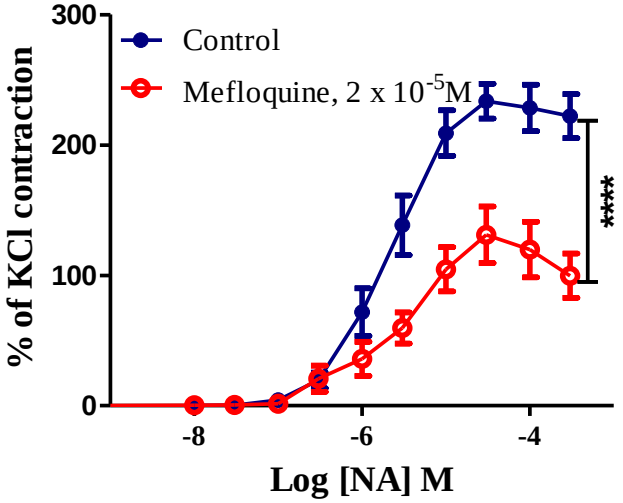


Figure 3.5 Effect of mefloquine ($2 x10^{-5}$M) on the response to noradrenaline in the porcine splenic artery. The contractions caused by noradrenaline were expressed as a percentage of the contractions produced by KCl. Data are presented as the mean value ± s.e.m. Differences between R_{max} values are shown by **** $P < 0.0001$ (Student's paired *t-test*, N=7).

3.3.3.3. Effect of probenecid on noradrenaline induced contraction in porcine splenic artery

Probenecid ($2x10^{-3}M$) significantly reduced the response to NA in the PSA. The responses to NA ($3x10^{-4}M$) were reduced from 158.83 ± 11.14% in the absence to 96.64 ± 14.14% in the presence of probenecid ($2x10^{-3}M$) ($p < 0.001$, Student's *t-test*, N =8) (Figure 3.6). However, probenecid did not alter the sensitivity of responses to NA in PSA ($p > 0.05$, Student's *t-test*) (Figure 3.6).

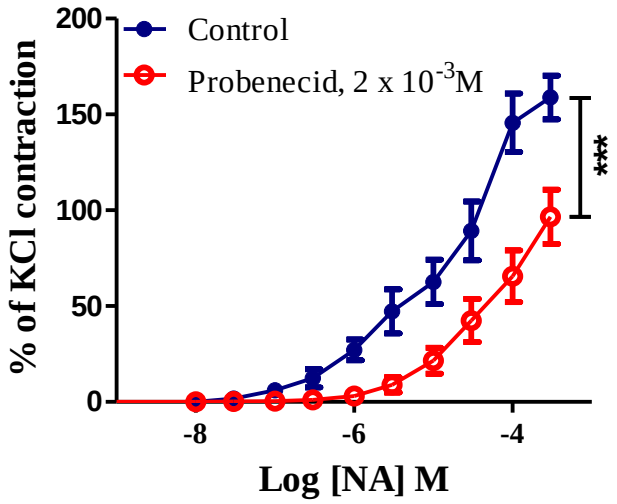


Figure 3.6 Effect of probenecid ($2x10^{-3}M$) on the response to noradrenaline in the porcine splenic artery. The contractions caused by noradrenaline were expressed as a percentage of the contractions produced by KCl. Data are presented as the mean value ± s.e.m. Differences between R_{max} values are shown by *** $P < 0.001$ (Student's paired *t-test*, N = 8).

3.3.3.4. Effect of carbenoxolone on noradrenaline-induced contraction in porcine splenic artery

Carbenoxolone, used at three different concentrations of 10^{-5}, $2x10^{-5}$ and 10^{-4}M did not alter the magnitude of NA-induced contraction in PSA. The maximum responses to NA were 149.9 ± 11.31% (control) and 145.8 ± 7.84% in the presence of carbenoxolone (10^{-5}M) ($p > 0.05$, Student's paired *t-test,* N = 5), the sensitivity (Log EC_{50}) was -6.25 ± 0.13 (control) and -6.75 ± 0.13 (carbenoxolone (10^{-5}M)) ($p > 0.05$, Student's paired *t-test*) (Figure 3.7a). The maximum responses to NA were 170.9 ± 34.65% (control) and 143.7 ± 17.17% in the presence of carbenoxolone ($2x10^{-5}$M) ($p > 0.05$, Student's paired *t-test,* N = 5), the sensitivity was -5.84 ± 0.27 (control) and -5.65 ± 0.14 (carbenoxolone ($2x10^{-5}$M)) ($p > 0.05$, Student's paired *t-test*) (Figure 3.7b). The maximum responses to NA was 175.7 ± 24.86% (control) and 156.5 ± 16.07% in the presence of carbenoxolone (10^{-4}M) ($p > 0.05$, Student's paired *t-test,* N = 5), the sensitivity was -6.07 ± 0.21 (control) and -5.80 ± 0.13 (carbenoxolone (10^{-4}M) ($P>0.05$, Student's paired *t-test*) (Figure 3.7c).

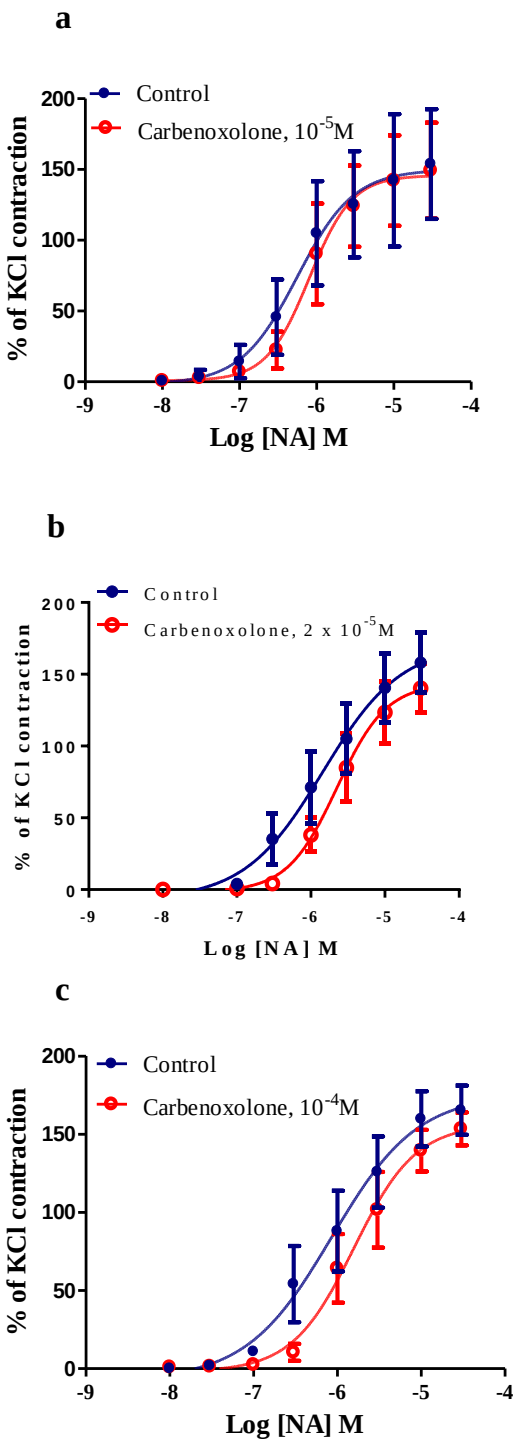


Figure 3.7 Effect of carbenoxolone (10^{-5}, $2x10^{-5}$ and10^{-4}M) on the response to noradrenaline in the porcine splenic artery. The contractions produced by noradrenaline were expressed as a percentage of the contractions caused by KCl. Data are presented as the mean value ± s.e.m. Carbenoxolone did not alter the contraction to noradrenaline in porcine splenic artery (P>0.05, Student's *t-test*, N = 5).

3.3.3.5. Effect of suramin and PPADS on noradrenaline-induced contraction in porcine splenic aretry

Suramin (10^{-4}M) did not reduce the magnitude of NA-induced contraction in PSA. The maximum responses to NA were 186.2 ± 76.80% in the absence and 132.9 ± 19.18% in the presence of suramin (P >0.05, Student's *t-test,* N =8) (Figure 3.8a). Suramin did not alter the sensitivity of responses to NA in PSA; log EC_{50} values were -5.99 ± 0.54 in the absence and in -6.41 ± 0.19 the presence of suramin (P >0.05, Student's *t-test*). Likewise, PPADS (10^{-5}M) did not alter the magnitude of NA-induced contraction in PSA. The maximum responses to NA were 175.0 ± 8.48% in the absence and 188.7 ± 14.21% in the presence of PPADS (10^{-5}M) (P >0.05, Student's *t-test,* N =8) (Figure 3.8b). Also, PPADS did not alter the sensitivity to NA; log EC_{50}values were -5.92 ± 0.10 in the absence and -5.79 ± 0.15 in the presence of PPADS (P >0.05, Student's *t-test*).

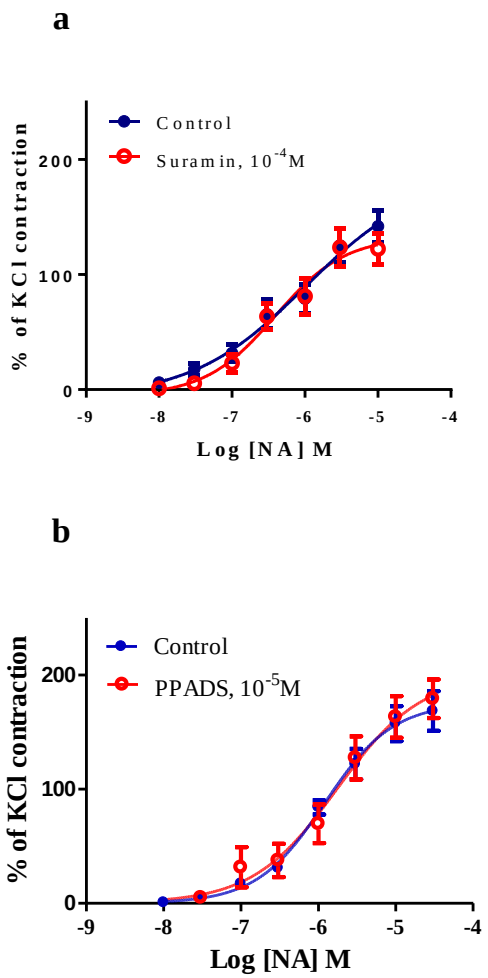


Figure 3.8 Effect of suramin (10^{-4}M) (a) and PPADS (10^{-5}M) (b) on the response to noradrenaline in the porcine splenic artery. The contractions caused by noradrenaline were expressed as a percentage of the contractions caused by KCl. Data are presented as the mean value ± s.e.m. Suramin (a) and PPADS (b) did not change the contraction to noradrenaline in the porcine splenic artery (P>0.05, Student's *t-test*, N =8).

3.3.3.6. Reproducibility of responses to EFS in porcine splenic artery

To examine whether the vasoconstriction response to EFS was reproducible in PSA, consecutive responses to EFS were obtained. EFS-induced contraction of PSA was reproducible as there were no significant differences between the responses (P > 0.05, Two-way ANOVA, N = 6) (Figure 3.9).

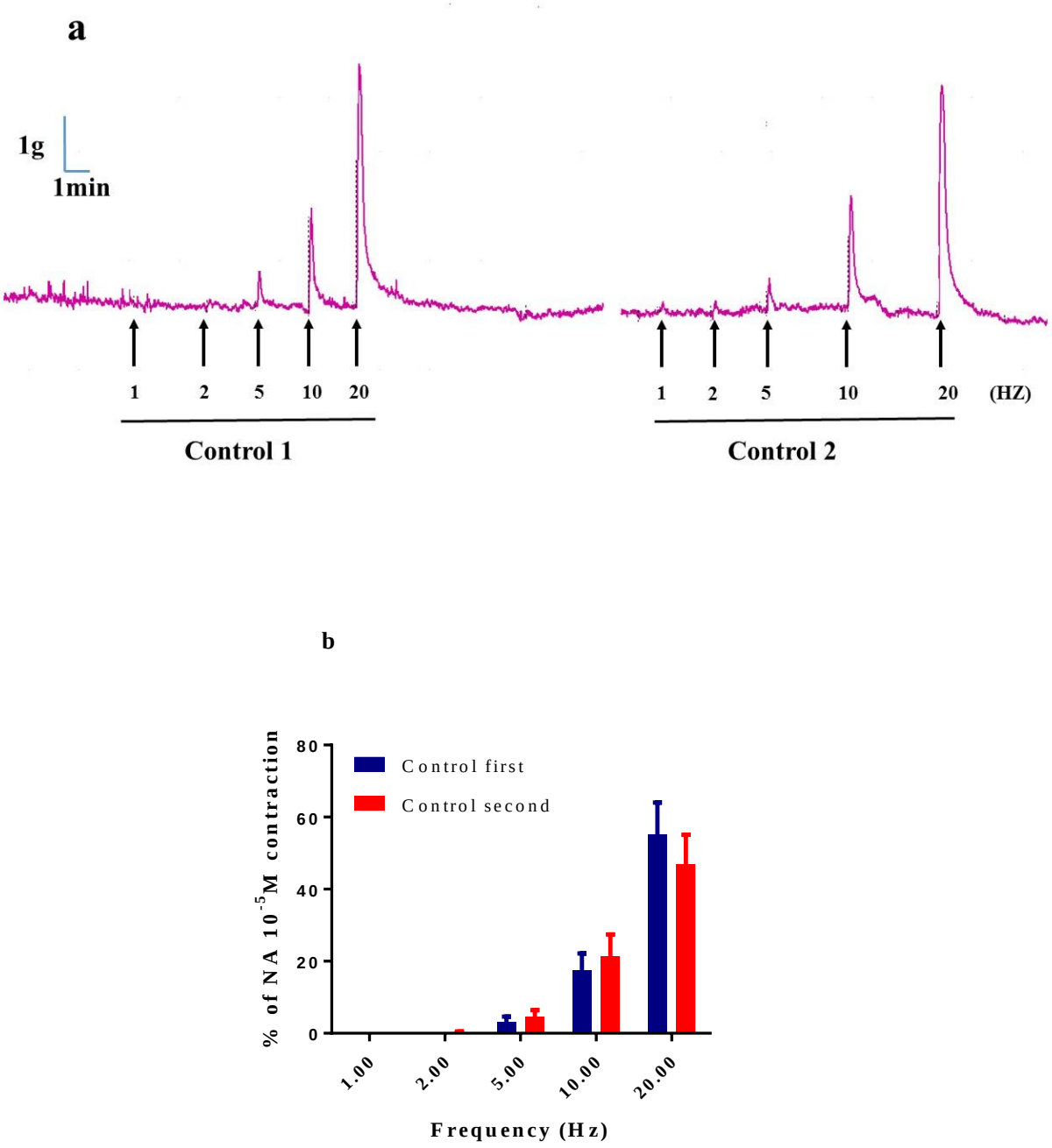


Figure 3.9 (a) Representative trace showing the vasoconstrictor responses to nerve stimulation in porcine splenic artery. (b) EFS responses to 5 different frequencies (1, 2, 5, 10, 20Hz, 90 V, 5ms, 10s), the contraction was expressed as a percentage of the response produced by noradrenaline (10^{-5}M). Each Colum represents the mean ± s.e.m. There was no significant difference between the responses ($P > 0.05$, Two-way ANOVA, N = 6).

3.3.3.7. Role of α_1-adrenoceptors and/or P2X receptors in mediating electrically-evoked vasocontractile responses in porcine splenic artery

Under basal tone conditions, EFS elicited frequency-dependent vasoconstrictor responses in the PSA (20V, pulse width 0.5msec) at frequencies 1, 2, 5, 10 and 20 Hz applied for 10s. Guanethidine, a sympatholytic compound ($5x10^{-6}$M), abolished vasoconstriction response evoked by EFS in the PSA at 10Hz and 20Hz ($p<0.05$, $p<0.0001$, Two-way ANOVA) (Figure 3.10).

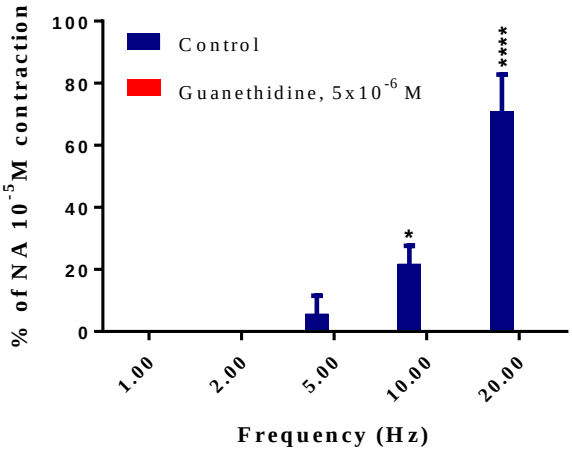


Figure 3.10 Electrical field stimulation responses to 5 different frequencies (1, 2, 5, 10, 20Hz, 90 V, 5ms, 10s) were expressed as a percentage of the response produced by noradrenaline (10^{-5}M). Each Column represents the mean ± s.e.m. Contractile responses were abolished in the presence of guanethidine ($5x10^{-6}$) (*P < 0.05, ****P < 0.0001, Two-way ANOVA, N = 4).

3.3.3.8. Effect of mefloquine on responses to EFS in the porcine splenic aretry

Mefloquine at $2x10^{-6}$ and $2x10^{-7}$M did not alter the contraction-induced by EFS ($p > 0.05$, Two-way ANOVA) (Figure 3.11a, b). Mefloquine at $2x10^{-5}$ M significantly reduced the vasoconstriction response evoked by EFS in the porcine splenic artery at 20Hz ($p < 0.0001$, Two-way ANOVA, N = 8) (Figure 3.11c).

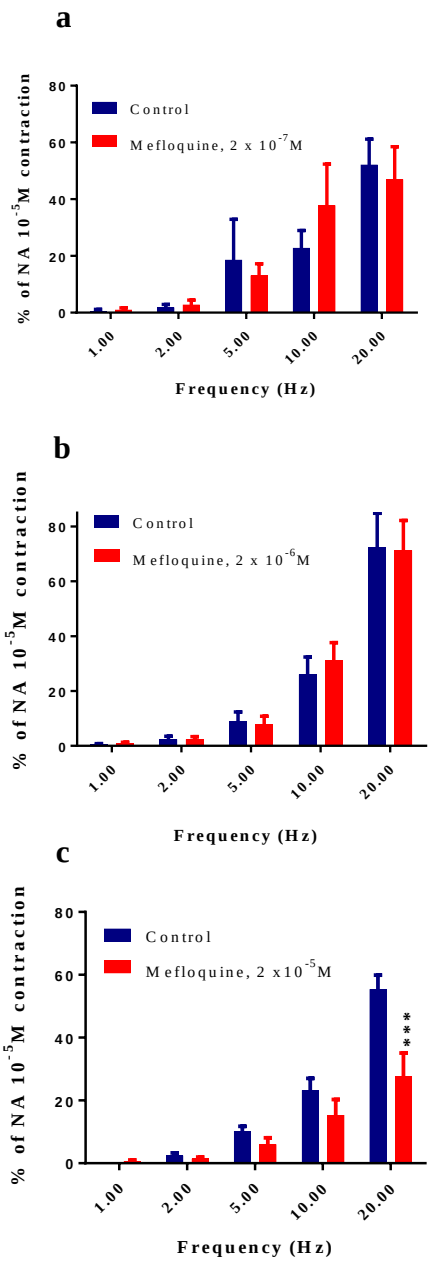


Figure 3.11. Effect of mefloquine ($2x10^{-7}$, $2x10^{-6}$, $2x10^{-5}$M) on the response to EFS in the porcine splenic artery. Responses to nerve stimulation were expressed as a percentage of the response produced by noradrenaline (10^{-5}M). Each column represents the mean ± s.e.m. Mefloquine at $2x10^{-7}$ (a) and $2x10^{-6}$M (b) did not alter the contraction-induced by EFS ($P > 0.05$, Two-way ANOVA, N = 8). Mefloquine at $2x10^{-5}$M (c) significantly reduced responses to EFS in porcine splenic artery at the highest frequency applied (20Hz) (***$P < 0.0001$, Two-way ANOVA, N = 8).

3.3.3.9. Effect of probenecid on responses to EFS in the porcine splenic artery

Probenecid ($2x10^{-3}M$) significantly reduced the contraction response evoked by EFS in the PSA at 10Hz and 20Hz ($P < 0.001$, Two-way ANOVA, N = 8) (Figure 3.12).

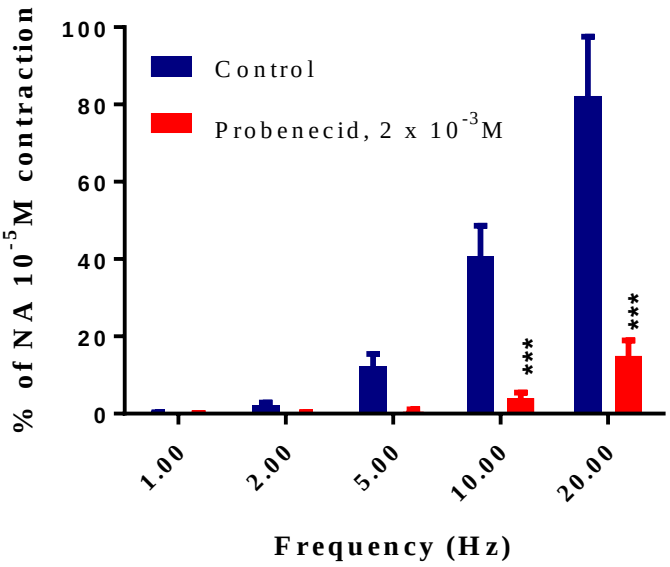


Figure 3.12. Effect of probenecid ($2x10^{-3}M$) on the response to EFS in the porcine splenic artery. Responses to nerve stimulation were expressed as a percentage of the response produced by noradrenaline (10^{-5}). Each column represents the mean ± s.e.m. Probenecid decreased the contraction to EFS at 10Hz and 20 Hz (***$P < 0.001$, Two-way ANOVA, N = 8).

3.3.3.10. Effect of carbenoxolone on responses to EFS in the porcine splenic artery

Carbenoxolone (10^{-4}M) did not alter the contraction to EFS in PSA (Figure 3.13).

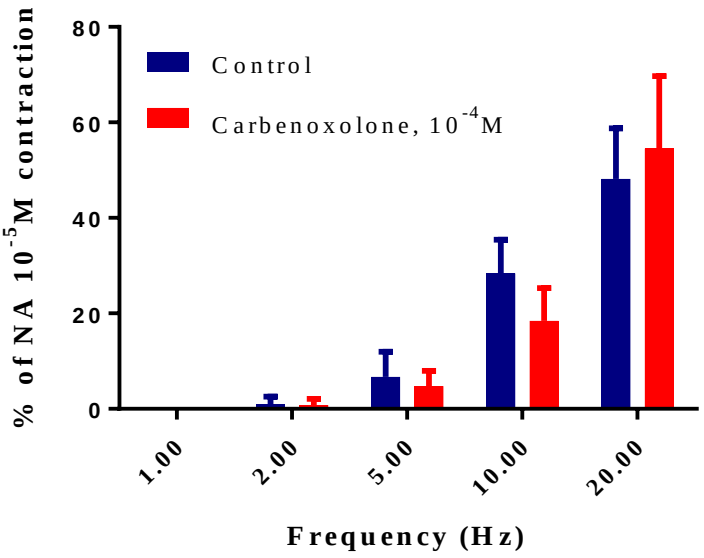


Figure 3.13. Effect of carbenoxolone (10^{-4}M) on the response to EFS in the porcine splenic artery. Responses to nerve stimulation were expressed as a percentage of the response produced by noradrenaline (10^{-5}). Each column represents the mean ± s.e.m. Carbenoxolone had no significant effect on the contraction to EFS ($P > 0.05$, Two-way ANOVA, N = 4).

3.3.3.11. Effect of suramin on responses to EFS in porcine splenic artery

Suramin ($3x10^{-4}$M) did not alter the contraction to EFS in PSA (Figure 3.14).

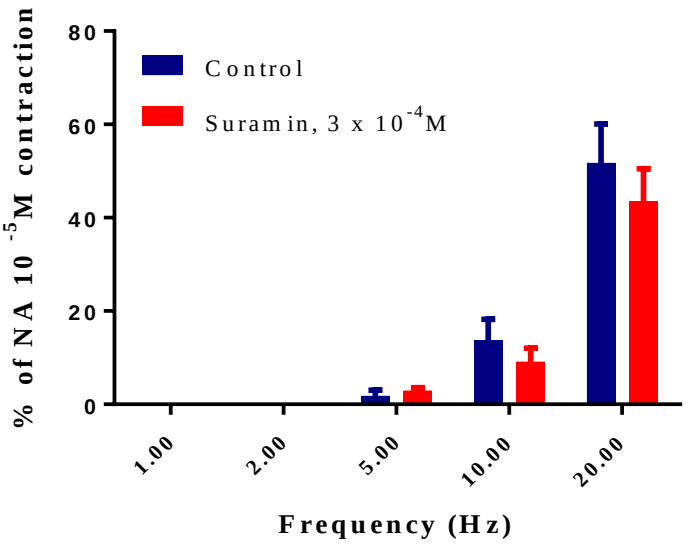


Figure 3.14. Effect of suramin ($3x10^{-4}$M) on the response to EFS in the porcine splenic artery. Responses to nerve stimulation were expressed as a percentage of the response produced by noradrenaline (10^{-5}M). Each column represents the mean ± s.e.m. Suramin had no significant effect on the contraction to ESF ($P > 0.05$, Two-way ANOVA, N = 5).

3.3.3.12. Effect of PPADS on responses to EFS in the porcine splenic artery

PPADS (10^{-5}M) did not alter the contraction to EFS in PSA (Figure 3.15).

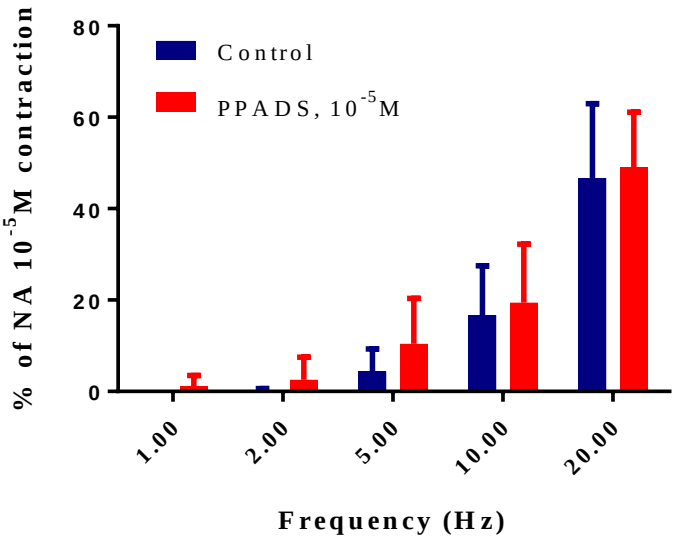


Figure 3.15. Effect of PPADS (10^{-5}M) on the response to EFS in the porcine splenic artery. Responses to nerve stimulation were expressed as a percentage of the response produced by noradrenaline (10^{-5}). Each column represents the mean ± s.e.m. PPADS had no significant effect on the contraction to ESF (P>0.05, Two-way ANOVA, N = 8).

