INVESTIGATION OF THE MECHANISMS OF
URIDINE 5' TRIPHOSPHATE INDUCED
CONTRACTION OF RAT MESENTERIC ARTERY
SMOOTH MUSCLE

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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July 2011
ABSTRACT

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Investigation of the mechanisms of uridine 5’ triphosphate induced contraction of rat mesenteric artery smooth muscle.

Uridine 5’ triphosphate (UTP) is a pyrimidine nucleotide which is released from a variety of cells including platelets and endothelial cells. The release of UTP can lead to vasoconstriction if there is damage to the endothelial layer and hence its actions on the vasculature are important areas of investigation.

The research presented in this thesis describes the measurement of isometric contractile responses of rat mesenteric arteries to UTP and of the electrophysiological measurements of ionic currents of smooth muscle cells isolated from these arteries.

UTP induced contractions were recorded using various pharmacological tools to investigate the possible signaling mechanisms leading to the contractile response. Particular attention was given to the identification of the ion channels involved in generating the UTP induced contraction. It was clear that the contraction was dependent on Ca\(^{2+}\) influx and my results indicated that this influx was only partly due to an increased activity of voltage-gated Ca\(^{2+}\) channels and that non-selective cation channels were also important. The involvement of PLC is likely as using U73122, a PLC blocker, significantly reduced the UTP induced response. However, the potential involvement of PKC is less convincing as several PKC isoform peptide inhibitors, linked to the carrier peptide Tat(47-57) to render them membrane permeable, failed to affect the contraction.

The ionic basis of the UTP induced contraction was studied by measuring the effect of UTP on various ionic currents measured in enzymatically isolated mesenteric artery smooth muscle cells using the whole-cell patch-clamp technique. UTP was found to inhibit both Kv and K\(_{\text{ATP}}\) currents, though in parallel to the contraction results, the UTP induced inhibition of these currents remained even in the presence of PKC block, suggesting a lack of involvement of PKC. Finally, application of UTP resulted in the activation of a non-selective current. An inhibition of K\(^{+}\) currents and the activation of a non-selective cation current would lead to membrane depolarization, the activation of voltage-gated Ca\(^{2+}\) channels and, dependent on the permeability of the non-selective channels, an additional route for Ca\(^{2+}\) influx and hence contraction.
Acknowledgements

I would like to thank my Mum Fatima Panhwar and Dad Faiz Muhammad Panhwar for their motivation and prayers. A very special thanks and love to my husband Aqeel Ahmed Qureshi, and children Safia, Manzoor and Ramsha, without their cooperation, love, and encouragement I could never achieved my goal.

I would like to thank my supervisor, Dr. Noel Davies for all his help and support over the last four years especially in electrophysiology, data analyses and during writing up my thesis. I also extend my thanks to Dr Bob Norman for the Tat-PKC inhibitor peptides. I am thankful to Mrs Diane Everitt for all her technical support from learning dissection of rat mesenteric arteries to myography technique. I would like to thank Dr Nina Storey and all my lab fellows Jenny Brignell, Yusuf Bhagatte, Helen Turrell, Carl Nelson, Richard Rainbow, and Sadaf Afreen for having a fantastic time together.

I would like to thank my employer, University of Sindh and my sponsor, Islamic development Bank for providing me opportunity and financial support for the entire course of study.
<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>EXPLANATIONS</th>
</tr>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Ang-II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BK$_{Ca}$</td>
<td>calcium activated potassium channels</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxyl terminus</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethylether)-N,N,N',N' tetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>potassium equilibrium potential</td>
</tr>
<tr>
<td>$E_K$</td>
<td>equilibrium potential for $K^+$</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>gadolinium</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N9-[2[ethanesulphonic acid]</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>$K_{ATP}$</td>
<td>ATP sensitive potassium channels</td>
</tr>
<tr>
<td>Kir</td>
<td>inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>Kv</td>
<td>voltage gated potassium channel</td>
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<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino terminus</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidyl inositol bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>S.E.M</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STOCs</td>
<td>spontaneous transient outward current</td>
</tr>
<tr>
<td>TRP channel</td>
<td>transient receptor potential channels</td>
</tr>
<tr>
<td>TRPC</td>
<td>canonical transient receptor potassium channels</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5' triphosphate</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
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</tbody>
</table>
# Table of Contents

## CHAPTER 1  INTRODUCTION .................................................................................. 10

1.1 **General Introduction** ................................................................................. 11
1.2 **The Cardiovascular System** ...................................................................... 12
1.3 **Arterioles: Structure and Function** .......................................................... 14
1.4 **Regulation of Peripheral Blood Flow** ......................................................... 15
    1.4.1 *Extrinsic regulation* ............................................................................ 15
    1.4.2 *Intrinsic regulation* ............................................................................ 17
    1.4.3 *The myogenic response of blood vessels* ........................................... 21
1.5 **Smooth Muscle** ....................................................................................... 22
    1.5.1 *Smooth muscle contraction* ............................................................... 23
    1.5.2 *Regulation of contraction in vascular smooth muscle* ...................... 24
    1.5.3 *Smooth muscle membrane potential and its role in regulating contraction* ........................................................................ 25
    1.5.4 *Ion channels and smooth muscle contraction* ................................... 27
    1.5.5 *Receptors involved in regulating smooth muscle contraction* .......... 38
1.6 **Signaling Pathways Involved** .................................................................... 41
    1.6.1 *G-protein coupled receptor (GPCR) signaling pathways* ................... 41
    1.6.2 *Ca\(^{2+}\) regulation* ........................................................................... 48
1.7 **Purinergic Signaling** .................................................................................. 49
    1.7.1 *Introduction to purinergic signaling* .................................................. 49
    1.7.2 *Purinergic signaling* ............................................................................ 51
1.8 **Aims and Overview of the Thesis** ............................................................. 54

## CHAPTER 2  MATERIAL AND METHODS ............................................................ 56

2.1 **Dissection of Rat Mesenteric Arteries** ...................................................... 57
2.2 **Myography** ............................................................................................ 60
CHAPTER 3 UTP INDUCED CONTRACTILE RESPONSE OF RAT MESENTERIC ARTERIES ..... 88

3.1 INTRODUCTION ............................................................................................................. 89
  3.1.1 Uridine 5' triphosphate ......................................................................................... 89

3.2 GENERAL CONDITIONS ............................................................................................... 91
  3.2.1 Contractile response ............................................................................................... 91
  3.2.2 Analysis ................................................................................................................ 91

3.3 RESULTS ..................................................................................................................... 92
  3.3.1 UTP induced contraction was sustained and reproducible ..................................... 92
  3.3.2 Cumulative dose response curve for UTP ............................................................... 94
  3.3.3 Role of extracellular Ca^{2+} influx in UTP induced contraction ............................ 96
  3.3.4 Involvement of voltage gated Ca^{2+} channels ....................................................... 98
  3.3.5 UTP induced contractions and activation of PLC ................................................ 101
  3.3.6 PKC involvement in the contractile response of mesenteric arteries to UTP .......... 103
3.3.7 Are non-selective cation channels involved in UTP induced contraction? .................. 116

3.4 DISCUSSION .............................................................................................................. 119

3.4.1 UTP induced sustained contraction depends on extracellular Ca\(^{2+}\) influx ............... 119
3.4.2 Voltage gated L-type Ca\(^{2+}\) channels and UTP induced vasoconstriction .................. 121
3.4.3 UTP activates PLC to initiate a signaling cascade leading to contraction ..................... 121
3.4.4 Contribution of PKC to UTP induced vasoconstriction ............................................ 122
3.4.5 Are non-selective cation channels involved in UTP induced contractions? .................. 124

3.5 SUMMARY ............................................................................................................... 125

CHAPTER 4 INVESTIGATION OF THE MODULATION OF IONIC CURRENTS BY UTP ....... 126

4.1 INTRODUCTION ........................................................................................................ 127

4.2 Kv CURRENT AND UTP ......................................................................................... 128

4.2.1 Isolation of Kv current .......................................................................................... 128
4.2.2 Modulation of Kv current by UTP ........................................................................ 128
4.2.3 Effect of low intracellular Ca\(^{2+}\) on Kv current modulation by UTP ....................... 136
4.2.4 Effect of PLC on Kv current modulation by UTP .................................................. 139
4.2.5 Does UTP modulate Kv current via PKC? ............................................................ 141
4.2.6 \(K_\text{ATP}\) current and UTP .................................................

4.3 MODULATION OF \(K_\text{ATP}\) CURRENT BY UTP ...................................................... 146

4.3.1 Effect of Ca\(^{2+}\) on \(K_\text{ATP}\) current modulation by UTP ........................................ 146
4.3.2 Does UTP modulate \(K_\text{ATP}\) current via PKC ......................................................... 150

4.4 NONSELECTIVE CATION CURRENT EVOKED BY UTP .................................... 153

4.5 ISOLATION OF VOLTAGE GATED Ca\(^{2+}\) CURRENT ...................................... 159

4.6 DISCUSSION ............................................................................................................. 161