3.3.3.13. Effect of mefloquine to 5-HT and U46619 in the porcine splenic artery

In order to investigate the specificity of mefloquine, responses to 5-HT and U46619 were obtained in the absence and presence of mefloquine ($2x10^{-5}$M). Mefloquine significantly reduced the maximum response to 5-HT in the PSA. The maximum responses to 5-HT were reduced from 125.78 ± 28.1% in control to 18.86 ± 8.47% in the presence of mefloquine ($p < 0.001$, Student's paired *t-test*; N = 4) (Figure 3.16a). In addition, mefloquine ($2x10^{-5}$M) reduced the response to U46619 in the PSA. The

maximum responses to U46619 were reduced from 117.7 ± 9.057% in control to 125.7 ± 6.2% in the presence of mefloquine (P < 0.001, Student *t-test*, N=4) (Figure 3.16b).

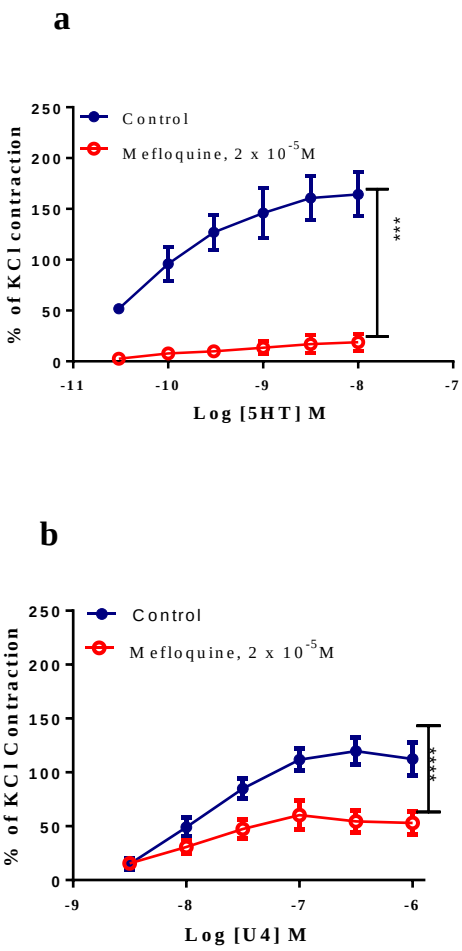


Figure 3.16 Effect of mefloquine ($2x10^{-5}$M) on the responses to 5-HT (a) and U46619 (b) in the rat aorta. The contractions caused by 5-HT and U46619 are represented as a percentage of the contractions caused by KCl (60mM). Data are presented as the mean value ± s.e.m. Differences between R_{max} values are shown by ***P < 0.001, (Student's paired *t-test*, N = 4).

3.3.3.14. The effect of α_{1A}-adrenoceptor antagonist on responses to phenylephrine in the porcine splenic artery

PSA was exposed to cumulative concentrations of phenylephrine (10^{-8} to $3x10^{-4}$M) in the absence and presence of three concentrations of WB 4101 (10^{-7}, $3x10^{-7}$ and 10^{-6}M), then a Schild plot (N = 6) was constructed. WB 4101 caused a concentration-dependent rightward displacement of the phenylephrine concentration-response curve. The data from six observations was drawn for which the slope was 1.21 ± 0.17, pA_2 value was 9.67 (Figure 3.17).

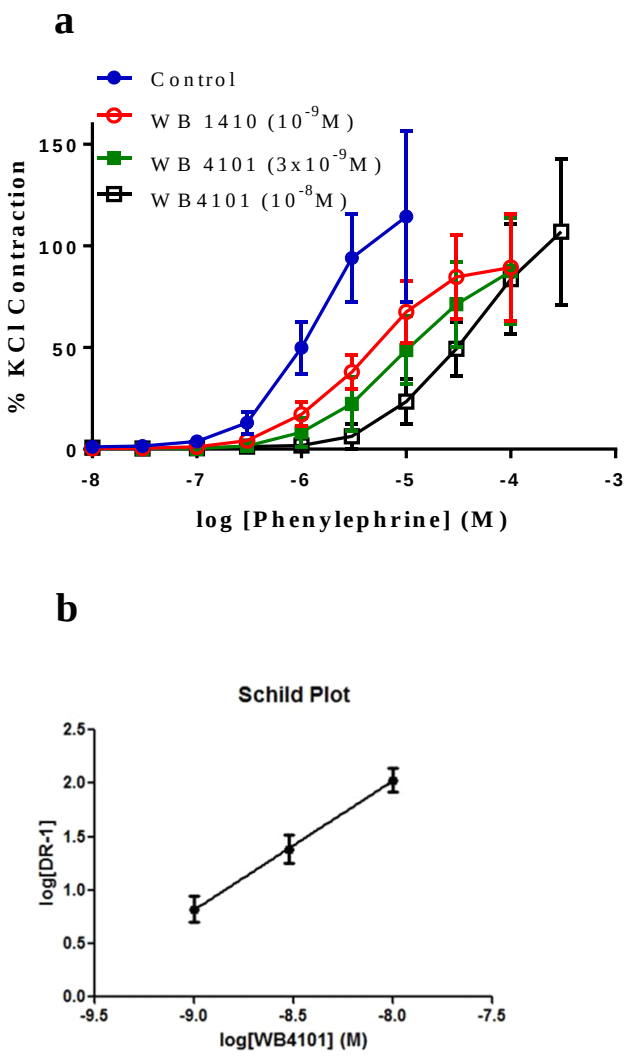


Figure 1.17 (a) The response to cumulative concentrations of phenylephrine (10^{-8} to $3x10^{-4}$M) were determined in the absence and presence of WB 4101 (10^{-7}, $3x10^{-7}$ and 10^{-6} M). The contractions caused by phenylephrine were expressed as a percentage of KCl-induced contraction. Data are presented as the mean value ± s.e.m (N = 6). (b) Schild plot was presented by plotteing the logarithm of dose ratio-1 (DR-1) against the logarithm of the concentration of the antagonist. (N = 6).

3.3.3.15. Effect of α_{1D}-adrenoceptor antagonist, BMY 7378, on responses to phenylephrine in the porcine splenic artery

PSA was exposed to cumulative concentrations of phenylephrine (10^{-8} to $3x10^{-4}$M) in the absence and presence of three concentrations of BMY 7378 (10^{-9}, $3x10^{-9}$ and 10^{-8} M), then a Schild plot (N = 6) was constructed. The data from six observations was drawn for which the slope was 1.08 ± 0.26 and the pA_2 value was 7.35 (Figure 3.18).

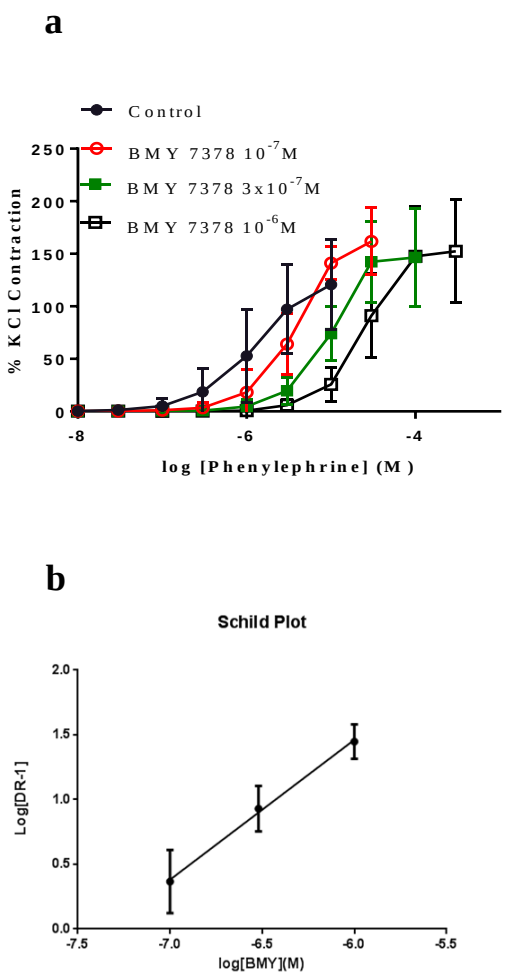


Figure 3.18 (a) The response to cumulative concentrations of phenylephrine (3×10^{-8} to 10^{-4}M) were determined in the absence and presence of BMY 7378 (10^{-8}, $3x10^{-8}$ and 10^{-7} M). The contractions caused by phenylephrine were expressed as a percentage of KCl-induced contraction. Data are presented as the mean value ± s.e.m (N = 6). (b) Schild plot was presented by plotting the logarithm of dose ratio-1 (DR-1) against the logarithm of the concentration of the antagonist. (N = 6).

3.4. Discussion

The aim of this chapter was to determine whether pannexins were present in the porcine splenic artery (PSA) and to examine their possible function. The results from the Western blot studies showed that pannexin1 and pannexin2 are both present in PSA. Inhibitors of pannexin channels (i.e. mefloquine and probenecid) significantly reduced the responses of PSA to (i) NA-induced contractions and (ii) the frequency-dependent response curves generated to nerve stimulation. In contrast, carbenoxolone, one of the first agents shown to directly inhibit pannexin1 currents (Bruzzone *et al* 2005), had no effect on responses to either exogenous NA or nerve stimulation in the PSA. Furthermore, neither suramin nor PPADS (P2 receptor antagonists) had any effect on responses to either exogenous NA or nerve stimulation. Thus, the present data do not support the hypothesis proposed by Billaud *et al* (2011), who suggested alpha-adrenoceptor stimulation involved the release of ATP through pannexin channels, to invoke a multicellular contraction by activating P2 purinoceptors. The actions of mefloquine may be non-selective since it also reduced responses to 5-HT and U46619.

3.4.1. Expression of pannexin in porcine splenic artery

Western blot analysis was carried out using anti-pannexin1 and anti-pannexin 2 antibodies. Both pannexin1 and pannexin2 expression was shown in PSA tissues. Both pannexin1 and pannexin2 were detected in PSA at 50kDa and 100kDa respectively, however pannexin3 was not detected. The primary antibody used was rabbit anti-pannexin antibody while goat anti-rabbit was used as a secondary antibody. Burns *et al* (2012) have shown the presence of pannexin1, pannexin2 and pannexin3 in rat middle cerebral artery.

3.4.2. Localization of pannexin1

Immunolabelling of pannexin1 in PSA shows pannexin1 expression in both endothelial and smooth muscle cells. Pannexin1 has been observed in both the endothelium and smooth muscle of smaller arteries and arterioles. It is restricted to the endothelium in rat aorta and femoral artery (Billaud *et al* 2011; Lohman *et al* 2012), and the smooth muscle cells of the rat middle cerebral artery (Burns *et al* 2012). Pannexin2 has been found in endothelial and smooth muscle cells of the rat middle cerebral artery (Burns *et al* 2012), and pannexin3 has been reported in small arteries less than 100 μm in diameter, particularly in the kidney (Lohman *et al* 2012). Pannexin1 expression has also been reported in human erythrocytes (Locovei *et al* 2006), although a more recent experiment to detect the protein in these cells with different antibodies was unsuccessful (Melhorn *et al* 2013)

Billaud *et al* (2011) suggested that pannexin1 contributes to α_1-adrenoreceptor-mediated vasoconstriction in thoracodorsal resistance arteries isolated from mice. Both pannexin1 inhibitors (i.e. mefloquine and probenecid) and purinergic receptor antagonists (i.e. suramin and reactive blue-2) were shown to reduce phenylephrine -induced vasoconstriction. Therefore, it has been speculated that phenylephrine binds to the α_{1D}-adrenoreceptor, causing pannexin1 to open to release ATP, which then acts as an intercellular messenger to cause P2 receptor-mediated contractions.

3.4.3. Effect of pannexin inhibition on responses to noradrenaline and EFS in the porcine splenic artery

Although the mechanism by which mefloquine and probenecid bind to pannexin channels remain speculative, one theory proposed is that probenecid (a blocker of organic anion transporters) may either access pannexin channels through the lipid bilayer or interact either with hydrophilic aspects of the protein (Silverman *et al* 2008). Probenecid is known to inhibit pannexin1 channels with an IC_{50} of ~150 μM, whereas it does not have an effect on connexin-based channels regardless of its concentration (Silverman *et al* 2008).

In the present study, at concentrations reportedly selective for pannexin channels, both probenecid and mefloquine reduced NA-induced contractions and those caused by activating the sympathetic nerves in the PSA. These data could be taken as evidence that pannexin channels are involved in conducting responses to NA in this blood vessel. This is similar to the observations in TDA from mice where 10 μM and 20 μM of mefloquine and 500μM of probenecid produced a considerable inhibitory effect on the response of TDA of mice to phenylephrine (a selective α1-adrenoreceptor agonist). However, there are justifiable grounds to question whether these compounds are acting selectively. Angus *et al* (2015) recently showed mefloquine (10–20 μM) to demonstrate a wide range of inhibitory actions. For example, mefloquine (3–10 μM) inhibited the contractions mediated by vasopressin, phenylephrine, endothelin-1, U46619, sympathetic nerve stimulation and K^+ (40 mM) in rat and mouse small mesenteric arteries. In the present study, the present study also showed that mefloquine inhibited responses to both 5-HT and the thromboxane mimetic, U46619. It is possible that pannexins are involved in transducing responses to any G-protein coupled receptor.

However, data presented in chapter 4 shows that inhibition of the influx of calcium is a more likely consequence, such that mefloquine produces a non-selective inhibition of contractile responses. Mefloquine (10 μm) has been shown to inhibit L-type Ca^{++} currents in single ventricular myocytes (Coker *et al* 2000).

Further evidence against a role for ATP released from inside cells via pannexin channels in the PSA is provided by the lack of effect of carbenoxolone. This compound has been shown to interact with pannexins. For example, Sridharan *et al* (2010) showed carbenoxolone (100 μM) to inhibit the hypotonically induced ATP release from human erythrocytes. Even at this concentration, carbenoxolone had no effect on NA responses, either exogenous, or after nerve activation. Similarly, Angus *et al* (2015) showed that carbenoxolone had no effect on agonist or nerve-induced activation of alpha-adrenceptors in rat small mesenteric resistance arteries.

Furthermore, while suramin (100 μM to 300 μM) has been shown to inhibit P2 receptors, leading to decreased contractility of TDA induced by phenylephrine (Bilaud *et al* 2011). Neither suramin nor PPADS had any effect on responses to exogenous NA in the PSA, ruling out a role for ATP-activation of P2 receptors in transducing an α_1-adrenoceptor-mediated response in the PSA. Interestingly, neither P2 receptor antagonist affected responses to nerve stimulation indicating that ATP is not a functional sympathetic neurotransmitter in this preparation, in contrast to some other blood vessels including rat mesenteric arteries (Rummery *et al* 2007; Pakdeechote *et al* 2007) and porcine mesenteric arteries (Shatarat *et al* 2014), although in the latter preparation, some pharmacological manipulation was required before a nerve-mediated purinergic response was observed.

3.4.4. α_1-adrenoceptor subclassification

Billaud *et al* (2011) reported that the pannexin channels formed a signalling domain with α_{1D}-adrenoceptors, therefore, to determine the subtype of α_1-adrenoceptor present in the PSA experiments were conducted with subtype selective α_1-adrenoceptor antagonists. The pA_2 values obtained for WB-4101 and BMY-7378 were 9.67 and 7.35 respectively. The slopes for either antagonist did not vary from unity showing that both antagonists competitively antagonise phenylephrine-induced contractile responses in the PSA (Barbieri *et al* 1998; Muramastsu *et al* 1990). These pA_2 values are in agreement with the values available in the literature for WB-4101 (9.46) and BMY-7378 (6.91) antagonists acting at α_{1A}-adrenoceptors and α_{1D}-adrenoceptors respectively (Barbieri *et al* 1998), and identify α-adrenoceptors in the PSA as being predominantly α_{1A}-adrenoceptors. In another study, WB-4101 antagonised α_{1A}–adrenoceptors in rat caudal arteries with a pA_2 value of 9.14 (Villalobos-Molina and Ibarra, 1996).

The aim of the next chapter therefore, was to examine whether pannexins were involved in mediating responses to NA in a preparation that contained α_{1D}-adrenoceptors, namely the rat thoracic aorta.

Chapter 4

4. Pannexin function in rat aorta

4.1.Introduction

The sympathetic nervous system plays a significant role in controlling blood pressure by acting at two types of adrenoceptors, i.e. α–and β–adrenoceptors (Nash, 1990). The α–adrenoceptors have been divided into two subtypes: α_1–adrenoceptors (located mostly on postjunctional vascular smooth muscle cells) and α_2–adrenoceptors (located on prejunctional sympathetic nerve endings and postjunctionally in some blood vessels) (Nash, 1990). The α_1-adrenoceptors are G protein-coupled receptors that signal thorough $G\alpha_{q/11,}$ leading to the activation of phospholipase Cβ with the subsequent production of inositol phosphates and diacylglycerol, leading to the release of calcium from the calcium stores and the activation of protein kinase C (O'Connell *et al* 2013). The α_1–adrenoceptors are currently classified into three subtypes: α_{1A}–adrenoceptors, α_{1B}–adrenoceptors and α_{1D}–adrenoceptors (Civantos Calzada and Aleixandre de Artinano 2001). The α_{1A}–adrenoceptor subtype has been implicated in the maintenance of vascular basal tone and of arterial blood pressure, whereas the α_{1B}–adrenoceptor subtype has been suggested to have a role in responding to exogenous agonists. α_{1D}–adrenoceptors are also found in the vasculature and this is the principal subtype present in rat mesenteric and carotid arteries and in the rat aorta (Villalobos-Molina & Ibarra, 1996). A recent study has shown that α_{1D}–adrenoceptors can form a complex with pannexin proteins, such that after activation, intracellular ATP is released to act as an intercellular signalling molecule, to consolidate adrenergic-mediated vascular contraction (Billaud *et al* 2011). In the previous chapter, purinoceptor antagonists had no effect on responses mediated by α_{1A}–adrenoceptors in porcine splenic arteries,

questioning a role for ATP, released via pannexin channels, subsequent to activating this receptor. Therefore, in this chapter, responses to noradrenaline were examined in the rat thoracic aorta, where the major α_1–adrenoreceptor subtype has been reported to be the α_{1D}–adrenoceptor (Kenney *et al* 1995). The first objective was, therefore, to determine the presence of pannexin protein in rate aorta by using Western blot and immunohistochemistry. Additionally, the role of pannexin channels and ATP (via P2 purinoceptors) in contributing to the vascular tone in RA in response to exogenous NA was assessed using pannexin blockers (mefloquine, probenecid, and BB-FCF), P2 receptor antagonists (suramin, PPADS and NF449) and apyrase (an enzyme that breaks down ATP).

4.2.Methods and Materials

4.2.1. Western blotting

Male Wistar rats weighing between 180-220g were sacrificed and the aorta was dissected as described in section 2.3. Samples were run on 7% SDS-PAGE gels using a Biorad Mini-protein 3 system as described in section 2.3. Subsequently, the nitrocellulose membrane was incubated overnight at 4^{o}C with the primary antibody Rabbit anti-pannexin1 (Abcam cat No. ab124969, Rabbit anti-pannexin2 Abcam cat No. ab55917 and Rabbit anti-pannexin 3 Abcam cat No. ab98093 at 1:1 000 concentration in 5% (w/v) milk solution and mouse monoclonal anti-GAPDH antibody (G8795 Sigma-Aldrich) (1:40 000 followed by incubation with secondary antibodies : IRDye®680LT Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) and IRDye®800CW Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences,Cambridge, UK) for 1 hour at room temperature. After that the

nitrocellulose membrane was analysed using Li-Cor Odyssey Imager Scanner and densities of the bands were determined using Odyssey (Application Software Version 3.0LI-COR Biosciences, Cambridge, UK).

4.2.2. Isometric tension recording

4.2.2.1. Rat aorta

The rat aorta was dissected and cut into 4 equal segments approximately 5mm in length as described in section 2.1. Experiments were carried out to investigate the contractile response of rat aorta to NA in the presence and absence of pannexin inhibitors (mefloquine, probenecid and Brilliant Blue FCF) and in the absence and presence of P2 purinoceptor antagonists (PPADS, suramin and NF449) or the ectonucleotidase (apyrase).

4.2.3. Immunohistochemistry

4.2.3.1. Paraffin embedding tissue and sectioning

Rat aorta were dissected and processed as described in section 2.4.1. Immunohistochemistry experiment was performed as described in section 2.4.1. The primary antibodies were diluted to 1:500 in (PBS 0.1%, 0.1% Tween-20,1%BSA) and incubated overnight, while the secondary antibody, rodamine red conjugate, was diluted t in (PBS 0.1%, 0.1% Tween-20, 1%BSA) to a dilution of 1:1000 and applied to the sections for 1 h. Following this they were incubated with DAPI (1in 500 dilution) for 5 min in the dark. Coverslips were applied using mounting medium (1% (v/v) 1, 4-

diazobicyclo-[2, 2, 2,]-octane (DABCO) fluorescence mounting medium-SS04. The slides were examined at 20 and 40 magnifications using a microscope to assess pannexin location. Sections were viewed, 20X magnification. The following filters were used to visualise the immunostains. DAPI - Excitation 350/50nm, emission 460/50 nm, Rhodamine Red - Excitation 545/20nm, emission 610/75 nm for the Goat anti-rabbit IgG (H+L) secondary antibody.

The general protocol for recording isometric tension

Rat thoracic artery segments were set up in organ baths for isometric tension recording as described in section 2.5. Segments were then exposed to increasing concentrations of NA (10^{-8}-10^{-4}M) raised in 3–fold increments to generate cumulative concentration curves (CCRC) to NA. After the CCRC was completed, it was compared to CCRC curves to NA generated in the presence of either, mefloquine ($2x10^{-5}$M), probenecid ($5x10^{-4}$M), suramin (10^{-4}M), PPADS (10^{-5}M), Brilliant Blue FCF (10^{-6},10^{-5}M), NF449 (10^{-7},10^{-6},10^{-5}M), or ectonucleotidase (apyrase) 1U/ML. Experiments were also carried out using 5-HT and U46619 as contractile agents. The effect of mefloquine ($2x10^{-5}$M) on 5-HT and U46619 responses were determined. In addition, experiments were carried out to determine whether either pannexin inhibitors or purinoceptor antagonists affected responses to KCl. To do this, tension was raised using KCl (60 mM) and the inhibitors/antagonists were added to the organ bath in increasing concentrations at 30 min intervals. Some tissues were not exposed to drugs and acted as controls.

To assess whether mefloquine affected Ca^{2+} entry mechanisms, arteries were set up as described above. Vessels were incubated in Ca^{2+}-free Krebs–Henseleit solution and left for 30 mins. Thereafter, the extracellular Ca^{2+} concentration was increased between

(1µM and 3 mM) in three-fold increments, to generate a cumulative concentration-response curve. This was also carried out in tissues exposed to mefloquine ($2x10^{-7}$, $2x10^{-6}$ or $2x10^{-5}$M).

4.2.4. Statistical analysis

As described in section 2.6.

4.3.Results

4.3.1. Pannexin1, 2 and 3 expression in rat thoracic aorta tissue

Western blot analysis on RA tissue using anti-pannexin1 antibodies showed a 50 kDa band in all samples. The 50 kDa bands observed during western blotting correspond to pannexin1 protein (http://www.abcam.com/pannexin-1-antibody-EPR5556-ab124969-references.html) (Figure 4.1).

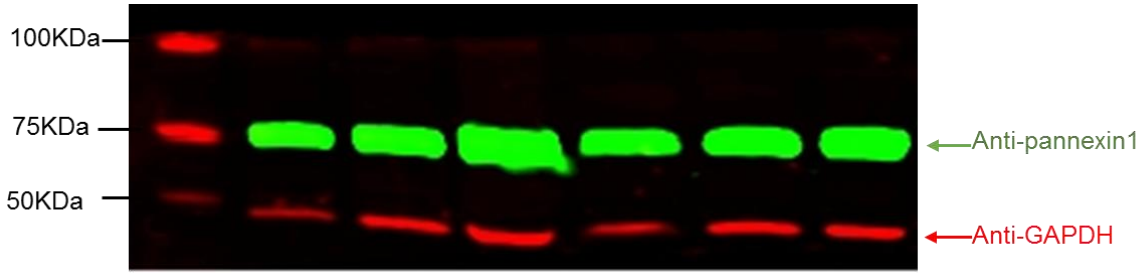


Figure 4.1 Western blot analysis to determine pannexin1 expression in rat aorta tissue. N = 6.

Western blot analysis on Rat aorta tissue using anti-pannexin2 antibodies showed 100kDa bands in all lanes. The 100kDa bands observed during western blotting correspond to the pannexin2 protein (http://www.abcam.com/pannexin-2-antibody-ab55917-references.html) (Figure 4.2). In addition, the expression of pannexin3 protin (http://www.abcam.com/pannexin-3-antibody-ab98093.html) was examined using anti-pannexin3 antibody, but the expression of pannexin3 was not evident in Rat aorta as I did not detect a band corresponding to pannexin3 (data not shown).

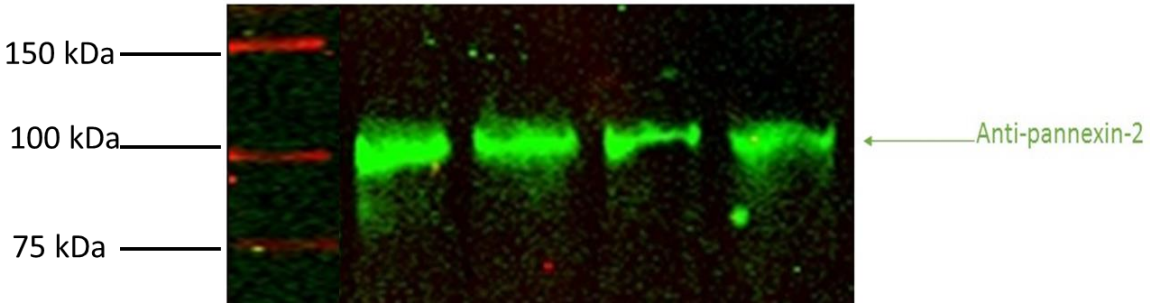


Figure 4.2 Western blot analysis to determine pannexin2 expression in Rat aorta tissue. N = 4.