4.6.1 Kv current and effect of UTP ............................................................................... 161
4.6.2 \(K_\text{ATP}\) current and UTP ...................................................................................... 163
4.6.3 NSCC current and UTP ........................................................................................ 164
4.6.4 Voltage gated Ca\(^{2+}\) current ................................................................................ 164
CHAPTER 5  OVERALL DISCUSSION .................................................................................166

5.1 DISCUSSION............................................................................................................167

5.1.1 UTP induced contraction depends largely on extracellular Ca\(^{2+}\) influx .............. 168

5.1.2 Voltage gated Ca\(^{2+}\) channels are partly involved in UTP induced contraction ........ 169

5.1.3 UTP mediates contraction via PLC activation......................................................... 170

5.1.4 Is PKC involved in generating the UTP induced contraction? ..................................... 171

5.1.5 UTP activates non selective cation channel ............................................................. 174

5.1.6 UTP modulates Kv current ................................................................................... 175

5.1.7 UTP modulates K\(_{ATP}\) current ............................................................................. 177

5.2 CONCLUSION AND FUTURE PERSPECTIVES ......................................................... 179

CHAPTER 6  REFERENCES ..............................................................................................181
Chapter 1  Introduction
1.1 General Introduction

The research presented in this thesis involves the study of the mechanism of UTP induced contraction in rat mesenteric arteries. UTP is a pyrimidine nucleotide which is released from various cells including endothelial cells and platelets. The release of UTP, as well as other nucleotides, increases upon shear stress and cardiac ischemia (Erlinge and Burnstock, 2008). The research presented in this thesis describes the measurement of isometric contractile responses of rat mesenteric arteries to UTP and of the electrophysiological measurements of ionic currents of smooth muscle cells isolated from these arteries. The smooth muscle which is present in the middle layer of the arterial vessel plays a major role in the contractions of blood vessels and maintenance of blood pressure.

It is now well understood that membrane potential plays an important role in the regulation of myogenic tone. K$^+$ channels are involved in regulating the membrane potential and hence the myogenic tone and blood flow. Various vasoconstrictors, including UTP, are known to decrease K$^+$ channel activity, reducing the K$^+$ efflux thereby causing contraction of vascular smooth muscle cells (Standen and Quayle, 1998). Because of this particular attention was given to K$\text{V}$ and K$\text{ATP}$ channel function under normal physiological conditions and in the presence of UTP.

In the rest of this chapter I aim to provide an introduction to some cardiovascular concepts with special reference to small resistance arteries, smooth muscle contraction and the regulation of membrane potential.
1.2 The cardiovascular system

The cardiovascular system was first demonstrated to be a circulatory system by William Harvey an English physiologist. It comprises the heart (pump) and a network of distributing and collecting blood vessels. The main function of the cardiovascular system is to transport oxygen and essential nutrients to the tissues and to remove CO₂ and other metabolites.

The heart is composed of four chambers; the right atrium, right ventricle, left atrium and left ventricle. Both atria receive blood whereas the ventricles act as pumps. The heart operates as two pumps, the right ventricle and the left ventricle, which act in series to maintain the pulmonary and systemic circulation respectively. The presence of valves in the heart ensures that the unidirectional flow of blood is maintained. The aorta along with other arteries ensures the rapid distribution of blood to the systemic circulation, while the pulmonary artery delivers blood to the lungs where exchange of carbon dioxide with oxygen takes place.

Blood vessels form an extensive network to transport blood throughout body by the pumping action of the heart. The network of blood vessels comprises arteries, arterioles, capillaries and veins. These vessels differ in their physical dimensions, morphology and functional specialization. The inner lumen of the blood vessels is lined with a layer of cells called the endothelium. With the exception of capillaries, the walls of blood vessels are made up of three layers: tunica intima, tunica media and tunica adventitia. The main components of all blood vessels are (with the exception of capillaries): endothelial cells, elastic fibers and connective tissue and smooth muscle cells. Capillaries comprise endothelial cells and a basal lamina.
Fig: 1.1 Figure representing the anatomical structure of an artery and a vein.

**Tunica intima** This is the inner most layers of blood vessels and consists of a single layer of endothelial cells surrounded by collagenous fibers and an elastic membrane called elastic interna.

**Tunica media** In the tunica media of large arteries, a compact layer of smooth muscle is arranged in a helical or spiral manner with a layer of connective tissue and matrix. The relative abundance of smooth muscle and other components of the tunica media may vary among different blood vessels. The proportion of smooth muscle is higher in the smaller arteries as compared to large arteries. However elastic fibers in the media decrease as the size of the arterial vessel decreases. Only a single layer of smooth muscle cells is present in small arteries of 15-100 µm internal diameters (Greger, 1996). In contrast to arteries veins are thin walled vessels containing a thinner and loosely arranged media.
**Tunica adventitia** The tunica adventitia is the outer layer of blood vessels which forms the attachment to nearby organs. It is made of elastic and collagenous fibrils arranged longitudinally. This layer may also contain fibroblasts and macrophages.