4.3.2. Pannexin1 expression assessed by immunohistochemistry in rat aorta

The immunostaining sections were viewed using a light microscope and a wide field fluorescence microscope (Leica DMRB). Sections were viewed at 20X and 40X magnifications to assess pannexin location in the arteries. Images were captured using Micro Manager. The following filters were used to visualise the immunostains. DAPI - Excitation 350/50nm, emission 460/50 nm, FITC - Excitation 480/40nm, emission 535/50 nm to visualise the Goat anti-rabbit Antibody and Rhodamine Red - Excitation

545/20nm, emission 610/75 nm for the Goat anti-rabbit IgG (H+L) secondary antibody (Figure 4.3).

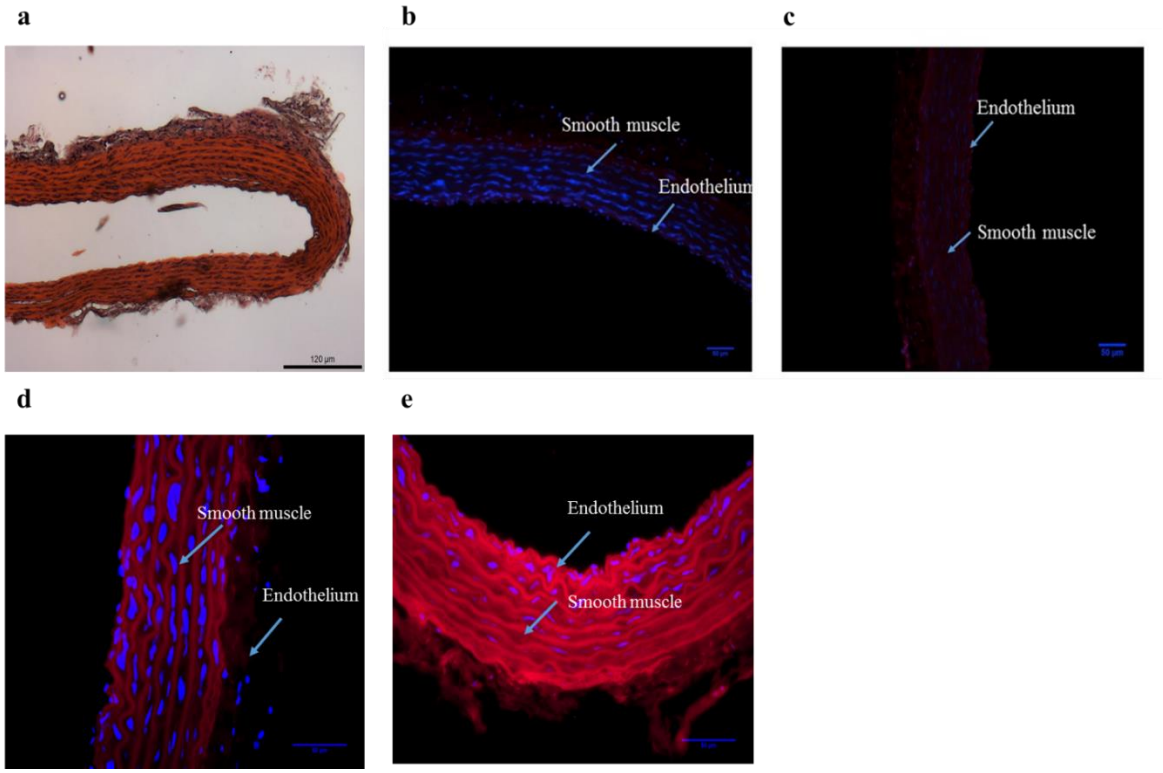


Figure 4.3. Light microscopic images of rat aorta at X20 (a) stained without primary antibody. Expression of pannexin1 shown by immunohistochemistry in the absence (b) and presence (c) of anti-pannexin1 specific antibody at X20. Expression of pannexin1 shown by immunohistochemistry in the absence (d) and presence (e) of anti-pannexin1 specific antibody at X40. Expression was evident in the endothelium and the smooth muscle cells of porcine splenic artery. N =6.

4.3.3. Isometric tension recording

4.3.3.1. Effect of noradrenaline on tension of rat aorta

To test whether the response to NA was reproducible in RA, consecutive concentration-response curves were obtained. NA induced a concentration-dependent contraction in the RA (Figure 4.4). The maximum responses did not significantly differ between the two curves; R_{max} values were 0.72± 0.06g and 0.72± 0.08g for curve one and two, respectively (Figure 4.4). In addition, the sensitivity to NA did not change between the curves; log EC_{50} values were -7.8±2.5 and -7.3 ± 2.8 for curve one and two, respectively ($p > 0.05$, Student's Paired *t-test*, N = 6).

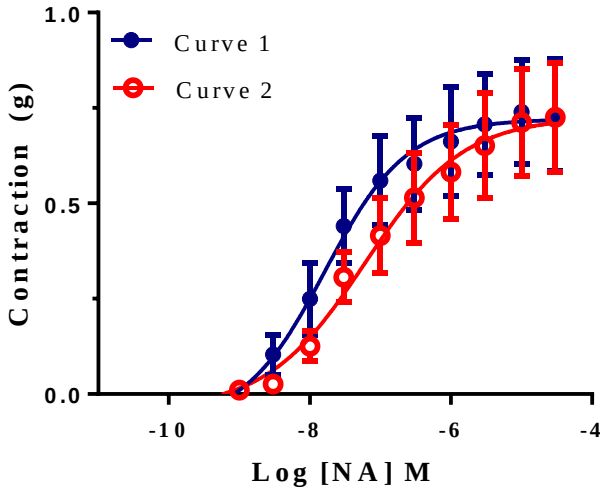


Figure 4.4 Responses to noradrenaline in rat thoracic aorta. Curve 1 is shown as closed circles, while curve 2 is shown as open circles. Data are presented as the mean value ± s.e.m. There was no significant difference between the concentration-response curves ($P > 0.05$, Student *t-test*, N = 6).

4.3.3.2. Effect of mefloquine on noradrenaline-induced-contraction in Rat aorta

Mefloquine, used at three different concentrations; $2x10^{-7}M$, $2x10^{-6}M$ and $2x10^{-5}M$, reduced the magnitude of NA-induced contraction in Rat aorta. The maximum responses to NA were reduced from 1.06 ± 0.11g in control to 0.82 ± 0.01g in the presence of mefloquine, $2x10^{-7}M$ ($p < 0.01$, Student's paired *t-test*; N = 5), but this concentration of mefloquine did not alter the sensitivity to NA in Rat aorta; log EC_{50} values were -7.08 ± 0.30 (control) and -6.68 ± 0.26 (mefloquine, $2x10^{-7}M$) (Student's paired *t-test*; N = 5) (Figure 4.5a). The maximum responses to NA were reduced from 1.31 ± 0.14g in control to 0.63 ± 0.13g in the presence of mefloquine, $2x10^{-6}M$ ($p < 0.0001$, Student's paired *t-test*; N = 5), but this concentration of mefloquine did not alter the sensitivity to NA in RA; log EC_{50} values were -6.74 ± 0.22 (control) and -7.14 ± 0.69 (mefloquine, $2x10^{-6}M$) (Student's paired *t-test*; N = 5) (Figure 4.5b). The maximum responses to NA were reduced from 1.16 ± 0.1g in control to 0.44 ± 0.03g in the presence of mefloquine, $2x10^{-5}M$ ($p < 0.001$, Student's paired *t-test*; N = 5), and the log EC_{50} value was reduced from -6.94 ± 0.23 (control) to -6.44±0.27 (mefloquine, $2x10^{-5}M$) ($p < 0.01$, Student's paired *t-test*; N = 7) (Figure 4.5c).

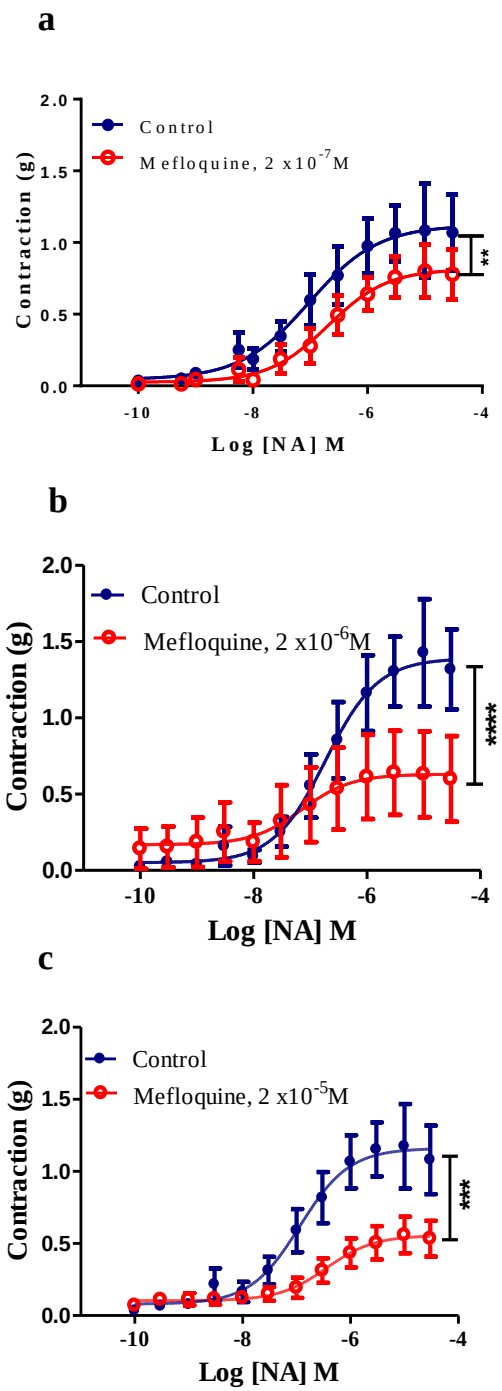


Figure 4.5 Effect of mefloquine at $2x10^{-7}$(a), $2x10^{-6}$ (b) and $2x10^{-5}$M (c) on the response to noradrenaline in the rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. Differences between R_{max} values are shown by **P < 0.01; ***P < 0.001, ****P<0.0001 (Student's paired *t-test*, N = 5-7).

4.3.3.3. Effect of probenecid on noradrenaline-induced contraction in rat aorta

Probenecid, used at three different concentrations of $2x10^{-5,}$ $2x10^{-4}$ and $2x10^{-3}$M, reduced the magnitude of NA-induced contraction in RA. The maximum responses to NA were reduced from 0.98 ± 0.12g in control to 0.70 ± 0.04g in the presence of probenecid ($2x10^{-5}$M) ($p < 0.01$, Student's paired *t-test*; N = 5), and the sensitivity (Log EC_{50}) was reduced from -7.41 ± 0.32 (control) to -6.75 ± 0.13 (probenecid, $2x10^{-5}$M) ($p < 0.05$, Student's paired *t-test*; N = 5) (Figure 4.6a). The maximum responses to NA were reduced from 1.41 ± 0.14g in control to 0.65 ± 0.052g in the presence of probenecid ($2x10^{-4}$M) ($p < 0.001$, Student's paired *t-test*; N = 5), and the sensitivity was reduced from -7.41 ± 0.28 (control) to -6.69 ± 0.17 (probenecid, $2x10^{-4}$M) ($p < 0.05$, Student's paired *t-test*; N = 5) (Figure 4.6b). The maximum responses to NA were reduced from 0.9 8± 0.11g in control to 0.43 ± 0.05g in the presence of probenecid ($2x10^{-3}$M) ($p < 0.0001$, Student's paired *t-test*; N = 5), and the sensitivity was reduced from -7.41 ± 0.32 (control) to -6.49 ± 0.21 (probenecid, $2x10^{-3}$M) ($p < 0.05$, Student's paired *t-test*; N = 5) (Figure 4.6c).

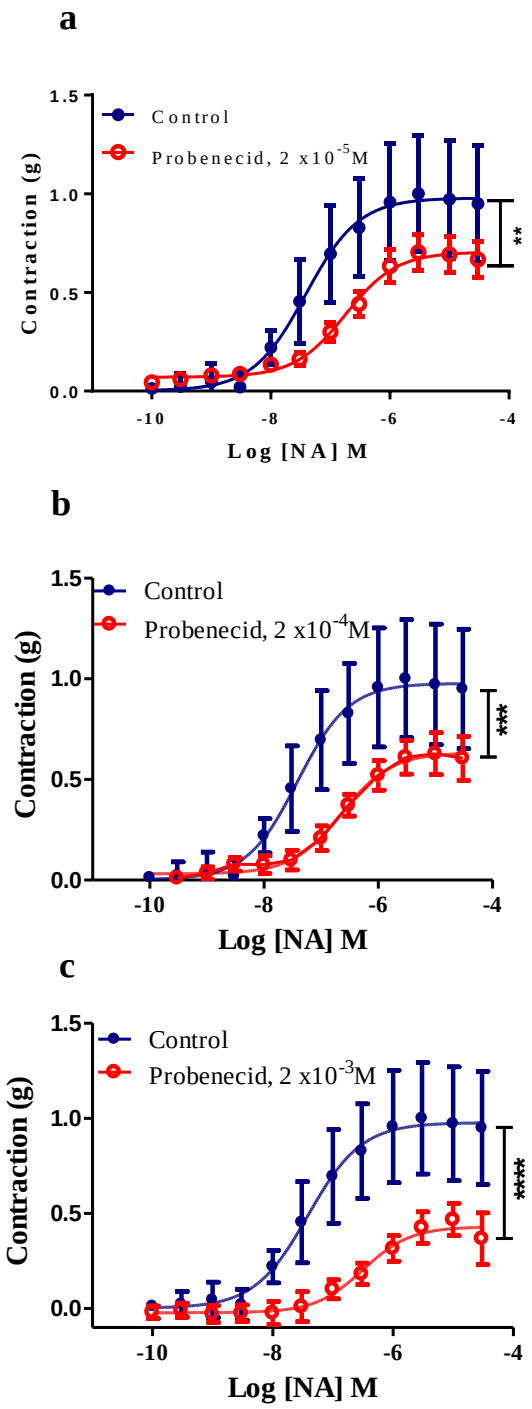


Figure 4.6 Effect of probenecid at $2x10^{-5}$ (a), $2x10^{-4}$ (b) and $2x10^{-3}$M (c) on the response to NA in the rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. Differences between R_{max} values are shown by **P < 0.01; ***P < 0.001, ****P < 0.0001 (Student's paired *t-test*, N = 5).

4.3.3.4. Effect of BB-FCF on noradrenaline-induced contraction in rat aorta

Brilliant Blue FCF (BB-FCF) is structurally similar to the purinergic receptor antagonist Brilliant Blue G (BBG), which is an inhibitor of ionotropic P2X7 receptors. It has also shown to be a selective inhibitor of pannexin1 channels (Wang *et al* 2013). BB-FCF at10^{-6} and 10^{-5}M had no effect on the magnitude or sensitivity of NA-induced contraction in RA (Table 4.1) (Figure 4.7a, b).

Table 4.1 Effects of BB-FCF on NA-induced contraction in Rat aorta

Treatment	R_{max}(g)	Log EC_{50}(M)
Control	0.80 ± 0.06	-7.19 ± 0.19
BB-FCF 10^{-6}M	0.75 ± 0.04	-7.17 ± 0.12
Control	0.87 ± 0.05	-7.25 ± 0.15
BB-FCF 10^{-5}M	0.88 ± 0.05	-7.25 ± 0.14

Data represent the mean ± s.e.m of 4 experiments

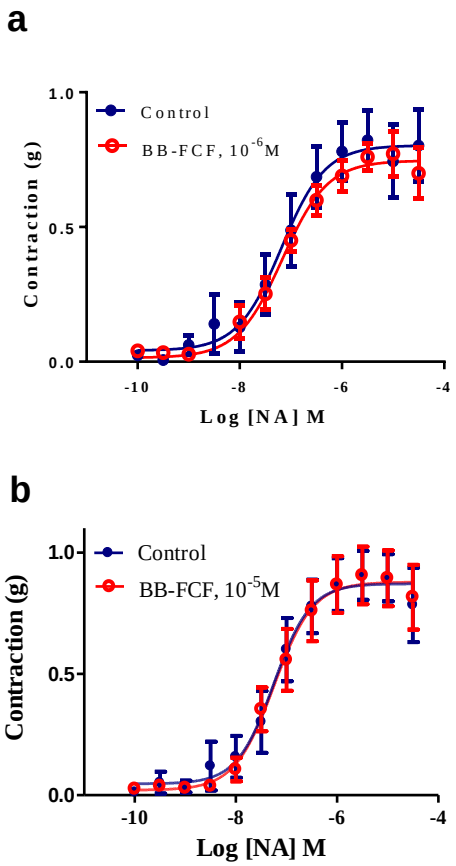


Figure 4.7 Effect of BB-FCF (a) 10^{-6} and (b) 10^{-5} M on the response to noradrenaline in rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. BB-FCF had no significant effect on the contraction induced by NA. ($P > 0.05$, Student *t-test*, N=4).

4.3.3.5. Effect of suramin and PPADS on noradrenaline-induced contraction in rat aorta

Suramin ($3x10^{-4}$M) and PPADS (10^{-5}M) had no effect on the magnitude of NA-induced contraction in RA (Table 4.2) (Figure 4.8a, b). Furthermore, neither suramin nor PPADS altered the sensitivity of responses to NA in RA (Table 4.2) (Figure 4.8a, b).

Table 4.2 Effects of Suramin and PPADS on NA-induced contraction in RA

Treatment	R_{max}(g)	Log EC_{50}(M)
Control	0.08 ± 0.07	-7.55 ± 0.30
Suramin$3x10^{-4}$M	0.71 ± 0.07	-7.78 ± 0.30
Control	0.78 ± 0.07	-7.55 ± 0.23
PPADS 10^{-5}M	0.67 ± 0.07	-7.93 ± 0.35

Data represent the mean ± s.e.m of 4 experiments

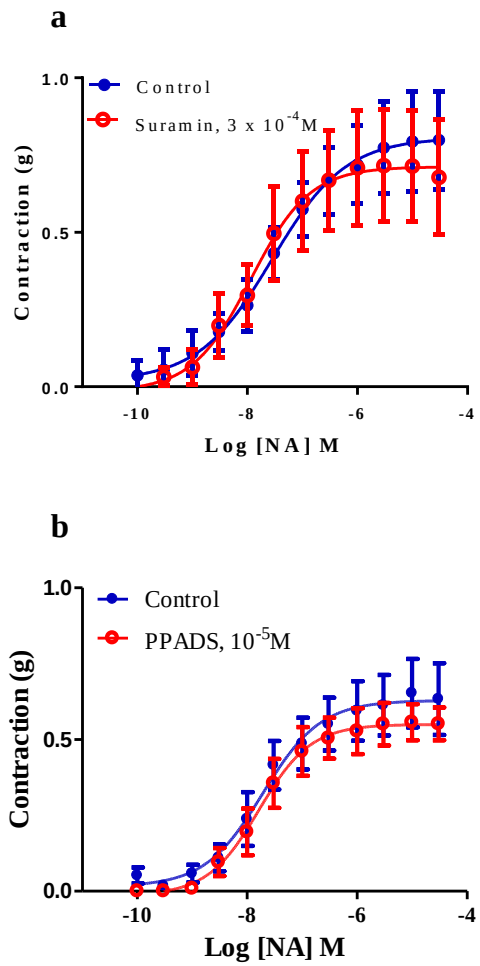


Figure 4.8 Effect of suramin $3x10^{-4}$ (a) and PPADS 10^{-5}M (b) on the response to noradrenaline in rat aorta. Controls are shown in closed circles while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. Suramin and PPADS had no significant effect on the contraction induced by NA. ($P > 0.05$, Student *t-test*, N=4).

4.3.3.6. Effect of NF449 on noradrenaline-induced contraction in rat aorta

NF449 at 10^{-7}, 10^{-6} and 10^{-5}M had no effect on the magnitude of NA-induced contraction in RA (Table 4.3) (Figure 4.9a, b, c). It also did not alter the sensitivity to NA in RA (Table 4.3) (Figure 4.9a, b, c).

Table 4.3 Effects of NF449 on NA-induced contraction in RA

Treatment	R_{max}(g)	Log EC_{50}(M)
Control	0.74 ± 0.07	-7.08 ± 0.27
NF449 10^{-7}M	0.55 ± 0.06	-6.94 ± 0.22
Control	1.07 ± 0.08	-6.92 ± 0 .18
NF449 10^{-6}M	0.88 ± 0.06	-6.66 ± 0.15
Control	0.99 ± 0.40	-7.09 ± 0.25
NF449 10^{-5}M	0.78 ± 0.09	-7.03 ± 0.21

Data represent the mean ± s.e.m of 4 experiments

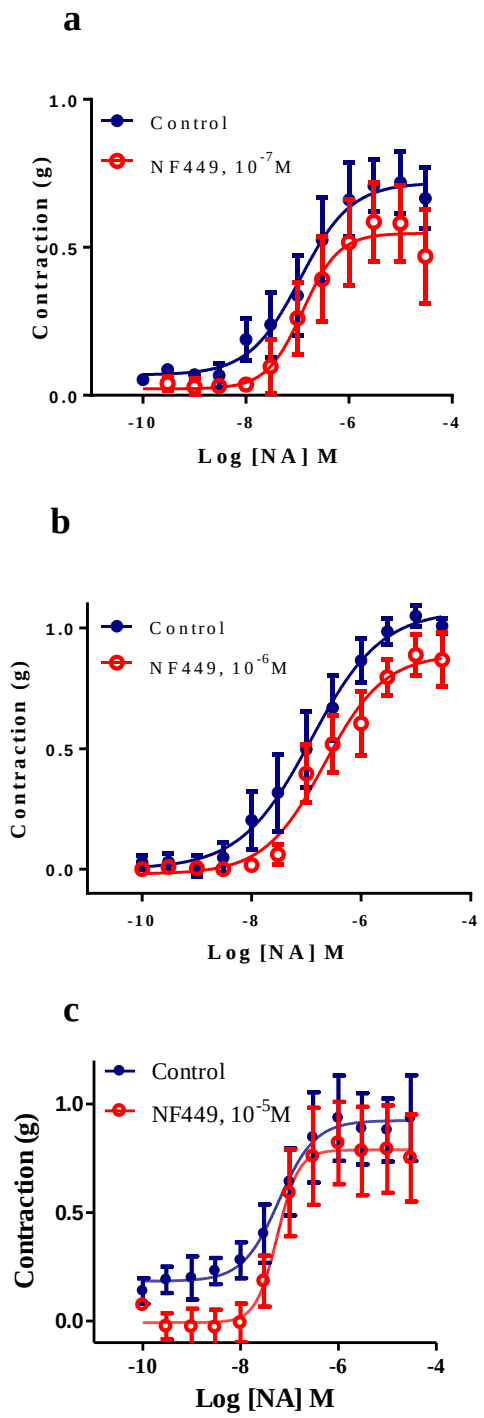


Figure 4.9 Effect of NF449 10^{-7} (a), 10^{-6} (b), and 10^{-5}M (c) on the response to noradrenaline in rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. ($P > 0.05$, Student *t-test*, N=4).

4.3.3.7. Effect of Apyrase on noradrenaline-induced contraction in rat aorta

Apyrase (1U/M) did not alter the magnitude of NA-induced contraction or the sensitivity to NA in RA (Figure 4.10). The maximum response to NA was 0.75 ± 0.04g in control and 0.70 ± 0.05g in the presence of apyrase 1U/M ($p > 0.05$, Student's paired *t-test*; N = 4), and log EC_{50} values were -6.73 ± 0.13 in the control and -6.49 ± 0.13 in the presence of apyrase 1U/M ($p > 0.05$, Student's paired *t-test*; N=4) (Figure 4.10).

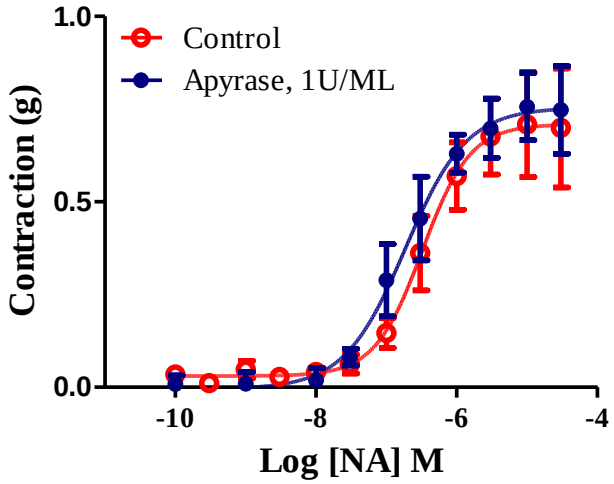


Figure 4.10 Effect of apyrase (1U/M) on the response to noradrenaline in the rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. Apyrase had no significant effect on the contraction induced by NA. ($P > 0.05$, Student *t-test*, N = 4).

4.3.3.8. Effect of mefloquine on 5-HT and U46619 in the rat aorta

In order to investigate the specificity of mefloquine, responses to 5-HT and U46619 were obtained in the absence and presence of mefloquine ($2x10^{-5}$M). Mefloquine reduced the maximum response and the sensitivity to 5-HT in the RA. The maximum response to 5-HT was reduced from 0.94 ± 0.12g in control to 0.32 ± 0.1g in the presence of mefloquine ($2x10^{-5}$M) ($p < 0.05$, Student's paired *t-test*; N = 5), log EC_{50} was -9.37 ± 0.17 in the absence and -9.08 ± 1.82 in the presence of mefloquine ($p < 0.05$, Student's paired *t-test*) (Figure 4.11b). In addition, mefloquine ($2x10^{-5}$M) abolished the response to U46619 in the RA. R_{max} was 0.78 ± 0.11g in the absence and 0.07 ± 0.007g in the presence of mefloquine ($P < 0.0001$, Student *t-test*, N=4) (Figure 4.11b).

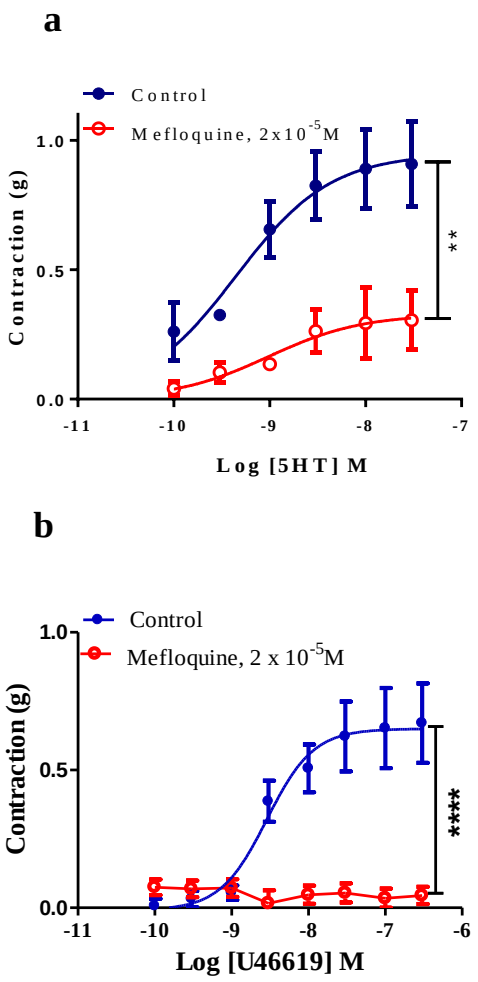


Figure 4.11 Effect of mefloquine ($2x10^{-5}$M) on the responses to (a) 5-HT and (b) U46619 in rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Each point represents the mean ± s.e.m. Differences between R_{max} values are shown by **P < 0.01, ****P < 0.0001 (Student's paired *t-test*, N = 5).

4.3.3.9. Effect of mefloquine and probenecid on KCl-induced contraction in rat aorta, while suramin has no effect

The effects of mefloquine, probenecid and suramin on the contractile response to the maximm response of the extracellular KCl (60 mM) in RA were examined. Mefloquine ($2x10^{-5}$M) and probenecid ($2x10^{-6}$M) reduced the magnitude of KCl-induced

contraction in RA. The KCl-induced tone was reduced from 5.04 ± 9.8% in control to -58.61 ± 16.90% in the presence of mefloquine $2x10^{-5}$M ($p < 0.001$, Student's paired *t-test*; N = 4) (Figure 4.12a). The KCl-induced tone was reduced from 5.04 ± 9.8% in control to -52.42 ± 15.53% in the presence of probenecid 3mM ($p < 0.001$, Student's paired *t-test*; N = 5) (Figure 4.12b). Suramin did not alter the KCl-induced contraction in RA. The KCl-induced tone was 5.04 ± 9.8% in the absence and -14.61% ± 14.59% in the presence of suramin ($3x10^{-4}$ M) ($P > 0.05$, Student's paired *t-test*; N = 4) (Figure 4.12c).

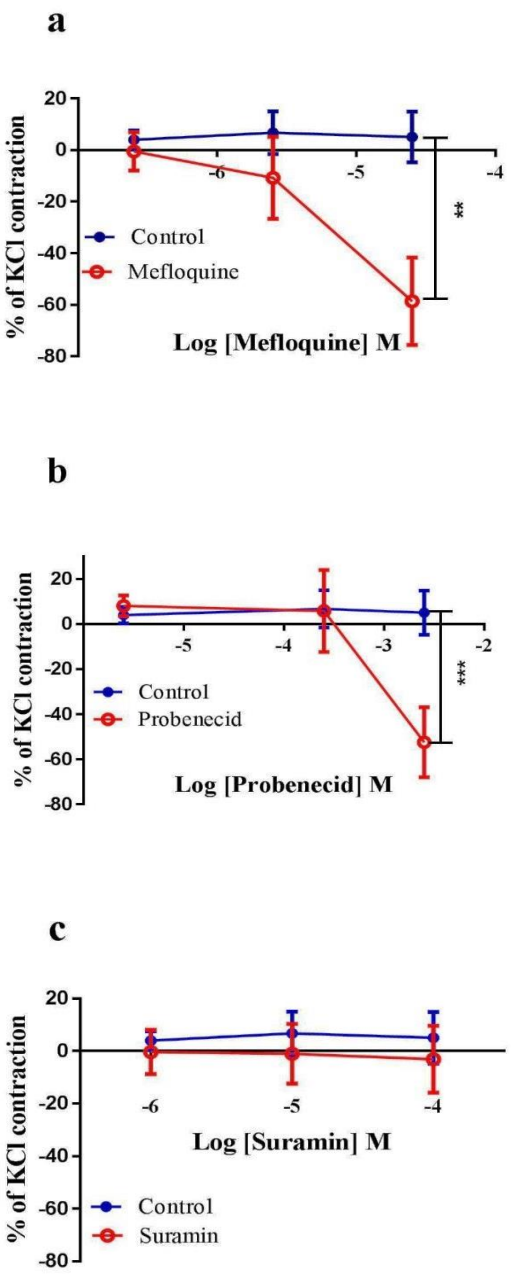


Figure 4.12. Effect of mefloquine (a), probenecid (b) and suramin (c) on KCl-induced tone in rat aorta. Controls are shown as closed circles, while drug treatment is shown as open circles. Data are shown as a percentage of the contractions induced by KCl. Data are presented as the mean value ± s.e.m. (a) Mefloquine ($2x10^{-5}$M) and (b) probenecid ($2x10^{-3}$M) significantly decreased the contraction to KCl in rat aorta. Differences between R_{max} values are shown by **P < 0.01, ***P < 0.001 (Student's paired *t-test,* N = 5). (c) Suramin had no significant effect on the contraction to KCl (P > 0.05, Student's paired *t-test*, N = 5).

4.3.3.10. Effect of mefloquine ($2x10^{-5}$M) on contraction to Ca^{2+} in the rat aorta

The effect of three different concentrations of mefloquine ($2x10^{-5}$, $2x10^{-6}$ and $2x10^{-7}$M) on the concentration-dependent contraction of Ca^{2+} ($1x10^{-6}$ to $3x10^{-3}$M, in three-fold increments) re-added to Ca^{2+}free buffer containing 60mM KCl was examined. Mefloquine at $2x10^{-5}$M reduced the magnitude of Ca^{2+}-induced contraction in RA ($P < 0.001$, One-way ANOVA). By contrast, lower concentrations of mefloquine ($2x10^{-6}$ and $2x10^{-7}$M) had no effect on the response to the re-addition of Ca^{2+} in RA (Figure 4.13). The maximum response to Ca^{2+} was reduced from 0.68 ± 0.1g in control to 0.03 ± 0.04g in the presence of mefloquine ($2x10^{-5}$M) ($p > 0.05$, One-way ANOVA; N = 4) (Figure 4.13a). The maximum responses to Ca^{2+} were 0.68 ± 0.1g in control and 0.7 ± 0.17g in the presence of mefloquine ($2x10^{-6}$M) ($p > 0.05$, One-way ANOVA; N = 4) (Figure 4.13). The maximum responses to Ca^{2+} were 0.68 ± 0.1g in control and 0.65 ± 0.09g in the presence of mefloquine ($2x10^{-7}$M) ($p > 0.05$, One-way ANOVA; N = 4) (Figure 4.13).

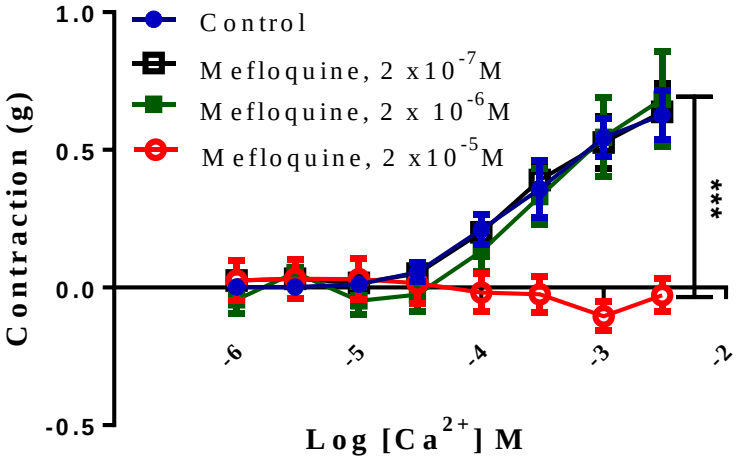


Figure 4.13 Effect of mefloquine ($2x10^{-7}$,$2x10^{-6}$and $2x10^{-5}$M) on the response to the re-introduction of Ca^{2+} in rat aorta. Each point represents the mean ± s.e.m. Mefloquine at $2x10^{-5}$M reduced significantly the maximum response to Ca^{2+} in rat aorta. Differences between R_{max} values are shown by ***P < 0.001 (One-way ANOVA, N=4). Mefloquine at $2x10^{-6}$ and $2x10^{-7}$M did not alter the maximum response to Ca^{2+} (One-way ANOVA, N=4).

4.4.Discussion

The aim of the present study was to determine whether pannexin channels and ATP contributed to the response to NA in a preparation containing α_{1D}–adrenoceptors, namely the rat aorta. Western blot analysis showed the presence of both pannexin1 and pannexin2. Conflicting data were obtained using three different pannexin inhibitors, i.e. mefloquine (a non-selective pannexin inhibitor), probenecid (a pannexin1 inhibitor) and Brilliant Blue FCF (a pannexin1 inhibitor) (Beckel *et al* 2015). While mefloquine and probenecid decreased the response to NA, BB-FCF had no effect. The lack of effect of P2 purinoceptor antagonists, suramin, PPADs and NF449 argues against a possible role

for ATP acting via P2 receptors in contributing to the NA-induced α_{1D}–adrenoceptor-mediated response in the rat aorta. Further investigation of the effects of mefloquine indicated that it also inhibited responses to raised KCl, 5-HT and U46619 and calcium-entry mechanisms suggesting that it produces non-selective actions and questions its use as a pannexin inhibitor.

Western blot analysis showed that both pannexin1 and pannexin2 are present in RA. Pannexin 1 fluorescence indicated the presence of pannexin1 in the tunica media, although this was clouded by autofluoresence in the control samples. Similarly, pannexin1 and pannexin2 have been reported to be expressed in several tissues, including in the CNS, eye, prostate, thyroid, liver, and kidney. For example, in the hippocampus, both pannexin1 and pannexin2 were expressed in the pyramidal cell layer and in individual neurons in the stratum oriens and stratum radiatum, but pannexin3 could only be detected in the skin. All 3 isoforms of pannexins are expressed in the pulmonary artery and alveoli. Pannexin1 is the predominant pannexin isoform in coronary arteries (except in arteries less than 100 μm) whereas pannexin3 is expressed in the juxtaglomerular apparatus and cortical arteriole. Billaud *et al* (2011) also showed that pannexin1 is expressed in vascular smooth muscle cells of TDA.

Billaud *et al* (2011) suggested that there is an interaction between pannexin1 and α_{1D}–adrenoceptors. Mefloquine (10-20 μM) and probenecid (0.5-2 mM) inhibited phenylephrine-induced vasoconstriction, suggesting that pannexin1 and α_{1D} – adrenoceptor are intimately coupled – both are part of the same protein complex. The existence of α_{1D}-adrenoceptors in rat aorta has been shown previously (Gisbert *et al* 2000) based upon the functional activity of a selective α_{1D}-adrenoceptor antagonist

(BMY 7378) which correlated with binding affinities for cloned α_{1D} -adrenoceptors (Kenny *et al* 1995; Saussy *et al* 1996; Hussain and Marshall, 1997).

In order to examine the involvement of pannexin in mediating the response to NA in RA (which contains both pannexin and α_{1D}–adrenoceptors), the magnitude of NA-induced contraction in RA were assessed in the presence of three pannexin inhibitors, i.e. mefloquine, probenecid, and BB-FCF. The results showed that while mefloquine and probenecid decreased the response to NA, BB-FCF had no effect. Mefloquine is a non-selective pannexin inhibitor. Probenecid has been shown to selectively block pannexin1 channels at concentrations up to 5 mM (Dando and Rorar, 2009; Lamkanfi *et al* 2009; Ransford *et al* 2009; Xia *et al* 2012; Silverman *et al* 2008), while BB-FCF has recently been shown to be a pannexin1 inhibitor (Beckel *et al* 2015; Wang *et al* 2013).

While mefloquine reduced responses to NA at three different concentrations (2×10^{-7}M, 2×10^{-6}M and 2×10^{-5}M), only the highest concentration inhibited the KCl response. Similarly, whereas all concentrations of probenecid (2×10^{-5}M, 2×10^{-4}M and 2×10^{-3}M) inhibited the NA-mediated contraction, only 2×10^{-3}M inhibited KCl-induced tone. Such differences in the results obtained from different concentrations could be because mefloquine and probenecid produce non-selective inhibition of vascular responses at high concentrations. Indeed, evidence for non-selective actions of mefloquine are presented below. This suggests that mefloquine and probenecid should only be used at relatively low concentrations as pannexin1 inhibitors in order to investigate the role of pannexin channels in vasoconstriction. The results with low concentrations of mefloquine and probenecid could be taken as evidence that pannexin channels participate in α_1-adrenoceptor-mediated vasoconstriction in the RA. However, even if

this were the case, there is no evidence to support the release of ATP through these channels to act as an intercellular messenger.

Billaud *et al* (2011) proposed that phenylephrine–induced α_{1D}–adrenoceptor-mediated vasoconstriction is caused by release of ATP through pannexin channels, based upon the sensitivity of responses to phenylephrine to P2 purinoceptor antagonists. The current results, however, do not support the 'α_1-adrenoceptor– pannexin1 channel ATP release' theory since P2 purinergic receptor antagonists (suramin, PPAD and NF449) and apyrase, an enzyme that breaks down ATP, had no significant inhibitory effect on NA-induced vasoconstriction in RA. This indicates that ATP is not involved via P2 receptors in mediating the response to the NA activation of α_{1D}–adrenoceptors in the rat aorta.

The results of the present study are in agreement with Angus *et al* (2015) who reported that responses to phenylephrine, U46619, vasopressin, endothelin-1, sympathetic nerve stimulation and K^+ were inhibited in rat and mouse small mesenteric arteries using mefloquine, Therefore, it has been argued that the release of ATP does not contribute to the contraction to phenylephrine after α_{1D}-adrenoceptor activation.

In the rat aorta, the effects of mefloquine indicated that it also inhibited responses to raised KCl, 5-HT and U46619. This confirms that mefloquine inhibits other agonist responses, possibly by inhibiting calcium-entry mechanisms. To investigate this further the present study showed that mefloquine, at high concentrations, blocked responses to the re-introduction of calcium into depolarised artery segments. Mefloquine has previously been reported to decrease L-type Ca^{2+} currents in single ventricular myocytes (Coker *et al* 2000), and so a similar mechanism is likely in the RA. It should be noted that inconsistent results have been reported in the literature regarding the influence of mefloquine on the responses to KCl with Billaud *et al* (2011) reporting that mefloquine

did not inhibit the response to KCl (40mM) in mouse TDA. Angus *et al* (2015) argued that mefloquine becomes a selective pannexin1 inhibitor only when used in concentrations lower than 0.1 μM.

In conclusion, in rat aorta, there is no conclusive evidence that pannexin/ATP is involved in α_{1D}–adrenoceptor-mediated responses in RA (which expresses both pannexin1 and α_{1D}-adrenoceptors). This indicates that the assumption reported by Billaud *et al* (2011) that pannexin1 is associated with α_{1D}-adrenoceptors (i.e. pannexin1 opens to release purines that act on purinergic receptors present on smooth muscle cell leading to enhanced vasoconstriction) is questionable.

Chapter 5

5. Role of pannexin channels in mediating endothelium-dependent vasorelaxation in porcine splenic artery and porcine coronary artery

5.1. Introduction

Cell-to-cell communication provides coordination of cellular processes in multicellular organisms. In the vascular system, the mechanisms underlying the multilayer communication between cells of different types include gap junctions (de Wit and Griffith, 2010, de Wit and Griffith, 2010, Paul, 1986, Burnstock and Ralevic, 2013) and paracrine action of signalling molecules, such as ATP (Burnstock and Ralevic 2014). Gap junctions are part of the connexin family of proteins that connect the cytoplasm of neighbouring cells by forming a transmembrane channel allowing ions and small molecules to pass through the channel. The gap junction proteins in invertebrates has the same function but differs in structure. This family of specific invertebrate gap junction proteins was designated as innexins (Phelan *et al* 1998). After innexins homologs were discovered in humans and other vertebrates, it was proposed to reclassify them along with their vertebrate homologs into a bigger family, named pannexins (Panchin *et al* 2000; Baranova *et al* 2004). Mammalian pannexins form hemichannels implicated in the release of ATP and other small molecules from the cytoplasm into the extracellular environment (Bruzzone *et al* 2003; Ishikawa *et al* 2011). Since pannexins are involved in intercellular communication that is essential for the functioning of the vascular system (de Wit and Griffith 2010), the presence (or absence) of pannexins may play an important role in blood flow regulation. Blood vessels contain two major functional components smooth muscle and endothelium. The endothelium

facilitates vasodilator effects of many blood-circulating hormones and locally acting autacoids (Vane , *et al*1994). In murine arteries pannexin1 is the primary expressed isoform (Lohman, *et al* 2012). Pannexin1 is present in endothelium of most arteries (Lohman *et al* 2012) and capillaries (Shestopalov and Panchin 2008), however the pattern of pannexin1 expression depends on the vessel size. In contrast to larger conduit arteries where pannexin1 is expressed primarily in the endothelium, in smaller resistance arteries it is also expressed in smooth muscle (Lohman *et al* 2012). In 2014 Dina *et al* studied the functional role of pannexin1 in facilitating vasodilator and anticontractile effects of the endothelium and suggested a major role for pannexin1in endothelium-dependent responses. They utilized pannexin1$^{-/-}$ mice as a test model for investigating the functional significance of pannexin1 and found that endothelial function was impaired in conduit arteries of pannexin1 knockout mice. In particular, they demonstrated that the EDHF component of endothelium-dependent responses was impaired in pannexin1 knockout mice. The aim of this study was to determine whether pannexins were involved in contributing to endothelium-dependent responses in two conduit arteries isolated from pigs, namely, the splenic artery and coronary artery.

5.2.Materials and methods

5.2.1. Western blotting

Porcine hearts were collected from the abattoir and processed as described in section 2.3. Samples were run on 7% SDS-PAGE gels using a Biorad Mini-protein 3 system as described in section 2.3. Subsequently, the nitrocellulose membrane was incubated overnight with the primary antibody with Rabbit anti-pannexin1 (Abcam cat No. ab124969) at 4°C , Rabbit anti-pannexin2 (Abcam cat No. ab55917) and Rabbit anti-

pannexin 3 (Abcam cat No. ab98093) at 1:1000 concentration in 5% (w/v) milk solution using and mouse monoclonal anti-GAPDH antibody (G8795 Sigma-Aldrich) (1:40 000) followed by incubation with appropriate secondary antibodies IRDye® 680LT Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) and IRDye® 800CW Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) for 1 hour at room temperature. After that membranes were analysed using Li-Cor Odyssey Imager Scanner and densities of the bands were determined using Odyssey.

5.2.2. Immunohistochemistry

5.2.2.1. Paraffin embedding tissue and sectioning

Porcine coronary arteries were dissected from the heart and processed as described in section 2.4.1. Immunohistochemistry experiment was performed as described in section 2.4.1. The primary antibodies were diluted in (PBS 0.1%, 0.1% Tween-20, 1%BSA) to a dilution of 1:500. And incubated overnight, while the secondary antibody, Rodamine Red conjugate (R-6394), was diluted to 1:1000 and applied to the sections for 1 h. Following this they were incubated with DAPI (1in 500 dilution) for 5 min in the dark. Coverslips were applied using mounting medium (1% (v/v) 1, 4-diazobicyclo-[2, 2, 2,]-octane (DABCO) fluorescence mounting medium-SS04. The slides were examined at 20 and 40 magnification using microscope to assess pannexin location. The following filters were used to visualise the immunostains; DAPI excitation 350/50nm, emission 460/50 nm, Rhodamine Red - Excitation 545/20nm, emission 610/75 nm for the Goat anti-rabbit IgG (H+L) secondary antibody.

5.2.3. Isometric tension

5.2.3.1. Tissue collection and sample preparation

Porcine splenic and coronary arteries (PSA and PCA) were dissected cut into 4 equal strips approximately 5mm in length as described in section 2.5.

5.2.3.2. Isometric tension recording

PSA and PCA were collected and processed as described in section 2.5. Briefly, following the addition of KCl twice, U46619 (1nM – 90nM) was employed to increase vascular tone to 50% of the KCl response. Once stable tone was achieved, concentration-response curves to bradykinin (10^{-10} - $3X10^{-6}$M) were constructed. Vasorelaxation to bradykinin was studied in PSA in the presence of the pannexin1 channel inhibitors mefloquine ($2x10^{-7}$, $2x10^{-6}$, $2x10^{-5}$M) and probenecid ($2x10^{-4}$, $2x10^{-5}$M) and compared to the control tissue.

Vasorelaxation to bradykinin was also studied in the presence of N^{G}-nitro-L-arginine methyl ester (L-NAME) (10^{-4}M)), a NO synthase inhibitor, to determine the NO-mediated component (Randall &Griffith, 1991). Indomethacin (10^{-5}M) was used to inhibit the synthesis of prostanoids and in the presence of (L-NAME) (10^{-4}M) and indomethacin (10^{-5}M) together, compared to the control segment.

Thereafter responses to bradykinin were studied in PCA in the absence or presence of the reportedly selective pannexin1 channel inhibitors mefloquine ($2x10^{-7}$, $2x10^{-6}$, $2x10^{-5}$M) probenecid ($3x10^{-7}$, $3x10^{-6}$, $3x10^{-5}$M) and carbenoxolone (10^{-4}M) in preparations exposed toboth L-NAME (10^{-4}M) and indomethacin (10^{-5}M) together to isolate the EDHF component of the response to Bradykinin.

5.2.4. Statistical analysis

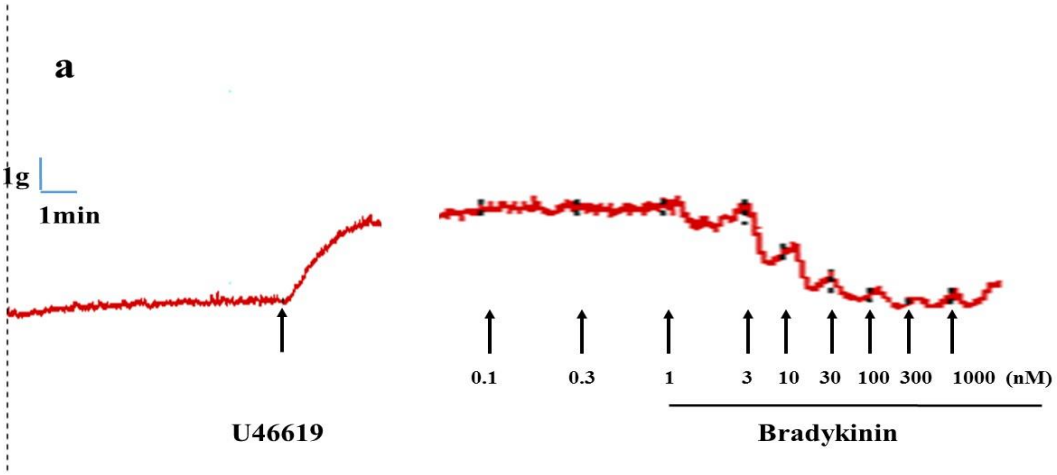
Statistical analysis was conducted as described in section 2.6. The relaxation to bradykinin was measured from the stabilised U46619-induced response and was expressed as a percentage of the U46619-induced contraction. Data were expressed as log concentration response curves. Values for all figures refer to mean ± s.e.m with 95% confidence.

5.3. Results

5.3.1. L-NAME and the combination of L-NAME and indomethacin decreased the vasorelaxation to bradykinin in porcine splenic artery

Bradykining induced concentration-dependent relaxation in PSA (Figure 5.1a). L-NAME (10^{-4}M) and the combination of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) significantly decreased the relaxation to bradykinin in PSA, while indomethacin (10^{-5}M) alone did not affect bradykinin-induced vasorelaxation in PSA. The maximum response to bradykinin was -83.62 ± 8.44% (control) and -25.79± 6.48% in the presence of L-NAME (10^{-4}M) ($P<0.05$, one way ANOVA, N = 6), the sensitivity was -9.09± 0.39 (control) and -8.56± 0.2 (L-NAME (10^{-4}M)) ($P>0.05$, one way ANOVA) (Figure 5.1b). The maximum response to bradykinin was -83.62 ± 8.44% (control) and -81.19± 12.88% in the presence of indomethacin (10^{-5}M) ($P>0.05$, one way ANOVA, N = 6), the sensitivity was -9.09 ± 0.39 (control) and -8.77± 0.60 (indomethacin (10^{-5}M)) ($P>0.05$, one way ANOVA) (Figure 5.1). The maximum response to bradykinin was -83.62 ± 8.44% (control) and -20.34 ± 7.13% in the presence of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) ($P<0.05$, one way ANOVA, N = 6), the sensitivity was -9.09±

0.39 (control) and -8.74± 0.44 (L-NAME and indomethacin) (P>0.05, one way ANOVA) (Figure 5.1b).



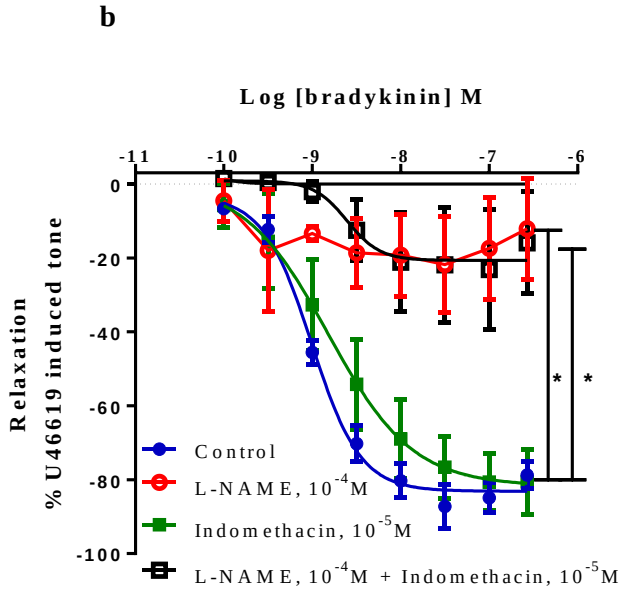


Figure 5.1 (a) Representative trace showing the vasorelaxation-induced by bradykinin in porcine splenic artery. (b) Effect of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) and their combination on the response to bradykinin in the porcine splenic artery. The relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=6. Differences between R_{max} values are shown by *P < 0.05 (one way ANOVA).

5.3.2. The effect of mefloquine on bradykinin-induced vasorelaxation in porcine splenic artery

Mefloquine, used at three different concentrations; $2x10^{-7}$, $2x10^{-6}$ and $2x10^{-5}$M did not alter the relaxation to bradykinin in PSA. The U46619 concentration used was significantly higher in the presence of mefloquine to produce the target tone (50-70% of the second KCl response). In the control tissue the concentration of U46619 used ranged from 10^{-9} and 10^{-7}M while in the presence of mefloquine concentrations as high as 10^{-6}M were required.