### 1.3 Arterioles: structure and function

Arterioles are small diameter blood vessels that form a network of microcirculation along with capillaries. Arterioles are comprised of one to two layers of smooth muscle that forms their muscular portion and are considered the stopcock of the circulation as they provide the primary site of vascular resistance. Arterioles branch out to capillaries which are the site of exchange of gases and nutrients from blood to the tissue.

The contractile activity of smooth muscle controls the tone of the resistance and terminal arteries and thereby plays an important role in the regulation of blood flow and blood pressure. Contraction of smooth muscle cells reduces the radius of the blood vessels and according to Pouiselle’s equation:

\[
Flow = \frac{\Delta P \cdot r^4}{8 \eta \cdot L}
\]

Where $\Delta P$ is the pressure difference, $r$ is the radius of the vessel, $\eta$ is blood viscosity and $L$ is length of the blood vessel. According to equation 1.1, the radius has a large effect on blood flow and hence a small decrease in radius will result in a large increase in the resistance to flow. The tonic contraction of vascular smooth muscle has great influence on the vascular resistance and maintains the local blood flow, arterial pressure, capillary filtration and central venous pressure (Levick, 2003).
1.4 Regulation of peripheral blood flow

The peripheral blood flow is regulated by two mechanisms, an extrinsic mechanism and an intrinsic or local control.

Fig: 1.2 Peripheral blood flow is under extrinsic and local or intrinsic control

1.4.1 Extrinsic regulation

1.4.1.1 Neuronal control

Smooth muscle cells of blood vessels are under the control of the autonomic nervous system and are innervated by post-ganglionic sympathetic nerve fibers. These sympathetic post ganglionic nerve fibers release noradrenaline and ATP.

Noradrenaline acts on different adrenergic receptors to cause either vasodilation or vasoconstriction. Noradrenaline evoked responses are mediated by α and β adrenergic receptors present on the smooth muscle of blood vessels. Noradrenaline has a higher affinity towards α receptors as compared to β receptors. Generally α₁-receptors are present on most blood vessels but in vessels supplying the heart, liver and skeletal
muscle β2 receptors are found. Stimulation of α1 receptors by noradrenaline causes vasoconstriction whereas β2 receptor stimulation leads to vasodilation of arteries in skeletal muscle and liver. β1 receptor stimulation by noradrenaline causes vasodilatation in rat basilar arteries possibly by activating ATP-dependent K⁺ channels (Kitazono et al., 1993). In mesenteric arteries the α1 adrenergic receptor response predominates.

The release of noradrenaline can be altered by several factors. Ang-II increases the release of noradrenaline by acting on presynaptic angiotensin receptors, thereby increasing the effectiveness of sympathetic transmission. Other modulators depress the release of noradrenaline, including noradrenaline itself acting on α2 receptors, adenosine, K⁺, and H⁺ (Levick, 2003). ATP is released as a co-transmitter with noradrenaline from the sympathetic varicosities in the blood vessels and acts on purinergic receptors. Other neurotransmitters released from nerve terminals present in blood vessel walls include substance P, which causes vasodilation via release of nitric oxide, and calcitonin gene related peptide (CGRP). CGRP is known to be involved in autoregulation and has been shown to causes vasodilation in, for example, mesenteric and ophthalmic arteries of rabbits (Nelson et al., 1990, Quayle et al., Zschauer et al., 1992).

1.4.1.2 Hormonal control

The control of blood circulation by hormones is comparatively less important than neuronal control under physiological conditions; nevertheless, hormones play an important role in the control of blood flow, especially so during pathological conditions such as hemorrhage.
Adrenaline, a methylated form of noradrenaline, is secreted from the adrenal gland situated above the kidneys. Adrenaline and noradrenaline are collectively known as catecholamines and their plasma concentrations under resting conditions are 0.1 to 0.5 nM and 0.5 to 3.0 nM respectively (Levick, 2003). At rest adrenaline has relatively little effect on the cardiovascular system. However, as the concentration of adrenaline rises, for example during exercise, it causes muscle vasodilation and skin vasoconstriction via activation of β2 and α2 receptors respectively. Angiotensin II and vasopressin are two peptide hormones that are involved in regulation of blood volume; both of these peptides act as powerful vasoconstrictors also.