The maximum response to bradykinin was -51.72 ± 7.80% (control) and -57.87 ± 25.69% in the presence of mefloquine ($2x10^{-7}$M) (P>0.05, One-way ANOVA, N = 6), the sensitivity was -8.78± 0.32 (control) and -8.156 ± 0.52 (mefloquine ($2x10^{-7}$M)) (P>0.05, One-way ANOVA) (Figure 5.2). The maximum response to bradykinin was -51.72 ± 7.806% (control) and -65.90 ± 9.84% in the presence of mefloquine ($2x10^{-6}$M) (P>0.05, One-way ANOVA, N = 6), the sensitivity was -8.785 ± 0.32 (control) and -7.85 ±1.41 (mefloquine ($2x10^{-6}$M)) (P>0.05, One-way ANOVA) (Figure 5.2). The maximum response to bradykinin was -51.72 ± 7.806% (control) and -58.84 ± 10.27% in the presence of mefloquine ($2x10^{-5}$M) (P>0.05, One-way ANOVA, N = 6), the sensitivity was -8.785 ± 0.32 (control) and -8.50 ± 0.24 (mefloquine ($2x10^{-5}$M)) (P>0.05, One-way ANOVA) (Figure 5.2).

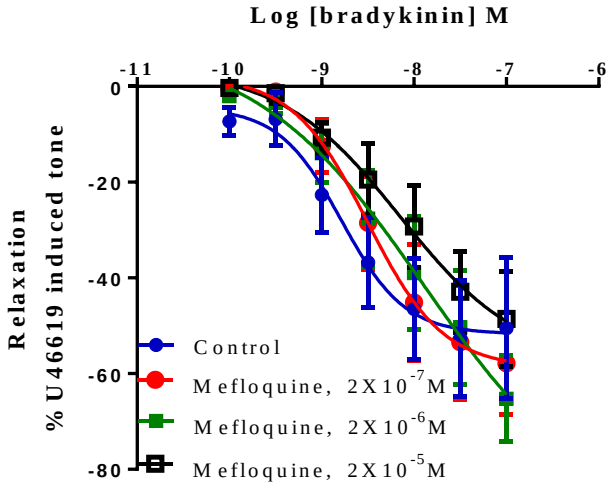


Figure 5.2 Effect of mefloquine ($2x10^{-7}$, $2x10^{-6}$ and $2x10^{-5}$M) on the response to bradykinin in the porcine splenic artery. The relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=6. Mefloquine did not alter the relaxation to bradykinin in porcine splenic artery (One-way ANOVA).

5.3.3. The effect of probenecid on bradykinin-induced vasorelaxation in porcine splenic aretry

Probenecid, used at two different concentrations; $2x10^{-5}$, $2x10^{-4}$M did not alter the relaxation-induced by bradykinin in PSA. The maximum response to bradykinin was -54.29 ± 4.50% (control) and -50.38 ± 7.25% in the presence of probenecid ($2x10^{-5}$M) (P>0.05, one way ANOVA, N = 7), the sensitivity was -8.35 ± 0.24 (control) and -8.21 ± 0.46 (probenecid ($2x10^{-5}$M)) (P>0.05, one way ANOVA) (Figure 5.3). The maximum response to bradykinin was -54.29 ± 4.50% (control) and -50.18 ± 5.03% in the presence of probenecid ($2x10^{-4}$M) (P>0.05, one way ANOVA, N = 7), the sensitivity was -8.35± 0.24 (control) and -8.32± 0.29 (probenecid ($2x10^{-4}$M)) (P>0.05, one way ANOVA)

(Figure 5.3). When using $2x10^{-3}$M probenecid, it was not possible to produce a sustained contraction with U46619.

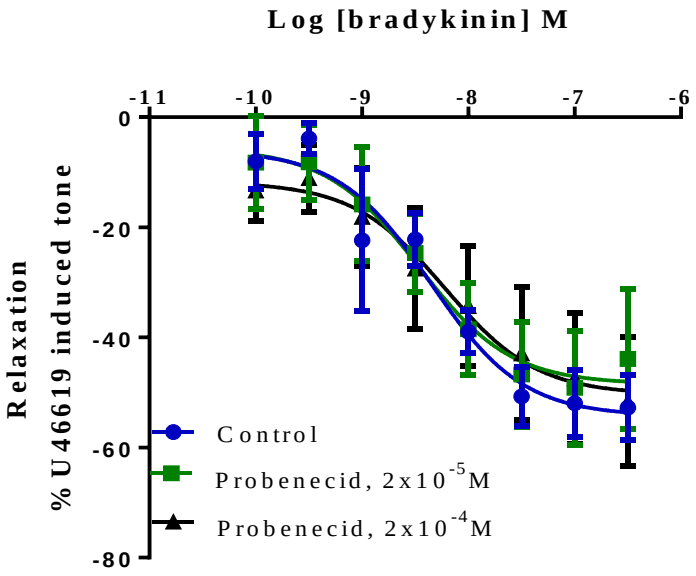


Figure 5.3 Effect of probenecid ($2x10^{-5}$ and $2x10^{-4}$M) on the response to bradykinin in the porcine splenic artery the relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=7. Probenecid did not alter the relaxation to bradykinin in porcine splenic artery (one way ANOVA).

Since there was no evidence for a large consistent EDHF response in the PSA, subsequent studies were attempted using the porcine coronary artery.

5.3.4. Pannexin1, pannexin 2 and pannexin 3 expression in porcine coronary artery

The expression of pannexin1 and pannexin2 was shown as a 50 kDa and 100 kDa bands respectively, in all samples of PCA. The lanes show coronary artery samples from 7 different pigs. The 50 kDa bands observed during western blotting correspond to

pannexin1 protein (http://www.abcam.com/pannexin-1-antibody-EPR5556-ab124969-references.html).While the 100 kDa bands correspond to pannexin2 (http://www.abcam.com/pannexin-2-antibody-ab55917-references.html) .In addition, the expression of pannexin3 (http://www.abcam.com/pannexin-3-antibody-ab98093.html) was examined using anti-pannexin3 antibody, but the expression of pannexin3 was not evident in PCA as we did not detect a band corresponding to pannexin3 (Figure 4.5).

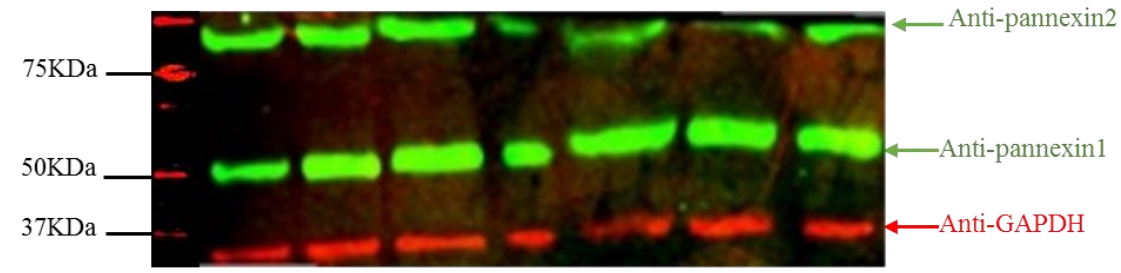


Figure 5.4 Western blot analysis to determine pannexin1, pannexin2 and pannexin3 expression in porcine coronary artery. N = 7.

5.3.5. Pannexin1 expression assessed by immunohistochemistry in porcine coronary artery

Immunolabelling of pannexin1 was carried out using anti-pannexin1 specific antibody, and a cross section of endothelium and smooth muscle cells of porcine coronary artery labelled for the presence of pannexin1 was visualised under a light microscope and a fluorescence microscope X20 and X40 magnifications. The image shows expression of pannexin1 in the endothelium and the smooth muscle cells of PCA (Figure 5.5).

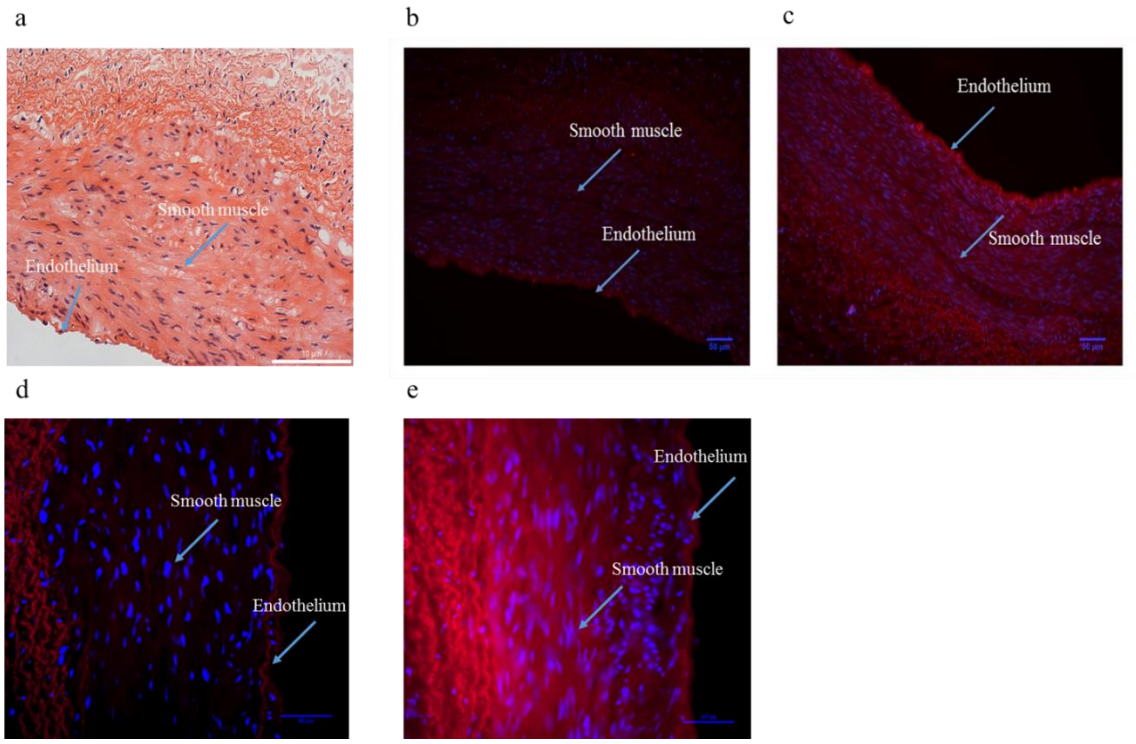


Figure 5.5. Light microscopic images of porcine coronary artery at X20 in the absence of a primary antibody (a) stained without primary antibody. Expression of pannexin1 shown by immunohistochemistry in the absence (b) and presence (c) of anti-pannexin1 specific antibody at X20. Expression of pannexin1 shown by immunohistochemistry in the absence (d) and presence (e) of anti-pannexin1 specific antibody at X40. Expression was evident in the endothelium and the smooth muscle cells of porcine coronary artery. N =6.

5.3.6. L-NAME and the combination of L-NAME and indomethacin decreased the vasorelaxation to bradykinin in porcine coronary artery

L-NAME (10^{-4}M) and the combination of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) significantly decreased the relaxation to bradykinin in PCA, while indomethacin (10^{-5}M) alone did not affect bradykinin -induced vasorelaxation in PCA. The maximum response to bradykinin was -80.27 ± 7.02% (control) and -25.15 ± 4.76% in the presence

of L-NAME (10^{-4}M) (P<0.001, one way ANOVA, N = 6), the sensitivity was -7.69 ± 0.21 (control) and -8.0± 0.2 (L-NAME (10^{-4}M)) (P>0.05, one way ANOVA) (Figure 5.6). The maximum response to bradykinin was -80.27 ± 7.02% (control) and -68.65 ± 2.88% in the presence of indomethacin (10^{-5}M) (P>0.05, one way ANOVA, N = 6), the sensitivity was -7.69 ± 0.21 (control) and -8.06 ± 0.56 (indomethacin (10^{-5}M)) (P>0.05, one way ANOVA) (Figure 5.6). The maximum response to bradykinin was -80.27 ± 7.02% (control) and -36.08 ± 4.8% in the presence of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) (P<0.001, one way ANOVA, N = 6), the sensitivity was -7.69 ± 0.21 (control) and -8.14 ± 0.11 (L-NAME and indomethacin) (P>0.05, one way ANOVA) (Figure 5.6).

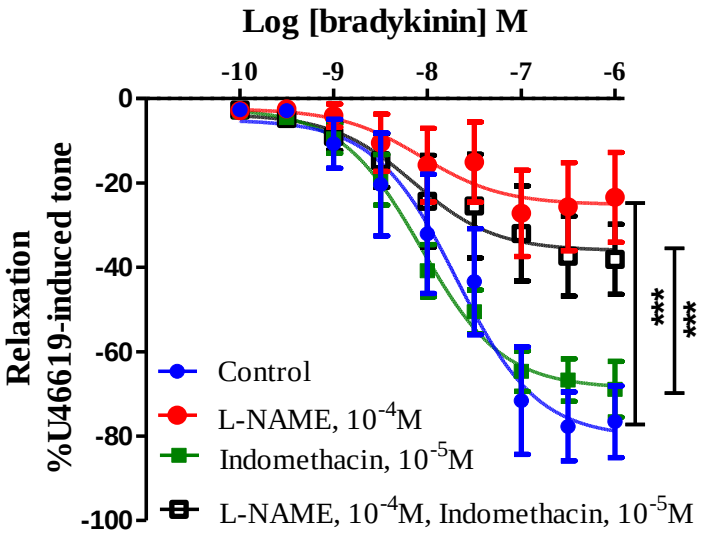


Figure 5.6 Effect of L-NAME (10^{-4}M) and indomethacin (10^{-5}M) and their combination on the response to bradykinin in the porcine coronary artery. The relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=6. Differences between R_{max} values are shown by *** P < 0.001 (one way ANOVA).

5.3.7. The effect of probenecid, carbenoxolone and mefloquine on bradykinin-induced vasorelaxation in porcine coronary artery

Mefloquine ($2x10^{-5}$M) decreased the relaxation to bradykinin, while probenecid ($2x10^{-3}$M) and carbenoxolone (10^{-4}M) did not affect bradykinin-induced vasorelaxation in PCA. The maximum response to bradykinin was -71.87± 9.54% (control) and -45.60 ± 6.48% in the presence of mefloquine ($2x10^{-5}$M) ($P<0.001$, one way ANOVA, N = 6), the sensitivity was -7.79 ± 0.24 (control) and -7.1 ± 3.4 (mefloquine ($2x10^{-5}$M)) ($P>0.05$, one way ANOVA) (Figure 5.7). The maximum response to bradykinin was -71.87± 9.54% (control) and -69.03 ± 5.67% in the presence of probenecid ($2x10^{-3}$M) ($P>0.05$, one way ANOVA, N = 6), the sensitivity was -7.79± 0.24 (control) and -8.26 ± 0.16 (probenecid ($2x10^{-3}$M)) ($P>0.05$, one way ANOVA) (Figure 5.7). The maximum response to bradykinin was -71.87 ± 9.54% (control) and -78.52 ± 6.21% in the presence of carbenoxolone (10^{-4}M) ($P>0.05$, one way ANOVA, N = 6), the sensitivity was -7.79 ± 0.24 (control) and -8.26± 0.14 (carbenoxolone (10^{-4}M) ($P>0.05$, one way ANOVA) (Figure 5.7).

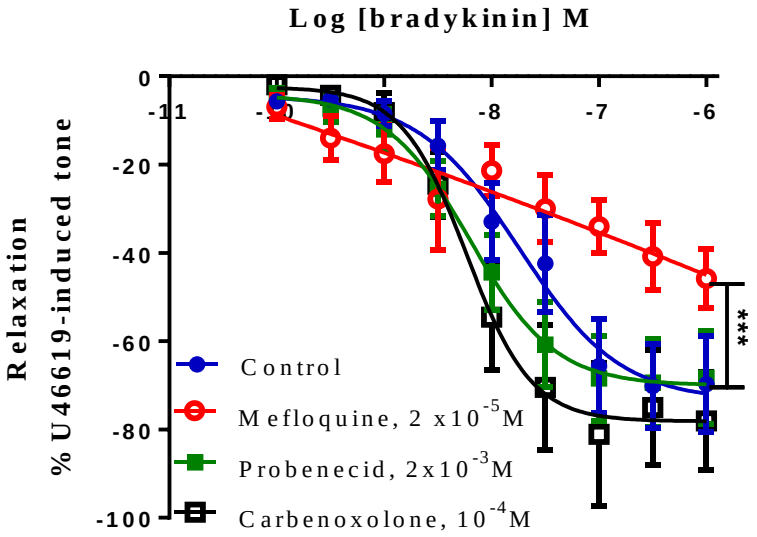


Figure 5.7 Effect of mefloquine ($2x10^{-5}$M), probenecid ($2x10^{-3}$M) and carbenoxolone (10^{-4}M) on the response to bradykinin in the porcine coronary artery. The relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=6. Differences between R_{max} values are shown by *** P < 0.001 (one way ANOVA).

5.3.8. The effect of probenecid, carbenoxolone and mefloquine on bradykinin-induced vasorelaxation in porcine coronery artery in the presence of L-NAME and indomethacin

Following treating the PCA with L-NAME (10^{-4}M) and indomethacin (10^{-5}M), mefloquine ($2x10^{-5}$M) decreased the relaxation to bradykinin, while probenecid ($2x10^{-3}$M) and carbenoxolone (10^{-4}M) did not affect bradykinin-induced vasorelaxation in PCA. The maximum response to bradykinin was -52.18 ± 8.07% (control) and -16.76 ± 3.5% in the presence of mefloquine ($2x10^{-5}$M) (P<0.01, one way ANOVA, N = 6), the sensitivity was -7.59 ± 0.25 (control) and -6.77 ± 0.61 (mefloquine ($2x10^{-5}$M)) (P>0.05, one way ANOVA) (Figure 5.8). The maximum response to bradykinin was -52.18 ±

8.07% (control) and -44.18 ± 8.70% in the presence of probenecid ($2x10^{-3}$M) (P>0.05, one way ANOVA, N = 6), the sensitivity was -7.59± 0.25 (control) and -7.77± 0.37 (probenecid ($2x10^{-3}$M)) (P>0.05, one way ANOVA) (Figure 5.8). The maximum response to bradykinin was -52.18 ± 8.07% (control) and -43.30± 7.34% in the presence of carbenoxolone (10^{-4}M) (P>0.05, one way ANOVA, N = 6), the sensitivity was -7.59± 0.25 (control) and -7.74 ± 0.32 (carbenoxolone (10^{-4}M)) (P>0.05, one way ANOVA) (Figure 5.8).

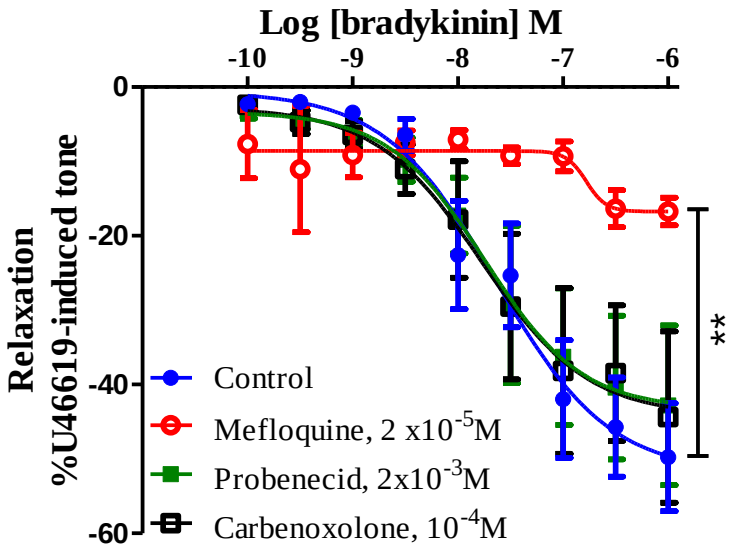


Figure 5.8 Effect of mefloquine ($2x10^{-5}$M), probenecid ($2x10^{-3}$M) and carbenoxolone (10^{-4}M) on the response to bradykinin in the porcine coronary artery in the presence of L-NAME and indomethacin. The relaxation caused by bradykinin was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± s.e.m, N=6. Differences between R_{max} values are shown by ** P < 0.01 (one way ANOVA).

5.4. Discussion

Although pannexin 1 has been shown previously to participate in endothelium-dependent vasodilation in pannexin1 knockout mice (Gaynullina *et al* 2014), the exact mechanism by which pannexin1 exerts its role remains speculative. This work addresses the theory that pannexin1 induces large vessel relaxations by regulating endothelium-derived hyperpolarization (EDH)-like mechanisms. The results showed that non-prostanoid non-nitric oxide component was involved in the bradykinin-mediated relaxation .Such experiments usually indicate the presence of an EDH- component of bradykinin-induced relaxation in porcine coronary arteries, studied after inhibition of NO-synthase and cyclooxygenase, was largely unaffected by pannexin inhibitors.

In PSA, bradykinin caused only a modest concentration-dependent relaxation in preparations exposed to L-NAME and indomethacin, indicating there was not much evidence for the presence of an EDH response whilst in the PCA, bradykinin caused a more consistent and slightly larger concentration-dependent relaxation in preparations exposed to L-NAME and indomethacin, indicating the possible presence of an EDH element in the response. Overall, the use of three different pannexin inhibitors produced conflicting effects. In the PSA, there was no evidence for an inhibitory action of pannexin inhibitors on bradykinin responses. In the PCA, the overall response showed that mefloquine inhibited the response to bradykinin, while probenecid and carbenoxolone had no effect on bradykinin .When the EDHF response was isolated, mefloquine reduced the bradykinin-induced EDHF response, while probenecid and carbenoxolone had no effect.

Studying the participation of pannexin1 in EDH mechanisms is of interest because it has been previously reported that the released of ATP from endothelial cells led to

vasorelaxation in an EDH-like manner (Kwon, 2001). Additionally, pannexins have been shown to form ATP-permeable 'hemichannels' or 'functional gap junctions' in some cell types (Bruzzone *et al* 2003; Ishikawa *et al* 2011; Sahu *et al* 2014; Dahl and Muller, 2014; Penuela *et al* 2012); such hemichannels may play a role in the EDH-like relaxation.

Pannexin1 mRNA has been shown to localize largely to endothelial cells of the murine saphenous artery (Gaynullina *et al* 2014). The existence of pannexin1 in endothelium was also established by immunohistochemistry in saphenous artery whole mounts; when pannexin1 was knocked out there was no compensatory up-regulation of pannexin2 or pannexin3.

Gaynullina *et al* (2015) reported that purinergic signalling is responsible for a considerable portion of the EDH-like response in the saphenous artery, since purinoceptor antagonists and apyrase significantly reduced EDH-like relaxations in wild type (WT) animals. Furthermore, knocking out pannexin1 protein reduced the presence of an EDHF response in the saphenous artery. They proposed the model presented in Figure 5.9.

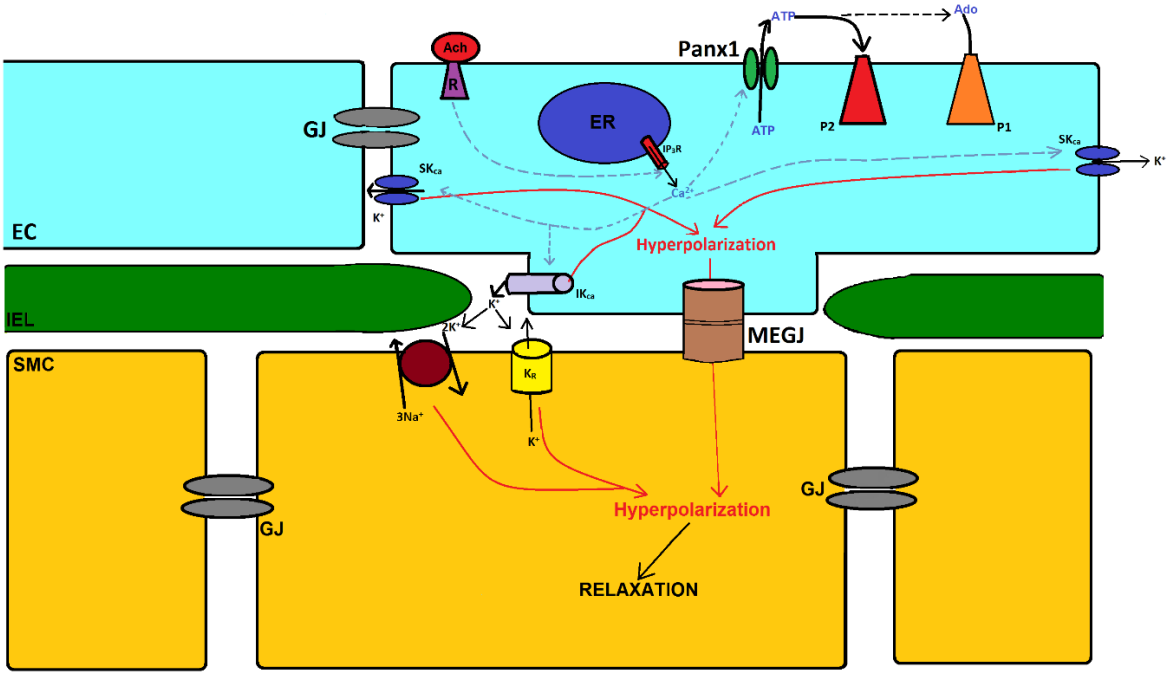


Figure 5.9 Diagram showing mechanisms of pannexin1 involvement in the functioning of EDHF-like mechanism during agonist-induced stimulation followed by smooth muscle relaxation. Intermediate conductance calcium activated potassium channels (IK_{Ca}) – endothelial intermediate conductance calcium-activated potassium channels; SK_{Ca} – small conductance calcium-activated potassium channels; R – receptor; ACh – acetylcholine; Ado – adenosine; ER – endoplasmic reticulum; IP3R – 1,4,5-inositol trisphosphate receptor; EC – endothelial cell; IEL – internal elastic lamina; SMC – smooth muscle cell; MEGJ – myoendothelial gap junctions; GJ – gap junctions; KIR – inwardly rectifying potassium channels. Adapted from Gaynullina *et al* 2015.

They proposed a possible mechanism involving the signalling of adenosine (triggered by ATP hydrolysis into adenosine). The extracellular ATP is hydrolysed to adenosine, which could bind to P1 receptors leading to vessel dilation. Additionally, it is recognised that extracellular ATP can stimulate P2Y receptors on endothelial cell surfaces, thus activating a supplementary increase in intracellular cytoplasmic Ca^{2+} (Yamamoto *et al* 2003) and further potentiating pannexin1-hemichannels (Locovei *et al* 2006). An

increase in cytoplasmic Ca^{2+} concentration is then expected following signalling pathways downstream of P1 receptors and P2 receptors, leading to an increase in the activation of intermediate conductance calcium activated potassium channels (IK_{Ca}) and calcium-sensitive potassium (KCa) channels and hyperpolarization of the endothelial cell. The resultant hyperpolarizing signal is then communicated to adjacent smooth muscle cells through the myoendothelial gap junctions or by the release of K^+ through intermediate conductance calcium activated potassium channels (IK_{Ca}). Gaynullina *et al* (2015) confirmed that the EDH-like components of ACh-induced relaxation are impaired in the absence of pannexin1.

The results from the present study indicate this is not a universal function of pannexins. In the PSA, responses to bradykinin were unaffected by three inhibitors that are known to act on pannexins, mefloquine, probenecid and carbenoxolone. There was only a small and inconsistent EDHF response in the PSA as evidenced by the response to bradykinin that remained after treatment with L-NAME and indomethacin. In the PCA, mefloquine reduced response to bradykinin, both under normal conditions and when an EDHF response was isolated. This could be taken as evidence that pannexins are involved in mediating the EDHF response consistent with Gaynullina *et al* (2015). However, probenecid and carbenoxolone had no effect on responses to bradykinin under either condition. Thus, overall, there is limited evidence to suggest that pannexins contribute to endothelium-dependent responses in either the PSA or the PCA. Interestingly, the lack of effect of carbenoxolone also indicates that the EDHF response in the PCA does not involve gap junctions (Gaynullina *et al* 2015). The mechanism of the EDHF response in the PCA is unknown and it might be speculated what other substance could affect the EDHF component in PCA such as K^+, H2O2 and hydrogen sulphide.

5.5. Conclusion

The data using three different pannexin inhibitors shows that there is no evidence to suggest pannexin channels are involved in mediating endothelium-dependent vasorelaxation in porcine splenic or coronary arteries.

Chapter 6

6. General discussion

Pannexin members (pannexin1, pannexin2, and pannexin3) are recently discovered membrane proteins present in in mammalian genomes (Bond and Naus, 2014; Panchin, 2000). The present study showed both pannexin1 and pannexin2 to be expressed in the PSA, PCA and RA**.** Immunolabelling of transverse sections of PSA, PCA and RA showed pannexin1 expression in both endothelial and smooth muscle cells. This is consistent with the observations of Billaud *et al* (2011) who provided evidence for the first time for a role of pannexin1 within the vasculature. They also used Western blotting and immunolabelling techniques to show pannexin1 to be expressed in endothelial cells and on the vascular smooth muscle cells in mouse TDA. They also showed colocalization of α_{1D}–adrenoceptors and pannexin1 antibodies. Additionally, coimmunoprecipitation from TDA lysates confirmed the association of α_{1D}–adrenoceptors and pannexin1. Immune–scanning electron microscopy techniques showed pannexin1 to be expressed in the plasma membrane of arterial smooth muscle cells of the murine systemic arterial network (aorta, carotid artery, femoral artery, renal artery, TDA, abdominal arteries, arterioles in the spinotrapezius muscle, and cremasteric arterioles). Throughout the arterial tree, pannexin1 was the only protein detected and was consistently expressed in endothelium regardless of artery size. In large arteries, pannexin1 is only present in endothelial cells. However, the expression of pannexin1 in smooth muscle cells was poorly detectable in larger conduit arteries (aorta/carotid/femoral). Some expression of pannexin1 in smooth muscle cells was detected in renal arteries, whereas in smaller arteries including TDA, abdominal artery, spinotrapezius arterioles and cremasteric arterioles, pannexin1 was found throughout the smooth muscle. Similar to the smaller systemic arteries, both small (luminal

diameter of 20–90 μm) and large (luminal diameter of 100–250 μm) coronary arteries also expressed pannexin1 in endothelium and smooth muscle cellsand no pannexin2 expression. Interestingly, in coronary arteries with a luminal diameter less than 100 μm, pannexin3 was detected in endothelium and smooth muscle (Lohman *et al* 2012).

Pannexin1 channel was shown to have a role in the spread of contractile responses through vascular smooth muscle cell by acting as a cation/small molecule channel, a mechanism that resembles how pannexin1 channels act within the CNS (Thompson, 2006). Pannexins have previously been shown to mediate paracrine intercellular communication through the release of purines (e.g. ATP or UTP). Recently, Billaud *et al* (2011) provided, for the first time, evidence for the expression and function of pannexin1 in the vasculature, in the thoracodorsal resistance arteries. The mechanism proposed include the phenylephrine-induced activation of α_1-adrenoceptors, leading to the release of ATP through pannexin1 channels, leading to vasoconstriction on neighbouring smooth muscle cells through the activation of P2 receptors. It has been concluded that pannexins may coordinate vascular smooth muscle cell constriction and thereby blood pressure regulation via the activation of purinergic receptors and the release of purines. Their evidence used chemical inhibitors including mefloquine and probenecid, but also knockout mice.

Billaud *et al* (2011) suggested that responses to noradrenaline involved pannexins in the TDA in mice. They showed that phenylephrine-induced contraction could be inhibited in the presence of pannexin1 inhibitors (mefloquine 10μM and 20μM (Lglesias *et al* 2010), probenecid (500μM and 300μM) (Pelegrin and Supernat, 2006) and pannexin1 peptide (200-300μM) (Billaud *et al* 2011). All of these pannexin inhibitors caused a significant decrease in phenylephrine-induced contraction. In addition, the purine-

degrading enzyme apyrase (1U/ml and 10 U/ml), and purinergic receptor antagonists (suramin 100µM and 300 µM) and reactive blue-2 (75µM and 100µM) (a compound that they claim is a known inhibitor of P2Y purinergic receptors) also altered $\alpha_{1D.}$-adrenoceptor-mediated responses in the mouse TDA (Billaud *et al* 2011). Billaud *et al* also showed that overexpression of pannexin1 caused an increased contractile response to phenylephrine.

This encouraged them to speculate that phenylephrine binds to α_{1D}-adrenoceptors causing the release of ATP through pannexin channels, in turn causing contraction. This ATP acts as an intercellular messenger on purinergic PY_2 receptors and causes contraction of the blood vessel. This proposed joint mechanism is demonstrated in Figure 1.7, which has been illustrated as an adaptation from Billaud *et al* 2011. However, the concurrent stimulation of P2X receptors by ATP was not ruled out as suramin is a known P2X receptor antagonist and significantly reduced the phenylephrine-induced contraction. Furthermore, the selectivity of reactive blue-2 for the P2Y receptor has been questioned (Glazel *et al* 2003). Therefore, there is not strong evidence about which purinergic receptors subtype is implicated in this response. Billaud *et al* conducted an electroporation of thoracodorsal resistance arteries with either pannexin1-green fluorescent protein or pannexin1 small interfering RNA and showed enhanced and decreased constriction, respectively. TDA were transfected either with pannexin1-green fluorescent protein or with pannexin1 small interfering RNA. When pannexin1 was overexpressed, the response of TDA to phenylephrine was increased by approximately30%, whereas under expression of pannexin1 induced a 45%decrease in constriction in response to phenylephrine. Also, transfection of TDA with control small interfering RNA did not affect phenylephrine-induced constriction. Immunolabeling of

transfected TDA revealed that pannexin1 expression was modified only in vascular smooth muscle cell and not in ECs (Billaud *et al* 2011).

Mefloquine is a drug that has been used clinically for the prevention and treatment of malaria. It does so by targeting the F (0) complex of the F (0) F (1) H (+)-ATPase (Martin *et al* 2002). Recently, Angus *et al* (2015) studied the specificity of melfloquine for pannexin1 by comparing the effect of mefloquine and carbenoxolone (a pannexin1 inhibitor) on the contractile responses in mesenteric and TDA of mice and rats. Angus *et al* (2015) argued that pannexin1 has no role in α_1-adrenoceptor mediated smooth muscle contraction, since mefloquine and carbenoxolone, at concentrations as low as 1μM, had no effect on the responses to phenylephrine and KCl. However, when mefloquine was used at a higher concentration (3-10 μM), it showed a non-selective inhibitory effect towards the responses to phenylephrine, U46619, vasopressin, sympathetic nerve stimulation and to KCl (40mM), carbachol and $CaCl_2$ induced contraction. This suggests that mefloquine becomes a non-selective inhibitor at concentrations above 1 μM. It has also been suggested that mefloquine inhibits Ca^{2+} channels in addition to being an anticholinergic (Unekwe *et al* 2010). Additionally, mefloquine has been shown to inhibit K^+ channels leading to prolonged cardiac repolarization (Borsini *et al* 2012).

In agreement with the Angus study the present study suggests that the use of mefloquine as a pannexin inhibitor is questionable because mefloquine (10–20 μM) produced a wide range of inhibitory actions, including inhibitory effects towards the contractions mediated by NA,U46619, sympathetic nerve stimulation, K^+ (40 mM), 5-HT, U46619, and L-type Ca^{++} channel-mediated responses.

Probenecid is a drug that has been used clinically for the long-term management of gout due to its inhibitory effect on the renal tubular transporter, a transporter that blocks the uptake of uric acid (Ballerini *et al* 2002). The literature also reports its effect on different transporters in different cells. For example, probenecid has been shown to inhibit ATP binding cassette (Ballerini *et al* 2002) but to selectively inhibit pannexin channels with no effect on connexin-based channels (Silverman *et al* 2008). The present study showed probenecid inhibited NA-induced contractile responses in both the PSA and the RA, possibly suggesting that pannexin1 and α_{1A}–adrenoceptors are coupled in the PSA and pannexin1 and α_{1D}–adrenoceptors are coupled in the RA. Although probenecid was also shown to inhibit KCl responses in this study, there was a degree of selectivity in that $2x10^{-5}$Mand 2 $x10^{-4}$M affected the NA-mediated contraction, but had no effect on KCl responses, while higher concentrations (2×10^{-3}M) affected both in RA.The mode of action and the mechanism by which probenecid inhibitors bind/affect to pannexin proteins are not clear, although it has been suggested that probenecid is water-soluble and thus it could interact with the hydrophilic part of the receptor protein (Silverman *et al* 2008).

Carbenoxolone is classified as a specific pannexin1 channel inhibitor (Alexander *et al* 2011, Pooenima *et al* 2011). Carbenoxolone (100 μM) has been shown to inhibit the hypotonically induced ATP release from human erythrocytes (Sridharan *et al* 2010). However, it was shown to have no effect on agonist or nerve-induced activation of alpha-adrenoceptors in rat small mesenteric resistance arteries (Angus *et al* 2015). The present study also showed carbenoxolone to have no effect on NA-induced contractile responses in the PSA, providing evidence against a role for pannexins in the PSA.

The inconsistency between different studies reported in the literature could be due to the non-selective impact of pannexin inhibitors coupled with the lack of effect of agents that interfere with purinergic signalling. For example, the present study provided evidence that both mefloquine and probenecid produced non-selective inhibition of vascular responses at high concentrations.While the present study proved the presence of both pannexin1 and pannexin2 in both endothelial and smooth muscle cells of the PSA and the RA tissuesit does not support the 'pannexin1 channel release ATP' hypothesis, suggested by Billaud *et al* (2011) in thoracodorsal resistance arteries, since the role of P2 receptors in mediating the contractile responses was ruled out in both the PSA and the RA. In PSA, pannexin1 inhibitors (probenecid and mefloquine) reduced both the sympathetic nerve stimulation and NA-induced contractile responses, potentially providing evidence for the involvement of pannexin1 channels in conducting responses to NA in these blood vessels through mediating the α_{1A}-adrenoceptor stimulated contractile responses, as opposed to the α_{1D}–adrenoceptors suggested by Billaud *et al* (2011). Similar data was obtained in the RA, a preparation that contains α_{1D}-adrenoceptors. However, no evidence was obtained for the involvement of ATP acting via P2 receptors in mediating either α_{1A}-adrenoceptors-mediated vasoconstriction in the PSA nor α_{1D}–adrenoceptor-mediated vasoconstriction in the RA.

Recently, it was reported that pannexin1 had a role in facilitating vasodilator and anticontractile effects of the endothelium (Gaynullina *et al* 2014). They used pannexin1^{-}/$^{-}$ mice as a test model for investigating the functional significance of pannexin1 and they concluded that endothelial function was impaired in conduit arteries of pannexin1 knockout mice. In particular, they demonstrated that the EDHF component of endothelium-dependent responses was impaired in pannexin1 knockout mice. The

exact mechanistic by which pannexin1 exerts its role remains speculative. However, the present study provided limited evidence for the involvement of pannexin channels in mediating the endothelium-derived hyperpolarization (EDH) response in PSA or PCA.

Future studies

More studies are warranted in the future to understand the mechanisms by which pannexin 1 channels are involved in the vasculature, as well as the mechanisms by which pannexin1 inhibitors exert their action. The non-selectivity issue related to the use of pannexin channel inhibitors could be overcome by evaluating the contractile response of tissue samples that do not express pannexin channels, i.e. by using pannexin1 knockout/knockdown techniques in preference to mefloquine and probenecid. For instance, the involvement of pannexin1 could be ruled out through inhibiting the pannexin1 gene in pannexin knockout mice using RNA interference experiments to genetically engineer pannexin knockout mice (Mocellin and Provenzano, 2004).

Aternatively, if RNA interference shows no reduction in responses then this will support the theory that the pannexin1 inhibitors we used lost selectivity at higher concentrations and that the reduction in responses seen was due to an alternative mechanism.

The involvement of pannexin1 channels in contractile responses could then only be confirmed if the results generated from pannexin1 inhibitors (i.e. mefloquine and probenecid) match those generated through RNA interference.

The findings presented in this thesis demonstrate the pannexin1 protein is present in large blood vessels but the data do not support a role for ATP released via pannexin channels in mediating responses to NA in these blood vessels. The data using three different pannexin inhibitors shows that there is no evidence to suggest pannexin

channels are involved in mediating endothelium-dependent vasorelaxation in porcine splenic or coronary arteries. It is clear that new tools are required to investigate the physiology of pannexins.

References

Abbracchio, M., Burnstock, G., Verkhratsky, A. & Zimmermann, H., (2009), 'Purinergic signalling in the nervous system, an overview', *Trends Neurosci*. 32, 19-29.

Abbracchio, M.P. & Burnstock, G., (1994), 'Purinoceptors, Are there families of P2X and P2Y purinoceptors?' *Pharmacology and Therapeutics*, 64(3), 445-475.

Abeele, F.V., Bidaux, G., Gordienko, D., Beck, B., Panchin, Y.V., Baranova, A.V. & Prevarskaya, N., (2006), 'Functional implications of calcium permeability of the channel formed by pannexin 1', *The Journal of Cell Biology*, 174(4), 535-546.

Ahlquist, R.P., (1948), 'A study of the adrenotropic receptors', *American Journal of Physiology*, 153(3), 586-600.

Alderton, W., Cooper, C., Knowlers, R., (2001), 'Nitric oxide synthases: structure, function and inhibitio', *Biochem. J.*, 357: 593-615.

Alexander, S., Mathie, A. & Peters J. A., (2015), 'Guide to receptors and channels (GRAC)', *Br J Pharmacol*, 158, S1–S239

Alexander, S., Mathie, A. & Peters J. A., (2009). Guide to receptors and channels (GRAC)', *Br J Pharmacol*, *158*, S1-S1.

Alexander, S., Mathie, A., and Peters, J., (2011), 'G protein-coupled receptors', *British Journal of Pharmacology*, 164, S5-S113.

Ambrosi, C., Gassmann, O., Pranskevich, J.N., Boassa, D., Smock, A., Wang, J. & Sosinsky, G.E., (2010), 'Pannexin 1 and pannexin 2 channels show quaternary similarities to connexons and different oligomerization numbers from each other', *Journal of Biological Chemistry*, 285(32), 24420-24431.

Angus, J. A., Broughton, A. & Mulvany, M. J., (1988), 'Role of alpha-adrenoceptors in constrictor responses of rat, guinea-pig and rabbit small arteries to neural activation', *J Physiol*, 403, 495-510.

Angus, J.A., Betrie, A.H. & Wright, C.E., (2015), 'Pannexin-1 channels do not regulate α 1-adrenoceptor-mediated vasoconstriction in resistance arteries', *European Journal of Pharmacology*, 750, 43-51.

Annane, D., Sanquer, S., Sébille, V., Faye, A., Djuranovic, D., and Raphaël, J., (2000), 'Compartmentalised inducible nitric-oxide synthase activity in septic shock', *The Lancet*, 355: 1143-1148.

Arnal, J., Dinh-Xuan, A., Pueyo, M., Darblade, B., and Rami, J., (1999), 'Endothelium-derived nitric oxide and vascular physiology and pathology', *Cell Mol Life Sci*, *55*, 1078.

Arunlakshana, O.T. & Schild, H.O., (1959), 'Some quantitative uses of drug antagonists', *British Journal of Pharmacology and Chemotherapy*, 14(1), 48-58.

Ballerini, P., Di Iorio, P., Ciccarelli, R., Nargi, E., D'Alimonte, I., Traversa, U., (2002), 'Glial cells express multiple ATP binding cassette proteins which are involved in ATP release', *Neuroreport,* 13: 1789-1792.

Bao, B.A., Lai, C.P., Naus, C.C. & Morgan, J.R., (2012), 'Pannexin 1 drives multicellular aggregate compaction via a signaling cascade that remodels the actin cytoskeleton', *Journal of Biological Chemistry*, 287(11), 8407-8416.

Bao, J. X., Gonon, F. & Stjarne, L., (1993), 'Frequency- and train length dependent variation in the roles of postjunctional alpha 1- and alpha 2- adrenoceptors for the field stimulation-induced neurogenic contraction of rat tail artery', *Naunyn Schmiedebergs Arch Pharmacol*, 347, 601-16.

Bao, L. & Dahl, G., (2006), 'Pannexin 1 in erythrocytes, function without a gap', *Proc Natl Acad Sci USA*, 103, 7655-7659.

Bao, L., Locovei, S. & Dahl, G., (2004), 'Pannexin membrane channels are mechanosensitive conduits for ATP', *FEBS Letters* 572, 65-68.

Baranova, A., Ivanov, D., Petrash, N., Pestova, A., Skoblov, M., Kelmanson, I., Shagin, D., Nazarenko, S., Geraymovych, E., Litvin, O., Tiunova, A., Born, T., Usman, N., Staroverov, D., Lukyanov, S. & Panchin Y., (2004), 'Themammalian pannexin family is homologous to the invertebrate innexin gap junction proteins', *G. enomics*, 83, 706-716.

Barbe, M. T., Monyer, H. & Bruzzone, R., (2006), 'Cell-cell communication beyond connexins, the pannexin channels', *Physiology (Bethesda)*, 21, 103-114.

Barbieri, A., Santagostino-Barbone, M.G., Zonta, F. & Lucchelli, A., (1998), 'Pharmacological characterization of α-adrenoceptors that mediate contraction in

splenic artery strips from the pig', *Naunyn-Schmiedeberg's Archives of Pharmacology*, 357(6), 654-661.

Barnes, T.M., (1994), 'OPUS, a growing family of gap junction proteins?', *Trends Genet*, 10, 303-305. 10. 1016/0168-9525(94)90023-X.

Beckel, JM., Daugherty, SL., Tyagi P., Wolf-Johnston, AS., Birder, LA., Mitchell, CH., (2015), 'Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder', *The Journal of physiology*, 593: 1857-1871.

Benham, C. D. & Tsien, R. W., (1987), 'A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle', *Nature*, 328, 275-8.

Bennett, M. & Pereda, A., (2006), *Brain Cell Biol*, 35 (1), 5.

Bennett, M., Garré, J., Orellana, J., Bukauskas, F., Nedergaard, M. & Sáez, J., (2012), 'Connexin and pannexin hemichannels in inflammatory responses of glia and neurons', *Brain Research*, 1487(0), 3-15.

Bergfeld, G.R. & Forrester, T., (1992), 'Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia', *Cardiovasc Res*, 26(1), 40-47.

Berman, R., Martin, P., Evans, W., and Griffith, T., (2002), 'Relative Contributions of NO and Gap Junctional Communication to Endothelium-Dependent Relaxations of Rabbit Resistance Arteries Vary with Vessel Size', *Microvascular Research*, 63: 115-128.

Bezzi, P. & Volterra, A., (2001), 'A neuron-glia signalling network in the active brain', *Curr Opin Neurobiol*, 11(3), 387-394.

Bhalla-Gehi, R., Penuela, S., Churko, J.M., Shao, Q. & Laird, D.W., (2010), Pannexin 1 and pannexin 3 delivery, cell surface dynamics, and cytoskeletal interactions. *J Biol Chem*, 285, 9147-9160.

Billaud, M., Chiu, Y.H., Lohman, A.W., Parpaite, T., Butcher, J.T., Mutchler, S.M. & Somlyo, A.V., (2015), 'A molecular signature in the pannexin 1 intracellular loop confers channel activation by the α1 adrenoreceptor in smooth muscle cells', *Science Signaling*, 8(364), ra17.

Billaud, M., Lohman, A., Straub, A., Looft-Wilson, R., Johnstone, S., Araj, C., Best, A., Chekeni, F., Ravichandran, K., Penuela, S., Laird, D. & Isakson, B., (2011), 'Pannexin

1 regulates alpha1-adrenergic receptor- mediated vasoconstriction', *Circ Res*, 109(1), 80-85.

Birch, D. J., Turmaine, M., Boulos, P. B. & Burnstock, G., (2008), 'Sympathetic innervation of human mesenteric artery and vein', *J Vasc Res*, 45, 323-32.

Blaschko, H., (1957), 'Formation of catechol amines in the animal body', *Br Med Bull*, 13, 162-5.

Bo, X. & Burnstock, G., (1993), 'Heterogeneous distribution of [3H]alpha, beta-methylene ATP binding sites in blood vessels', *J Vasc Res*, 30, 87-101.

Boassa, C., Ambrosi, F., Qiu, G., Dahl, G., Gaietta, G. & Sosinsky, J., (2007), 'Pannexin1 channels contain a glycosylation site that targets the hexamer to the plasma membrane', *Biol Chem*, 282 (43), 31733.

Bond, S. & Naus, C.C., (2014), 'The pannexins, past and present', *Frontiers in Physiology*, 5, 58.

Bond, S.R., Lau, A., Penuela, S., Sampaio, A.V., Underhill, T.M., Laird, D.W. & Naus, C.C., (2011), 'Pannexin 3 is a novel target for Runx2, expressed by osteoblasts and mature growth plate chondrocytes', *Journal of Bone and Mineral Research*, 26(12), 2911-2922.

Bradley, E., Law, A., Bell, D. & Johnson, C. D., (2003), 'Effects of varying impulse number on cotransmitter contributions to sympathetic vasoconstriction in rat tail artery', *Am J Physiol Heart Circ Physiol*, 284, H2007-14.

Bredt, D., Hwang, P., and Snyder, S., (1990), 'Localization of nitric oxide synthase indicating a neural role for nitric oxide', *Nature*, 347: 768-770.

Brock, J. A. & Cunnane, T. C., (1999), 'Effects of Ca^{2+} concentration and Ca^{2+} channel blockers on noradrenaline release and purinergic neuroeffector transmission in rat tail artery', *Br J Pharmacol*, 126, 11-8.

Bruzzone, R., Barbe, M.T., Jakob, N.J. & Monyer, H., (2005), 'Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes', *Journal of Neurochemistry*, 92(5), 1033-1043.

Bruzzone, R., Hormuzdi, S.G., Barbe, M.T., Herb, A. & Monyer, H., (2003), 'Pannexins, a family of gap junction proteins expressed in brain', *Proceedings of the National Academy of Sciences*, 100(23), 13644-13649.

Bruzzone, S., Guida, L., Zocchi, E., Franco, L. & De Flora, A., (2001), 'Connexin 43 hemi channels mediate Ca^{2+}-regulated transmembrane NAD+ fluxes in intact cells', *FASEB J*, 15(1), 10-12.

Bryan, R., You, J., Golding, E., and Marrelli, S., (2005), 'Endothelium-derived Hyperpolarizing Factor', *Anesthesiology*, 102: 1261-1277.

Buchholz, J. N. & Duckles, S. P., (1992), 'In vitro measurement of endogenous norepinephrine release from small blood vessels with short stimulation trains', *J Pharmacol Toxicol Methods*, 28, 137-41.

Buchthal, F. & Folkow, B., (1948), 'Interaction between acetylcholine and adenosine triphosphate in normal, curarised, and denervated muscle', *Acta Physiologica* Scandinavica 15, 150-160.

Buhimschi, I., (1998), 'The nitric oxide pathway in pre-eclampsia: pathophysiological implications', *Human Reproduction Update*, 4: 25-42.

Bunse, S., Schmidt, M., Hoffmann, S., Engelhardt, K., Zoidl, G. & Dermietzel, R., (2011), 'Single cysteines in the extracellular and transmembrane regions modulate pannexin 1 channel function', *J Membr Biol*, 244, 21-33.

Bunse, S., Schmidt, M., Prochnow, N., Zoidl, G. & Dermiet-zel, R., (2010), 'Intracellular cysteine C346 is essentially involved in regulating PANX1 channel activity', *J Biol Chem*, 285, 38444-38452.

Burns, A., Phillips, S. & Sokoya, E., (2012), 'Pannexin protein expression in the rat middle cerebral artery', *J Vasc Res*, 49(2), 101-110.

Burnstock, G. & Kennedy, C., (1985), Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol*, 16, 433-40.

Burnstock, G. & Prosser, C. L., (1960), 'Conduction in smooth muscles, comparative electrical properties', *Am J Physiol*, 199, 553-9.

Burnstock, G. & Ralevic, V., (2014), 'Purinergic signaling and blood vessels in health and disease', *Pharmacological reviews*, 66(1), 102-192.

Burnstock, G., (1972), 'Purinergic nerves', *Pharmacol Rev*, 24, 509-81.

Burnstock, G., (1976), 'Do some nerve cells release more than one transmitter?', *Neuroscience*, 1, 239-48.

Burnstock, G., (1978), 'A basis for distinguishing two types of purinoceptors in cell membrane receptors', in Straub, R. W. & Sods L., (eds.), *Cell membrane receptors for drugs and hormones, a multidisciplinary approach*. New York, Raven Press, 107-18.

Burnstock, G., (2006a), 'Pathophysiology and therapeutic potential of purinergic signaling', *Pharmacol Rev* 58(1): 58-86.

Burnstock, G., (2006b), 'Purinergic signalling', *Br J Pharmacol*, 147, Suppl 1, S172-181.

Burnstock, G., (2011), 'Introductory overview of purinergic signalling. Frontiers in Bioscience', 3, 896-900. .

Burnstock, G., (2012), 'Purinergic signalling, it's unpopular beginning, its acceptance and its exciting future', *Bioessays*, 34, 218-225.

Burnstock, G. & Ralevic, V (2013), 'Purinergic signaling and blood vessels in health and disease', *Pharmacol Rev*, 66, 102-192. 10. 1124/pr. 113. 008029.

Burnstock, G. & Ralevic, V (2014),'Purinergic signaling and blood vessels in health and disease', *Pharmacol Rev* , 66**,** 102-192.

Burnstock, G., Campbell, G., Satchell, D. & Smythe, A., (1970), 'Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by nonadrenergic inhibitory nerves in the gut', *British Journal of Pharmacology*, 40 668-688.

Bylund, D. B., Eikenberg, D. C. P., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., Molinoff, P. B., Ruffolo, R. R., Jr. & Trendelenburg, U., (1994), 'International Union of Pharmacology nomenclature of adrenoceptors', *Pharmacol Rev*, 46, 121-36.

Calver, A., Collier, J., Moncada, S., and Vallance, P., (1992), 'Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension', *Journal Of Hypertension*, 10: 1025-1032.

Calzada, B.C. & de Artiñano, A.A., (2001), 'Alpha-adrenoceptor subtypes', *Pharmacological Research*, 44(3), 195-208.

Campbell, W.B., Gebremedhin, D., Pratt, P.F. & Harder, D.R., (1996), 'Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors', *Circulation Research*, 78, 415-423.

Cea, L.A., Riquelme, M.A., Vargas, A.A., Urrutia, C. & Sáez, J.C., (2014), 'Pannexin 1 channels in skeletal muscles', *Frontiers in Physiology*, *5*.

Celetti, S.J., Cowan, K.N., Penuela, S., Shao, Q., Churko, J. & Laird, D., (2010), 'Implications of pannexin 1 and pannexin 3 for keratinocyte differentiation', *J Cell Sci*, 123(8), 1363-1372.

Chekeni, F.B., Elliott, M.R., Sandilos, J.K., Walk, S.F., Kinchen, J.M., Lazarowski, E.R., Armstrong, A.J., Penuela, S., Laird, D.W., Salvesen, G.S., Isakson, B., Bayliss, D. & Ravichandran, K., (2010), 'Pannexin 1 channels medi-ate "find-me" signal release and membrane permeability during apoptosis', *Nature*, 467, 863-867.

Chen, G., Suzuki, H., & Weston, A., (1988), 'Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels', *British Journal of Pharmacology*, 95, 1165-1174.

Cherian, P., Siller-Jackson, A., Gu, S., Wang, X., Bonewald, L., Sprague, E. & Jiang, J., (2005), 'Mechanical strain opens connexin 43 hemichannels in osteocytes, a novel mechanism for the release of prostaglandin', *Mol Biol Cell*, 16(7), 3100-3106.

Cheung, D., MacKay, G., and Burnette, E., (1999), 'Regulation of vascular tone by endothelium-derived hyperpolarizing factor', *Clin Exp Pharmacol Physiol*, 26: 172-175.

Civantos, C.B. & de Artinano, A., (2001), 'Alpha-adrenoceptor subtypes', *Pharmacological Research*, 44, 195-208.

Clancy J., McVicar A., (2009). 'Physiology and Anatomy for Nurses and Healthcare Practitioners: A homeostatic approach'. 3rd edn: London, Uk.

Coker, S.J., Batey, A.J., Lightbown, I.D., Díaz, M.E. & Eisner, D.A., (2000), 'Effects of mefloquine on cardiac contractility and electrical activity *in vivo*, in isolated cardiac

preparations, and in single ventricular myocytes', *British Journal of Pharmacology*, 129(2), 323-330.

Collo, G., Neidhart, S., Kawashima, E., Kosco-Vilbois, M., North, R. A. & Buell, G., (1997), 'Tissue distribution of the P2X7 receptor', *Neuropharmacology*, 36, 1277-1283.

Cotrina, M. L., Lin, J. H-C., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C. C. G. & Nedergaard, M., (1998), 'Connexins regulate calcium signalling by controlling ATP release', *Proceedings of the National Academy of Sciences*, 95, 15735-15740.

Cruikshank, S.J., Hopperstad, M., Younger, M., Connors, B.W., Spray, D.C. & Srinivas, M., (2004), 'Potent block of Cx36 and Cx50 gap junction channels by mefloquine', *Proceedings of the National Academy of Sciences of the United States of America*, 101(33), 12364-12369.

D'Hondt, P.R., De Smedt, H., Vinken, M., De Vuyst, E., De Bock, M., Wang, N., Rogiers, V., Leybaert, L., Himpens, B. & Bultynck, G., (2011), 'Pannexin channels in ATP release and beyond, an unexpected rendezvous at the endoplasmic reticulum', *Cell Signal*, 23(2), 305-316.

Dahl, G. & Keane, R.W., (2012), 'Pannexin, from discovery to bedside in 11+/-4 years?', *Brain Res*, 1487, 150-159.

Dahl, G. & Locovei, S., (2006), 'Pannexin: to gap or not to gap, is that a question?' *IUBMB Life*, 58(7), 409.

Dahl, G. & Muller, K.J., (2014), 'Innexin and pannexin channels and their signaling', *FEBS letters*, 588(8), 1396-1402.

Dale, H. H., (1906), 'On some physiological actions of ergot', *J Physiol*, 34, 163-206.

Dando, R. & Roper, S.D., (2009), 'Cell-to-cell communication in intact taste buds through ATP signalling from pannexin 1 gap junction hemichannels', *J. Physiol*. 587, 5899-5906. doi, 10. 1113/jphysiol. 2009. 180083.

De wit, C. & Griffith, T. M.,(2010), 'Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses', *Pflügers Archiv-European Journal of Physiology*, 459**,** 897-914.

Dixon, R. A., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D., (1986), 'Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin', *Nature*, 321, 75-9.

Dornyei, G., Kaley, G. & Koller, A., (1997), 'Release of nitric oxide and prostaglandin H2 to acetylcholine in skeletal muscle venules', *American Journal of Physiology-Heart and Circulatory Physiology* 272, H2541-H2546.

Drury, A. N. & Szent-Györgyi, A., (1929), 'The physiological activity of adenine compounds with special reference to their action upon the mammalian heart', *Journal of Physiology*, 68, 213-237.

Drury, A.N. (1936), 'The physiological activity of nucleic acid and its derivatives', *Physiol Rev*, 16, 292–325.

Dubyak, G. R., (1991), 'Signal transduction by P2-purinoceptors for extracellular ATP', *American Journal of Respiratory Cell and Molecular Biology*, 4, 295-300.

Dunn, W. R., Mcgrath, J. C. & Wilson, V. G., (1989), 'Expression of functional postjunctional alpha 2-adrenoceptors in rabbit isolated distal saphenous artery - a permissive role for angiotensin II?', *Br J Pharmacol*, 96, 259-61.

Dvoriantchikova, G., Ivanov, D., Panchin, Y. & Shestopalov, V., (2006), 'Expression of pannexin family of proteins in the retina', *FEBS Lett*, 580, 2178-2182.

Edvinsson, L., Ekblad, E., Hakanson, R. & Wahlestedt, C., (1984), 'Neuropeptide Y potentiates the effect of various vasoconstrictor agents on rabbit blood vessels', *Br J Pharmacol*, 83, 519-25.

Edwards, G., (2004), 'Potassium and potassium clouds in endothelium-dependent hyperpolarizations', *Pharmacological Research*, 49, 535-541.

Edwards, G., Dora, K.A., Gardener, M.J., Garland, C.J. & Weston, A.H., (1998), 'K^+ is an endothelium-derived hyperpolarizing factor in rat arteries', *Nature*, 396, 269-272.

El-Bermani, A. W., (1978), 'Pulmonary noradrenergic innervation of rat and monkey, a comparative study', *Thorax*, 33, 167-74.

Ellis, J. L. & Burnstock, G., (1990), 'Neuropeptide Y neuromodulation of sympathetic co-transmission in the guinea-pig vas deferens', *Br J Pharmacol*, 100, 457-62.

Ellsworth, M.L., Forrester, T., Ellis, C.G. & Dietrich, H.H., (1995), 'The erythrocyte as a regulator of vascular tone', *Am J Physiol Heart Circ Physiol.* 269, H2155 - H2161.

Emorine, L. J., Marullo, S., Briend-Sutren, M. M., Patey, G., Tate, K., Delavier-Klutchko, C. & Strosberg, A. D., (1989), 'Molecular characterization of the human beta 3-adrenergic receptor', *Science*, 245, 1118-21.

Emorine, L. J., Marullo, S., Briend-Sutren, M. M., Patey, G., Tate, K., Delavier-Klutchko, C. & Strosberg, A. D., (1989), 'Molecular characterization of the human beta 3-adrenergic receptor', *Science*, 245, 1118-21.

Evans, R. J. & Surprenant, A., (1992), 'Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP', *Br J Pharmacol*, 106, 242-9.

Fagura, M.S., Lydford, S.J. & Dougall, I.G., (1997), 'Pharmacological classification of α1-adrenoceptors mediating contractions of rabbit isolated ear artery, comparison with rat isolated thoracic aorta', *British Journal of Pharmacology*, 120(2), 247-258.

Falck, AA. (1962), 'New evidence for the localization of noradrenalin in the adrenergic nerve terminals', *Med Exp Int J Exp Med*, 6**,** 169-72.

Fedan, J. S., Hogaboom, G. K., O'Donnell, J. P., Colby, J. & Westfall, D. P., (1981), 'Contribution by purines to the neurogenic response of the vas deferens of the guinea pig', *Eur J Pharmacol*, 69, 41-53.

Forstermann, U., Pollock, J., Schmidt, H., Heller, M., and Murad, F., (1991), 'Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells', *Proceedings Of The National Academy Of Sciences* 88, 1788-1792.

Förstermann, U., Schmidt, H., Pollock, J., Sheng, H., Mitchell, J., & Warner, T., Nakane, M. & Murad, F. (1991), 'Isoforms of nitric oxide synthase Characterization and purification from different cell types', *Biochemical Pharmacology*, 42, 1849-1857.

Frielle, T., Collins, S., Daniel, K. W., Caron, M. G., Lefkowitz, R. J. & Kobilka, B. K., (1987), 'Cloning of the cDNA for the human beta 1-adrenergic receptor', *Proc Natl Acad Sci USA*, 84, 7920-4.

Furchgott, R ., & Zawadzki, J., (1980). 'The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine'. *Nature*, 288(5789), pp.373-376.

Fukui, D., Yang, X. P. & Chiba, S., (2005), 'Neurogenic double-peaked vasoconstriction of human gastroepiploic artery is mediated by both alpha1- and alpha2-adrenoceptors', *Br J Pharmacol*, 144, 737-42.

Furness, J. B., (1973), 'Arrangement of blood vessels and their relation with adrenergic nerves in the rat mesentery', *J Anat*, 115, 347-64.

Gaddum, J. H. & Kwiatkowski, H., (1939), 'Properties of the substance liberated by adrenergic nerves in the rabbit's ear', *J Physiol*, 96, 385-91.

Gardiner, S., Compton, A., Bennett, T., Palmer, R., and Moncada, S., (1990), 'Regional haemodynamic changes during oral ingestion of NG-monomethyl-l-arginine or NG-nitro-l-arginine methyl ester in conscious Brattleboro rats', *British Journal of Pharmacology 101*, 10-12.

Garland, C.J., Hiley, C.R. & Dora, K.A., (2011), 'EDHF, spreading the influence of the endothelium', *British Journal of Pharmacology*, 164(3), 839-852.

Garthwaite, J., (1995), 'Neural nitric oxide signalling', *Trends In Neurosciences*, 18: 51-52.

Gauthier, K., Edwards, E., Falck, J., Reddy, D., and Campbell, W., (2005), '14,15-Epoxyeicosatrienoic Acid Represents a Transferable Endothelium-Dependent Relaxing Factor in Bovine Coronary Arteries', *Hypertension*, 45: 666-671.

Gaynullina, D., Shestopalov, V., Panchin, Y., and Tarasova, O., (2015), 'Pannexin 1 facilitates arterial relaxation via an endothelium-derived hyperpolarization mechanism'. FEBS Letters 589: 1164-1170.

Gaynullina, D., Tarasova, O.S., Kiryukhina, O.O., Shestopalov, V.I. & Panchin, Y., (2014), 'Endothelial function is impaired in conduit arteries of pannexin 1 knockout mice', *Biology Direct*, 9(1), 1.

Gebremedhin, D., Harder, D., Pratt, P., and Campbell, W., (1998), 'Bioassay of an Endothelium-Derived Hyperpolarizing Factor from Bovine Coronary Arteries: Role of a Cytochrome P450 Metabolite', *J Vasc Res*, 35: 274-284.

Gisbert, R., Noguera, M.A., Ivorra, M.D. & D'Ocon, P., (2000), 'Functional evidence of a constitutively active population of α1D-adrenoceptors in rat aorta', *Journal of Pharmacology and Experimental Therapeutics*, 295(2), 810-817.

Glänzel, M., Bültmann, R., Starke, K., & Frahm, A., (2003), 'Constitutional isomers of Reactive Blue 2 - selective P2Y-receptor antagonists?', *European Journal Of Medicinal Chemistry*, *38*, 303-312.

Glass, R., Loesch, A., Bodin, P. & Burnstock, G., (2002), 'P2X4 and P2X6 receptors associate with VE-cadherin in human endothelial cells', *Cell Mol Life Sci*, 59, 870-81.

Goodall, M. & Kirshner, N., (1958), 'Biosynthesis of epinephrine and norepinephrine by sympathetic nerves and ganglia', *Circulation*, 17, 366-71.

Gordon, J. L., (1986), 'Extracellular ATP, effects, sources and fate', *Biochem J*, 233, 309-19.

Gordan R. Gwathmey J.K., & Xie L-H., (2015). 'Autonomic and endocrine control of cardiovascular function'. *World Journal of Cardiology* 7: 204-214.

Gossman, D.G. & Zhao, H.B., (2008), 'Hemichannel-mediated inositol 1, 4, 5-trisphosphate (IP3), release in the cochlea, a novel mechanism of IP3 intercellular signaling', *Cell Commun Adhes*, 15(4), 305-315.

Green, H.N. & Stoner, H.B., (1950), *Biological Actions of Adenine Nucleotides*. H.K. Lewis & Co, London.

Griffith, T., (2004), 'Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis?', *British Journal Of Pharmacology*, 141: 881-903.

Guimaraes, S. & Moura, D., (2001), 'Vascular adrenoceptors, an update', *Pharmacol Rev*, 53, 319-56.

Halcox, J.P., Narayanan, S., Cramer-Joyce, L., Mincemoyer, R. & Quyyumi, A.A., (2001), 'Characterization of endothelium-derived hyperpolarizing factor in the human

forearm microcirculation', *American Journal of Physiology-Heart and Circulatory Physiology*, 280, H2470-2477.

Hansen, M.A., Dutton, J.L., Balcar, V.J., Barden, J.A. & Bennett, M.R., (1999), 'P2X (purinergic), receptor distributions in rat blood vessels', *J Auton Nerv Syst*, 75, 147-55.

Hassinger, D., Guthrie, P., Atkinson, P., Bennett, M. & Kater, S., (1996), 'An extracellular signaling component in propagation of astrocytic calcium waves', *Proc Natl Acad Sci USA*, 93, 13268-13273.

He, G.W., (2002), 'Nitric oxide and endothelium-derived hyperpolarizing factor in human arteries and veins', *Journal of Cardiac Surgery*, 17, 317-323.

Hensley, K., Tabatabaie, T., Stewart, C., Pye, Q., and Floyd, R. (1997), 'Nitric Oxide and Derived Species as Toxic Agents in Stroke, AIDS Dementia, and Chronic Neurodegenerative Disorders', *Chem. Res. Toxicol*, 10: 527-532.

Hieble, J. P., Bylund, D. B., Clarke, D. E., Eikenburg, D. C., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P. & Ruffolo, R. R., JR., (1995), 'International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors, consensus update', *Pharmacol Rev*, 47, 267-70.

Hisadome, K., Koyama, T., Kimura, C., Droogmans, G., Ito, Y. & Oike, M., (2002), 'Volume regulated anion channels serve as an auto/paracrine nucleotide release pathway in aortic endothelial cells', *Journal of General Physiology* 119, 511-520.

Holton P (1959), The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *Journal of Physiology*, 145, 494-504.

Huang, Y., Maruyama, Y., Dvoryanchikov, G., Pereira, E., Chaudhari, N. & Roper, S., (2007), 'The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds', *Natl Acad Sci USA*, 104(15), 6436.

Hussain, M.B. & Marshall, I., (1997), 'Characterization of a1-adrenoceptor subtypes mediating contractions to phenilephrine in rat thoracic aorta, mesenteric artery and pulmonary artery', *Br J Pharmacol*, 122, 849-858.

Iglesias, R., Spray, D.C. & Scemes, E., (2010), 'Mefloquine blockade of pannexin 1 currents, resolution of a conflict', *Cell communication & adhesion*, 16(5-6), 131-137.

Inscho, E. W., Mitchell, K. D. & Navar, L. G., (1994), 'Extracellular ATP in the regulation of renal microvascular function', *FASEB J*, 8, 319-28.

Ishikawa, M., Iwamoto, T., Nakamura, T., Doyle, A., Fukumoto, S. & Yamada, Y., (2011), 'Pannexin 3 functions as an ER Ca^{2+} channel, hemichannel, and gap junction to promote osteoblast differentiation', *The Journal of Cell Biology*, 193(7), 1257-1274.

Iwamoto, T., Nakamura, T., Doyle, A., Ishikawa, M., deVega, S., Fukumoto, S. & Yamada, Y., (2010), 'Pannexin 3 reg-ulates intracellular ATP/cAMP levels and pro-motes chondrocyte differentiation', *J Biol Chem*, 285, 18948-18958.

Jacobson, KA. Balasubramanian, R., Deflorian, F., Gao ZG (2012), 'G proteincoupled adenosine (P1) and P2Y receptors: ligand design and receptor interactions', *Purinergic signalling* 8(3): 419-436.

Jie, K., Van Brummelen, P., Vermey, P., Timmermans, P. B. & Van Zwieten, P. A., (1984), 'Identification of vascular postsynaptic alpha 1- and alpha 2-adrenoceptors in man', *Circ Res*, 54, 447-52.

Jie, K., Van Brummelen, P., Vermey, P., Timmermans, P. B. & Van Zwieten, P. A., (1987), 'Modulation of noradrenaline release by peripheral presynaptic alpha 2-adrenoceptors in humans', *J Cardiovasc Pharmacol*, 9, 407-13.

Kar, R., Batra, N., Riquelme, M.A. & Jiang, J.X., (2012), 'Biological role of connexin intercellular channels and hemichannels', *Archives of Biochemistry and Biophysics*, 524(1), 2-15.

Katz, S. and Krum, H., (2001), 'Acetylcholine-mediated vasodilation in the forearm circulation of patients with heart failure: indirect evidence for the role of endothelium-derived hyperpolarizing factor', *The American Journal Of Cardiology*, 87: 1089-1092.

Kennedy, C. & Burnstock, G., (1985), 'Evidence for two types of P2- purinoceptor in longitudinal muscle of the rabbit portal vein', *Eur J Pharmacol*, 111, 49-56.

Kennedy, C., Chootip, K., Mitchell, C., Syed, N.I. & Tengah, A., (2013), 'P2X and P2Y nucleotide receptors as targets in cardiovascular disease', *Future Medicinal Chemistry*, 5(4), 431-449.

Kennedy, C., Todorov, L. D., Mihaylova-Todorova, S. & Sneddon, P., (1997), 'Release of soluble nucleotidases, a novel mechanism for neurotransmitter inactivation?', *Trends Pharmacol Sci*, 18, 263-6.

Kenny, B., Chalmers, D., Philpott, P. & Naylor, A., (1995), 'Characterization of an alpha 1D-adrenoceptor mediating the contractile response of rat aorta to noradrenaline', *Br J Pharmacol*, 115(6), 98.

Kenny, L.C., Baker, P.N., Kendall, D.A., Randall, M.D. & Dunn, W.R., (2002), 'Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia', *Clinical Science*, 103(1), 67-73.

Kienitz, M., Bender, K., Dermietzel, R., Pott, L. & Zoidl, G., (2011), 'Pannexin 1 constitutes the large conductance cation channel of cardiac myocytes', *J Biol Chem*, 286, 290-298.

King, B. F., Townsend-Nicholson, A., Wildman, S. S., Thomas, T., Spyer, K. M. & Burnstock, G., (2000), 'Coexpression of rat P2X2 and P2X6 subunits in *Xenopus* oocytes', *Journal of Neuroscience*, 20, 4871-4877.

Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J. & Regan, J. W., (1987), 'Cloning, sequencing, and expression of the gene coding for the human platelet alpha 2-adrenergic receptor', *Science*, 238, 650-6.

Kwon, S.C., (2001), 'Mechanisms of NO-resistant relaxation induced by acetylcholine in rabbit renal arteries', *Journal of Veterinary Medical Science*, 63(1), 37-40.

Lazrowski, E. R., & Boucher, R .,(2001), 'UTP as an Extracellular Signaling Molecule' . *Physiology*, 16**,** 1-5.

Lacy, P., Pilkington, G., Hanvesakul, R., Fish, H., Boyle, J., and Thurston, H., (2000), 'Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric small arteries', *British Journal Of Pharmacology*, 129: 605-611.

Lagercrantz, H., (1971), 'Isolation and characterization of sympathetic nerve trunk vesicles', *Acta Physiol Scand Suppl*, 366, 1-44.

Lai, C., Bechberger, J. & Naus, C., (2009), 'Pannexin 2 as a novel growth regulator in C6 glioma cells', *Oncogene*, 28, 4402-4408.

Lai, C., Bechberger, J., Thompson, R., MacVicar, B., Bruzzone, R. & Naus C., (2007), 'Tumor-Suppressive Effects of pannexin 1 in C6 Glioma Cells', *Cancer Res*, 67 (4), 1545. .

Lamkanfi, M., Malireddi, R.K.S. & Kanneganti, T.D., (2009), 'Fungal zymosan and mannan activate the cryopyrin inflammasome', *J Biol Chem*, 284, 20574-20581. doi, 10. 1074/jbc. M109. 023689.

Lamont, C., Vial, C., Evans, R. J. & Wier, W. G., (2006), 'P2X1 receptors mediate sympathetic postjunctional Ca^{2+} transients in mesenteric small arteries', *Am J Physiol Heart Circ Physiol*, 291, H3106-13.

Lands, A. M., Arnold, A., Mcauliff, J. P., Luduena, F. P. & Brown, T. G., JR., (1967), 'Differentiation of receptor systems activated by sympathomimetic amines', *Nature*, 214, 597-8.

Lee, T. J., Su, C. & Bevan, J. A., (1976), 'Neurogenic sympathetic vasoconstriction of the rabbit basilar artery', *Circ Res*, 39, 120-6.

Lewis, C. J. & Evans, R. J., (2001), 'P2X receptor immunoreactivity in different arteries from the femoral, pulmonary, cerebral, coronary and renal circulations', *J Vasc Res*, 38, 332-40.

Lis, C. J. & Evans, R. J., (2000), 'Comparison of P2X receptors in rat mesenteric, basilar and septal (coronary), arteries', *J Auton Nerv Syst*, 81, 69-74.

Liu, S. F., McCormack, D. G., Evans, T. W. & Barnes, P. J., (1989), 'Characterization and distribution of P2-purinoceptor subtypes in rat pulmonary vessels', *J Pharmacol Exp Ther*, 251, 1204-10.

Locovei, S., Wang, J. & Dahl, G., (2006), 'Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium', *FEBS Lett*, 580, 239-244.

Loesch, A. & Burnstock, G., (2000), 'Ultrastructural localisation of ATPgated P2X2 receptor immunoreactivity in vascular endothelial cells in rat brain', *Endothelium*, 7, 93-8.

Lohman, A.W., Billaud, M., Straub, A.C., Johnstone, S.R., Best, A.K., Lee, M. & Isakson, B.E., (2012), Expression of pannexin isoforms in the systemic murine arterial network. *Journal of Vascular Research*, 49(5), 405-416.

Lohman, AW. Billaud, M. & Isakson, B.E., (2012), Mechanisms of ATP release and signalling in the blood vessel wall. *Cardiovascular Research*, 95(3), 269-280.

Lomasney, J. W., Lorenz, W., Allen, L. F., King, K., Regan, J. W., Yang-Feng, T. L., Caron, M.G. & Lefkowitz, R. J., (1990), 'Expansion of the alpha 2-adrenergic receptor family, cloning and characterization of a human alpha 2-adrenergic receptor subtype, the gene for which is located on chromosome 2', *Proc Natl Acad Sci USA*, 87, 5094-8.

Lundberg, J.M., Terenius, L., Hokfelt, T. & Goldstein, M., (1983), 'High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man', *Neurosci Lett*, 42, 167-72.

Maclean, M.R., McCulloch, K.M., Macmillan, J.B. & McGrath, J.C., (1993), 'Influences of the endothelium and hypoxia on neurogenic transmission in the isolated pulmonary artery of the rabbit', *Br J Pharmacol*, 108, 150-4.

Madjar, H., Docherty, J.R. & Starke, K., (1980), 'An examination of pre- and postsynaptic alpha-adrenoceptors in the autoperfused rabbit hind limb', *J Cardiovasc Pharmacol*, 2, 619-27.

Maroto, R. & Hamill, O.P., (2001), 'Brefeldin A block of integrin-dependent mechanosensitive ATP release from *Xenopus* oocytes reveals a novel mechanism of mechanotransduction', *Journal of Biological Chemistry*, 276, 23867-23872.

Martin-Galiano, A.J., Gorgojo, B., Kunin, C.M. & de la Campa, A.G., (2002), 'Mefloquine and new related compounds target the F (0), complex of the F (0) F (1), H (+)-ATPase of Streptococcus pneumoniae', *Antimicrobial Agents and Chemotherapy*, 46, 1680-1687.

Mathewson, A., Dunn, W., (2014), 'A Comparison of Responses to Raised Extracellular Potassium and Endothelium-Derived Hyperpolarizing Factor (EDHF) in Rat Pressurised Mesenteric Arteries', *Plos ONE*, 9: e111977.

Matoba, T., Shimokawa, H., Kubota, H., Morikawa, K., Fujiki, T., and Kunihiro, I., (2002), 'Hydrogen Peroxide Is an Endothelium-Derived Hyperpolarizing Factor in

Human Mesenteric Arteries', *Biochemical And Biophysical Research Communications*, 290: 909-913.

McCulloch, A.I., Randall, M.D., (1998), 'Sex differences in the relative contributions of nitric oxide and EDHF to agonist-stimulated endotheliumdependent relaxations in the rat isolated mesenteric arterial bed', *Br J Pharmacol*, 123(8), 1700-1706.

McGeown J.G., (2002). 'Physiology: a clinical core text of human physiology with self-assessment'. Churchill Livingstone: UK.

Melhorn M.I., Brodsky A.S., Estanislau J., Khoory J.A., Illigens B., Hamachi I., Kurishita, Y., Fraser, A.D., Nicholson-Weller, A., Dolmatova, E., Duffy, H.S. & Ghiran, I.C., (2013), 'CR1-mediated ATP release by human red blood cells promotes CR1 clustering and modulates the immune transfer process', *J Biol Chem*, 288, 31139-31153 10. 1074/jbc. M113. 486035.

Metcalfe, M.J., Baker, D.M., Turmaine, M. & Burnstock, G., (2007), 'Alterations in purinoceptor expression in human long saphenous vein during varicose disease', *Eur J Vasc Endovasc Surg*, 33, 239-50.

Mihich, E., Clarke, D.A. & Philips, F.S., (1954), 'Effect of adenosine analogs on isolated intestine and uterus', *Journal of Pharmacology and Experimental Therapeutics*, 111, 335- 342.

Miura, H., Bosnjak, J.J., Ning, G., Saito, T., Miura, M. & Gutterman, D.D., (2003), 'Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles', *Circulation Research* 92, e31-e40.

Muraki, K., Imaizumi, Y., and Watanabe, M., (1998), 'Effects of UTP on membrane current and potential in rat aortic myocytes', *European Journal of Pharmacology*, 360, 239-247.

Muramatsu, I., Ohmura, T., Kigoshi, S., Hashimoto, S. & Oshita, M., (1990), 'Pharmacological subclassification of α1-adrenoceptors in vascular smooth muscle', *British Journal of Pharmacology*, 99(1), 197-201.

Nash, D.T., (1990), 'Alpha-adrenergic blockers, mechanism of action, blood pressure control, and effects of lipoprotein metabolism', *Clinical Cardiology*, 13, 764-772.

Nielsen, H., Thom, S. M., Hughes, A. D., Martin, G. N., Mulvany, M. J. & Sever, P. S., (1989), 'Postjunctional alpha 2- adrenoceptors mediate vasoconstriction in human subcutaneous resistance vessels', *Br J Pharmacol*, 97, 829-34.

Nilsson, H., Goldstein, M. & Nilsson, O., (1986), 'Adrenergic innervation and neurogenic response in large and small arteries and veins from the rat', *Acta Physiol Scand*, 126, 121-33.

O'Connell, T.D., Jensen, B.C., Baker, A.J. & Simpson, P.C., (2014), 'Cardiac alpha1-adrenergic receptors, novel aspects of expression, signaling mechanisms, physiologic function, and clinical importance', *Pharmacological Reviews*, 66, 308-333.

Ohbuchi, T., Yokoyama, T., Saito, T., Ohkubo, J. I., Suzuki, H., Ishikura, T. & Ueta, Y., (2011), 'Possible contribution of pannexin channel to ATP-induced currents in vitro in vasopressin neurons isolated from the rat supraoptic nucleus', *Brain Research*, 1394, 71-78.

Ohta, M., Toyama, K., Gutterman, D.D., Campbell, W.B., Lemaître, V., Teraoka, R. & Miura, H., (2013), 'Ecto-5′-nucleotidase, CD73, is an endothelium-derived hyperpolarizing factor synthase', *Arteriosclerosis, Thrombosis, And Vascular Biology*, 33(3), 629-636.

Pakdeechote, P., Rummery, N.M., Ralevic, V. & Dunn, W.R., (2007), 'Raised tone reveals purinergic-mediated responses to sympathetic nerve stimulation in the rat perfused mesenteric vascular bed', *Eur J Pharmacol*, 563, 180-6.

Palmer, RM., Ferrige, AG. Moncada, S., (1987), 'Nitric release accounts for the biological activity of endothelium-derived relaxing factor', *Nature*, 327:524-526.

Panchin, Y., Kelmanson, I., Matz, M., Lukyanov, K., Usman, N. & Lukyanov, S., (2000), 'Ubiquitous family of putative gap junction molecules', *Curr Biol*, 10, R473-R474.

Parkington, H., Chow, J., Evans, R., Coleman, H., and Tare, M., (2002), 'Role for endothelium-derived hyperpolarizing factor in vascular tone in rat mesenteric and hindlimb circulations*in vivo*', *The Journal Of Physiology*, 542: 929-937.

Paul, D.L., (1986), 'Molecular cloning of cDNA for rat liver gap junction protein', *J Cell Biol*, 103, 123-134. 10. 1083/jcb. 103. 1. 123.

Pelegrin, P. & Surprenant, A., (2006), 'Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor', *EMBO J*. 25(21), 5071.

Penuela, S., Bhalla, R., Gong, Q., Cowan, N., Celetti, J., Cowan, J., Bai, D., Shao, Q. & Laird D., (2007), 'Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins', *J Cell Sci*, 120(21), 3772-373.

Penuela, S., Bhalla, R., Nag, K. & Laird, D.W., (2009), 'Glycosylation regulates pannexin intermixing and cellular localization', *Mol Biol Cell*, 20, 4313-4323.

Penuela, S., Celetti, S., Bhalla, R., Shao, Q. & Laird, D., (2008), 'Distribution Profiles of Pannexin 1 and Pannexin 3', *Cell Commun Adhes*, 15 (1), 133.

Penuela, S., Gehi, R. & Laird, D.W., (2012.) 'The cellular life of pannexins'. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, 1, 621-632.

Penuela, S., Gehi, R. & Laird, D.W., (2013), 'The biochemistry and function of pannexin channels', *Biochim Biophys Acta* 2012. doi, 10. 1016/j. bbamem. 01. 017.

Poornima, V., Madhupriya, M., Kootar, S., Sujatha, G., Kumar, A. & Bera, A.K., (2012), 'P2X7 receptor-pannexin 1 hemichannel association, effect of extracellular calcium on membrane permeabilization', *Journal of Molecular Neuroscience*, 46(3), 585-594.

Powell, C. E. & Slater, I. H., (1958), 'Blocking of inhibitory adrenergic receptors by a dichloro analog of isoproterenol', *J Pharmacol Exp Ther*, 122, 480-8.

Qiu, F. & Dahl, G, (2009), 'A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP', *Am. J. Cell Physiol*, 296(2), C250.

Gardiner, S., Compton, A., Bennett, T., Palmer, R. and Moncada, S. (1990). 'Regional haemodynamic changes during oral ingestion of NG-monomethyl-l-arginine or NG-nitro-l-arginine methyl ester in conscious Brattleboro rats'. *British Journal of Pharmacology*, 101(1), pp.10-12.

Qu, Y., Misaghi, S., Newton, K., Gilmour, L., Louie, S., Cupp, J., Dubyak, G., Hackos, D. & Dixit, V., (2011), 'Pannexin 1 is required for ATP release during apoptosis but not for inflammasome activation', *J Immunol*, 186, 6553-6561.

Ralevic, V. & Burnstock, G., (1990), 'Postjunctional synergism of noradrenaline and adenosine 5'-triphosphate in the mesenteric arterial bed of the rat', *Eur J Pharmacol*, 175, 291-9.

Ralevic, V. & Burnstock, G., (1998), Receptors for purines and pyrimidines. *Pharmacol Rev*, 50, 413-92.

Ralevic, V. & Kendall, D. A., (2002), 'Cannabinoids inhibit pre- and postjunctionally sympathetic neurotransmission in rat mesentericarteries', *Eur J Pharmacol*, 444, 171-81.

Ralevic, V., (2009), 'Purines as neurotransmitters and neuromodulators in blood vessels', *Curr Vasc Pharmacol*, 7, 3-14.

Rang, H.P., Ritter, J.M., Flower, R.J. & Henderson, G., (2014), *Rang & Dale's Pharmacology*, Elsevier Health Sciences.

Ransford, G., Fregien, N., Qiu, F., Dahl, G., Conner, G. & Salathe, M., (2009), 'Pannexin 1 contributes to ATP release in airway epithelia', *Am J Respir Cell Mol Biol*, 41(5), 525-534.

Ray, A., Zoidl, G., Wahle, P. & Dermietzel, R., (2006), 'Pannexin expression in the cerebellum', *Cerebellum*, 5, 189-192.

Ray, F.R., Huang, W., Slater, M. & Barden, J.A., (2002), 'Purinergic receptor distribution in endothelial cells in blood vessels, a basis for selection of coronary artery grafts', *Atherosclerosis*, 162, 55-61.

Regan, J.W., Kobilka, T.S., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J. & Kobilka, B.K., (1988), 'Cloning and expression of a human kidney cDNA for an alpha 2-adrenergic receptor subtype', *Proc Natl Acad Sci USA*, 85, 6301-5.

Reisin, I.L., Prat, A.G., Abraham, E.H., Amara, J.F., Gregory, R.J., Ausiello, D.A. & Cantiello, H.F., (1994), 'The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel', *Journal of Biological Chemistry* 269, 20584-20591.

Richardson, P.J. & Brown, S.J., (1987), 'ATP release from affinity purified rat cholinergic nerve terminals', *J Neurochem*, 48, 622-30.

Roberts, R.E., Tomlinson, A.E., Kendall, D.A. & Wilson, V.G., (1998), 'Alpha2-adrenoceptor-mediated contractions of the porcine isolated ear artery, evidence for a

cyclic AMP-dependent and a cyclic AMP-independent mechanism', *Br J Pharmacol*, 124, 1107-14.

Roman, R.M., Wang, Y., Lidofsky, S.D., Feranchak, A.P., Lomri, N., Scharschmidt, B.F. & Fitz, J.G., (1997), 'Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume', *Journal of Biological Chemistry*, 272, 21970-21976.

Rummery, N.M., Brock, J A., Pakdeechote, P., Ralevic, V. & Dunn, W.R., (2007), 'ATP is the predominant sympathetic neurotransmitter in rat mesenteric arteries at high pressure', *J Physiol*, 582, 745-54.

Rutishauser, S., (1994), *Physiology and anatomy, a basis of nursing and health care*. Churchill Livingstone, UK.

Sabirov, R.Z., Okada, Y., (2004), 'ATP-conducting maxi-anion channel, a new player in stress-sensory transduction', *Japanese Journal of Physiology* 54, 7-14.

Sahu, G., Sukumaran, S. & Bera, A.K., (2014), 'Pannexins form gap junctions with electrophysiological and pharmacological properties distinct from connexins', *Scientific reports*, 4, 4955, doi: 10.1038/srep04955.

Samoilova, M., Li, J., Pelletier, M., Wentlandt, K., Adamchik, Y., Naus, C. & Carlen, P., (2003), Epileptiform activity in hippocampal slice cultures exposed chronically to bicuculline: increased gap junctional function and expression', *Neurochem J*, 86(3), 687. .

Sandow, S., (2002). 'Involvement of Myoendothelial Gap Junctions in the Actions of Endothelium-Derived Hyperpolarizing Factor', *Circulation Research*, 90: 1108-1113.

Santiago, M., Veliskova, J., Patel, N., Lutz, S., Caille, D., Charollais, A., Meda, P. & Scemes, E., (2011), 'Targeting pannexin 1 improves seizure outcome', *PLoS One*, 6, e25178.

Saussy Jr, D.L., Goetz, A.S., Queen, K.L., King, H.K., Lutz, M.W. & Rimele, T.J., (1996), 'Structure activity relationships of a series of buspirone analogs at alpha-1 adrenoceptors, Further evidence that rat aorta alpha-1 adrenoceptors are of the alpha-1D-subtype', *J Pharmacol Exp Ther*, 278, 136-144.

Scemes, E., Spray, D. & Meda, P., (2009), 'Connexins, pannexins, innexins, novel roles of "hemi-channels"' *Pflugers Arch* 457(6), 1207.

Schror, K., (1990), 'Thromboxane A2 and platelets as mediators of coronary arterial vasoconstriction in myocardial ischaemia', *Eur Heart J*, 11(B), 27-34. .

Schwiebert, L. M., Rice, W. C., Kudlow, B. A., Taylor, A. L. & Schwiebert, E. M., (2002), 'Extracellular ATP signalling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells', *Am J Physiol Cell Physiol*, 282, C289-301.

Seeley, R., Tate, P., and Stephens, T., (2008), 'Anatomy & physiology (Dubuque, IA: McGraw-Hill) '.

Seifert R, Schultz G., (1989), 'Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides', *Trends Pharmacol Sci*, 10(9): 365-369.

Seror, C., Melki, M., Subra, F., Raza, S., Bras, M., Saïdi, H., Nardacci, R., Voisin, L., Paooletti, A., Law, F., Martins, I., Amendola, A., Abdul-Sater, A., Ciccosanti, F., Delelis, O., Niedergang, F., Thierry, S., Said-Sadier, N., Lamaze, C., Métivier, D., Estaquier, J., Fimia, G., Falasca, L., Casetti, R., Modjtahedi, N., Kanellopoulos, J., Mouscadet, J., Ojcius, D., Piacentini, M., Gougeon, M., Kroemer, G. & Perfettini, J., (2006), 'Extracel-lular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection', *J Exp Med*, 208, 1823-1834.

Shatarat, A., Dunn, W.R. & Ralevic, V., (2014), 'Raised tone reveals ATP as a sympathetic neurotransmitter in the porcine mesenteric arterial bed', *Purinergic Signalling*, 10(4), 639-49.

Sheppard, H.A.A., (1986), 'Use of the fluorescent day, Fast blue, to label sympathetic postganglionc neurons supplying mesenteric arteries and enteric neurones of the rat', *Journal Of the Autonomic Nervous System*, 18, 73-82.

Sherwood, L., (1997), *Human physiology, from cells to systems*. 3rd edn. Belmont, CA, Wadsworth Publishing Company.

Shestopalov, V. & Panchin, Y., (2008), 'Pannexins and gap junction protein diversity', *Cell Mol Life Sci*. 65, 376-394.

Shimokawa, H., Yasutake, H., Fujii, K., Owada, M., Nakaike, R., and Fukumoto, Y., (1996), 'The Importance of the Hyperpolarizing Mechanism Increases as the Vessel Size Decreases in Endothelium-Dependent Relaxations in Rat Mesenteric Circulation', *Journal Of Cardiovascular Pharmacology*, 28: 703-711.

Silverman, W., De Rivero Vaccari, J.P., Locovei, S., Qiu, F., Carlsson, S., Scemes, E., Keane, R. & Dahl, G., (2009), 'The pannexin 1 channel activates the inflammasome in neurons and astrocytes', *J Biol Chem*, 284, 18143-18151.

Silverman, W., Locovei, S. & Dahl, G., (2008), 'Probenecid, a gout remedy, inhibits pannexin 1 channels', *American Journal of Physiology-Cell Physiology*, 295(3), C761-C767.

Simonsen, U., Prieto, D., Hernandez, M., Saenz De Tejada, I. & Garcia-Sacristan, A., (1997), 'Adrenoceptor-mediated regulation of the contractility in horse penile resistance arteries', *J Vasc Res*, 34, 90-102.

Sneddon, P. & Burnstock, G., (1984), 'Inhibition of excitatory junction potentials in guinea-pig vas deferens by alpha, beta-methylene-ATP, further evidence for ATP and noradrenaline as cotransmitters', *Eur J Pharmacol*, 100, 85-90.

SoRelle, R., (1998), 'Nobel Prize Awarded to Scientists for Nitric Oxide Discoveries', *Circulation*, 98, 2365-2366.

Sorge, R.E., Trang, T., Dorfman, R., Smith, S.B., Beggs, S., Ritchie, J. & Herbert, T.A., (2012), 'Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity', *Nature medicine*, 18(4), 595-599.

Sosinsky, G., Boassa, D., Dermietzel, R., Duffy, H., Laird, D., MacVicar, B., Naus, C., Penuela, S., Scemes, E., Spray, D., Thompson, R., Zhao, H. & Dahl, G., (2011), 'Pannexin channels are not gap junction hemichannels', *Channels (Austin)*, 5193-197.

Sridharan M., Adderley S.P., Bowles E.A., Egan T.M., Stephenson A.H., Ellsworth M.L., and Sprague R.S., (2010), 'Pannexin 1 is the conduit for low oxygen tension-induced ATP release from human erythrocytes', *Am J Physiol Heart Circ Physiol*, 299, H1146-H1152. 10. 1152/ajpheart. 00301. 2010.

Starke, K., (1981), 'Alpha-adrenoceptor subclassification', *Rev Physiol Biochem Pharmacol*, 88, 199-236.

Stridh, M., Tranberg, M., Weber, S., Blomstrand, F. & Sandberg, M., (2008), 'Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular calcium, likely involvement of connexin hemichannels', *J Biol Chem*, 283(16), 10347-10356.

Su, C. & Bevan, J. A., (1970), 'The release of H3-norepinephrine in arterial strips studied by the technique of superfusion and transmural stimulation', *J Pharmacol Exp Ther*, 172, 62-8.

Suadicani, S. O., Brosnan, C. F. & Scemes, E., (2006), 'P2X7 Receptors mediate ATP release and amplification of astrocytic intercellular Ca^{2+} signalling', *Journal of Neuroscience* 26, 1378-1385.

Sumi, Y., Woehrle, T., Chen, Y., Yao, Y., Li, A. & Junger, W. G., (2010), 'Adrenergic receptor activation involves ATP release and feedback through purinergic receptors', *American Journal of Physiology-Cell Physiology*, 299(5), C1118-C1126.

Swayne, L., Sorbara, C. & Bennett, S., (2010), 'Pannexin 2 is expressed by postnatal hippocampal neural progenitors and modulates neuronal commitment', *J Biol Chem*, 285, 24977-24986.

Takeuchi, K., Shinozuka, K., Akimoto, H., Ishii, R. & Hashimoto, T., (1994), 'Methoxamine-induced release of endogenous ATP from rabbit pulmonary artery', *Eur J Pharmacol*, 254, 287-90.

Taylor, S. and Weston, A., (1988), 'Endothelium-derived hyperpolarizing a new endogenous inhibitor from the vascular endothelium', *Trends In Pharmacological Sciences*, 9: 272-274.

Templeton, A. G., Macmillan, J., Mcgrath, J. C., Storey, N. D. & Wilson, V. G., (1989), 'Evidence for prazosin-resistant, rauwolscine-sensitive alpha-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail', *Br J Pharmacol*, 97, 563-71.

Tewari, M. and Seth P., (2015), 'Emerging role of P2X7 receptors in CNS health and disease', *Ageing Res Rev*, 24, 328-42.

Thompson, R., (2006), 'Ischemia Opens Neuronal Gap Junction Hemichannels', *Science*, 312, 924-927.

Thompson, R., Jackson, M., Olah, M., Rungta, R., Hines, D., Beazely, M., MacDonald, J. & MacVicar, B., (2008), 'Activation of pannexin-1 hemichannels augments aberrant bursting in the hippocampus', *Science*, 322, 1555-1559.

Toda, N. & Okamura, T., (1990), 'Mechanism underlying the response to vasodilator nerve stimulation in isolated dog and monkey cerebral arteries', *The American Journal of Physiology*, 259, H1511-1517.

Toda, N. and Okamura, T., (1990), 'Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle', *Biochemical and Biophysical Research Communications*, 170, 308-313.

Toda, N., (1981), 'Non-Adrenergic, Non-Cholinergic Innervation In Monkey And Human Cerebral Arteries', *British Journal of Pharmacology*, 72, 281-283.

Todorov, L.D., Mihaylova-Todorova, S., Westfall, T.D., Sneddon, P., Kennedy, C., Bjur, R. A. & Westfall, D P., (1997), 'Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation', *Nature*, 387, 76-9.

Turmel, P., Dufresne, J., Hermo, L., Smith, C.E., Penuela, S., Laird, D.W. & Cyr, D.G., (2011), 'Characterization of pannexin 1 and pannexin 3 and their regulation by androgens in the male reproductive tract of the adult rat', *Molecular Reproduction and Development*, 78(2), 124-138.

Unekwe, P., Ogamba, J., Chilaka, K., & Okonkwo, J., (2010), 'Effect of mefloquine on the mechanical activity of the mouse isolated rectal smooth muscle', *Nigerian Journal Of Physiological Sciences* 22.

Urakami-Harasawa, L., Shimokawa, H., Nakashima, M., Egashira, K., and Takeshita, A., (1997), 'Importance of endothelium-derived hyperpolarizing factor in human arteries', *Journal Of Clinical Investigation* 100: 2793-2799.

Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A. & Buell, G., (1994), 'A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP', *Nature*, 371, 516-9.

Vane, J.,Mitchell, J., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J. and Willoughby, D. (1994). 'Inducible isoforms of cyclooxygenase and nitric-oxide

synthase in inflammation.' *Proceedings of the National Academy of Sciences*, 91(6), pp.2046-2050.

Vallance, P., (2001), 'Endothelial function and nitric oxide: clinical relevance', *Heart*, 85: 342-350.

Vallance, P., (2003), 'Nitric oxide: therapeutic opportunities', *Fundam Clin Pharmacol*, 17: 1-10.

Vallance, P., Collier, J., and Moncada, S., (1989), 'Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man', *The Lancet*, 334: 997-1000.

Vanhoutte, P., (2004), 'Endothelium-dependent hyperpolarizations: the history', *Pharmacological Research*, 49: 503-508.

Verderio, C., Bruzzone, S., Zocchi, E., Fedele, E., Schenk, U., De Flora, A. & Matteoli, M., (2001), 'Evidence of a role for cyclic ADP-ribose in calcium signalling and neurotransmitter release in cultured astrocytes', *J Neurochem* 78(3), 646-657. .

Vial, C., (2002), 'P2X1 Receptor-Deficient Mice Establish the Native P2X Receptor and a P2Y6-Like Receptor in Arteries', *Molecular Pharmacology*, 62, 1438-1445.

Villalobos-Molina, R. & Ibarra, M., (1996), 'α 1-Adrenoceptors mediating contraction in arteries of normotensive and spontaneously hypertensive rats are of the α 1D or α 1A subtypes', *European Journal of Pharmacology*, 298(3), 257-263.

Vogt, A., Hormuzdi, S. & Monyer, H., (2005), 'Pannexin 1 and pannexin 2 expression in the developing and mature rat brain', *Mol Brain Res*, 141, 113-120.

Von Kugelgen, I., (2006), 'Pharmacological profiles of cloned mammalian P2Y-receptor subtypes', *Pharmacol Ther*, 110, 415-32.

Von Kügelgen, I., Allgaier, C., Schobert, A. & Starke, K., (1994), 'Co-release of noradrenaline and ATP from cultured sympathetic neurons', *Neuroscience*, 61, 199-202.

Wang, J. & Dahl, G., (2010), 'SCAM analysis of Panx1 suggests a peculiar pore structure', *J Gen Physiol*, 136, 515-527.

Wang, J., Jackson, DG., Dahl, G., (2013), 'The food dye FD&C Blue No. 1 is a selective inhibitor of the ATP release channel pannexin1', *The Journal of General Physiology*, 141: 649-656.

Wang, J., Ma, M., Locovei, S., Keane, R. & Dahl, G., (2007), 'Modulation of membrane channel currents by gap junction protein mimetic peptides, size matters', *Am J Physiol* Cell Physiol, 293, C1112-C1119.

Wang, L., Karlsson, L., Moses, S., Hultgardh-Nilsson, A., Andersson, M., Borna, C., Gudbjartsson, T., Jern, S. & Erlinge, D., (2002), 'P2 receptor expression profiles in human vascular smooth muscle and endothelial cells', *J Cardiovasc Pharmacol*, 40, 841-53.

Wang, X.H., Streeter, M., Liu, Y.P. & Zhao, H.B., (2009), 'Identification and characterization of pannexin expression in the mammalian cochlea', *J Comp Neurol*, 512, 336-346.

Westfall, D. P., Sedaa, K. & Bjur, R. A., (1987), 'Release of endogenous ATP from rat caudal artery', *Blood Vessels*, 24, 125-7.

Westfall, D.P., Stitzel, R. E. & Rowe, J. N., (1978), 'The postjunctional effects and neural release of purine compounds in the guinea-pig vas deferens', *Eur J Pharmacol*, 50, 27-38.

Wise, A., Watson-Koken, M. A., Rees, S., Lee, M. & Milligan, G., (1997), 'Interactions of the alpha2A-adrenoceptor with multiple Gifamily G-proteins, studies with pertussis toxin-resistant G-protein mutants', *Biochem J*, 321(3), 721-8.

Xia, J., Lim, J.C., Lu, W., Beckel, J.M., Macarak, E.J., Laties, A.M. & Mitchell, C.H., (2012), 'Neurons respond directly to mechanical deformation with pannexin-mediated ATP release and autostimulation of P2X7 receptors', *The Journal of Physiology*, 590(10), 2285-2304.

Yaginuma, H., Kawai, S., Tabata, K., Tomiyama, K., Kakizuka, A., & Komatsuzaki, T., Noji, H. & Imamura, H., (2014), 'Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging', *Sci. Rep*, 4, 6522.

Yamamoto, K., Sokabe, T., Ohura, N., Nakatsuka, H., Kamiya, A. & Ando, J., (2003), 'Endogenously released ATP mediates shear stress-induced Ca^{2+} influx into pulmonary artery endothelial cells', *American Journal of Physiology-Heart and Circulatory Physiology*, 285(2), H793-H803.

Ye, Z., Wyeth, M., Baltan-Tekkok, S. & Ransom B., (2003), 'Functional hemichannels in astrocytes, a novel mechanism of glutamate release', *J Neurosci*, 23(9), 3588-3596.

Yen, M. & Saier Jr., M., (2007), 'Gap junctional proteins of animals, the innexin/pannexin superfamily', *Prog Biophys Mol Biol*, 94, 5-14.

Zappalà A., Li Volti, G., Serapide, M.F., Pellitteri, R., Falchi, M., La Delia, F., Cicirata, V. & Cicirata, F. (2007) 'Expression of pannexin2 protein in healthy and ischemized brain of adult rats', *Neuroscience*. Sep;148(3) 653-667. doi:10.1016/j.neuroscience.2007.06.028. PMID: 17692470.

Zappala, A., Cicero, D., Serapide, M., Paz, C., Catania, M., Falchi, M., Parenti, R., Panto, M., La Delia, F. & Cicirata, F., (2006), 'Expression of pannexinl in the CNS of adult mouse: cellular localization and effect of 4- aminopyridine-induced seizures', *Neuroscience*, 141 (1), 167.

Zappala, A., Li Volti, G., Serapide, M., Pellitteri, R., Falchi, M., La Delia, F., Cicirata, V. & Cicirata, F., (2007), 'Expression of pannexin2 protein in healthy and ischemized brain of adult rats', *Neuroscience*, 148 (3), 653.

Zhong, H. & Minneman, K. P., (1999), 'Alpha1-adrenoceptor subtypes', *Eur J Pharmacol*, 375, 261-76.

Zimmermann, H., (2006), 'Ectonucleotidases in the nervous system', *Novartis Found Symp*, 276, 113-28; discussion 128-30, 233-7, 275-81.