1.4.2 Intrinsic regulation

Intrinsic regulation or local control refers to the mechanisms that regulate vascular tone to meet the metabolic needs of nearby tissues. Additionally, local control also describes the responses to pathological conditions such as vasospasm and inflammation. The close interaction between the endothelial layer and smooth muscle exerts an important form of local regulation of vascular tone.

1.4.2.1 Endothelial factors

Various vasodilators such as nitric oxide (NO), endothelium derived hyperpolarizing factor (EDHF) and prostacyclin (PGI2), are synthesized and released from endothelial cells. In addition, endothelial cells also synthesize and release endothelin-1 (ET-1), which is a very powerful peptide vasoconstrictor.

Nitric oxide An increase in shear stress due to an increase in blood velocity stimulates the production of endothelial nitric oxide (NO) via protein kinase B induced activation of the enzyme endothelial nitric oxide synthase (eNOS). NO is thereby synthesized and
released from the endothelial cells and acts to relax adjacent smooth muscle cells resulting in vasodilatation.

**EDHF** EDHF is an additional vasodilator, with properties different from either NO or prostacyclin, released by endothelium in response to increased shear stress (Mombouli and Vanhoutte, 1997).

### 1.4.2.2 Metabolic vasodilators

In order to supply oxygen to fulfill the metabolic demand of cardiac and skeletal muscle during elevated metabolism, the local blood flow to these regions increases. This increase in blood flow due to vasodilation in response to increased metabolism is called functional hyperaemia or metabolic hyperaemia. Small resistance blood vessels are most sensitive to metabolic control. The exact vasodilators causing metabolic hyperaemia may vary between tissues and are listed below:

**Acidosis** Carbon dioxide and lactic acid produced in metabolically active tissue causes local tissue acidosis resulting in vasodilatation. Acidosis based vasodilatation is particularly important in cerebral arteries and there is also a weak response to metabolic acidosis in skeletal and cardiac muscles.

**Hypoxia** is an important cause of vasodilatation but has opposing effects on the systemic and pulmonary circulation. Hypoxia dilates systemic arterioles whereas it constricts pulmonary arteries and large arteries. Hypoxia may be systemic or local. Systemic hypoxia at low oxygen pressure of <40 mmHg or 5 kPa causes vasodilation to increase tissue perfusion to restore oxygen supply. However, local hypoxia of skeletal
muscles during exercise leads to the formation of adenosine that is a contributing chemical agent to metabolic hyperaemia.

*Adenosine* Adenosine monophosphate is a breakdown product of ATP (adenosine triphosphate) that is largely produced during hypoxic conditions. AMP is further metabolized into adenosine by a nucleotidase in the interstitial fluid of skeletal muscle and myocardium. The adenosine formed causes vasodilation by activating adenosine receptors on vascular smooth muscle.

*K+ ions* The interstitial K+ concentration rises during exercise and neuronal activity and contributes to metabolic hyperemia. It is believed that during muscle contraction there is an initial rise of K+ concentration from 4 to 9 mM that causes hyperpolarization due to increased opening of inward rectifier potassium channels and activation of Na+-K+ pump (Levick, 2003).

1.4.2.3 *Autacoids*

Under various pathological conditions these chemicals are released and perform their specialized functions. These agents, called autacoids, that include histamine, bradykinin, serotonin, prostaglandins, thromboxane and leukotrienes are vasoactive agents that are released under various pathological conditions.

*Histamine* is released from mast cells and basophilic leukocytes under conditions such as trauma, allergic reactions and asthma. Histamine acts on H₁ and H₂ receptors of blood vessels to cause vasodilation or vasoconstriction respectively. H₁ receptor activation causes vasoconstriction via a Gq linked IP₃ signaling pathway; whereas, H₂ receptor activation causes vasodilatation via a cAMP dependent pathway.
**Bradykinin** causes vasospasm and is produced during inflammation from its plasma protein precursor kininogen by the action of kallikrein. Bradykinin causes vasodilatation of small resistance arteries and increases the permeability of veins. Its effect is also mediated by the release of other vasodilators like NO, EDHF and prostacyclin from endothelial cells.

**Serotonin** is produced from its amino acid precursor tryptophan in platelets, intestine and some neurons and is released during inflammation and causes vasoconstriction and increases the permeability of veins leading to inflammatory swelling.

**Prostaglandins and thromboxanes** are eicosanoids and are produced from arachidonic acid by the action of cyclooxygenase. Prostaglandins can be subdivided into the F category (PGF), which are vasoconstrictors, and the E category recognized for their inflammatory vasodilatory actions and reactive hyperaemia.

Thromboxane A2 is a blood clotting factor synthesized by platelets and released during blood clotting by the action of cyclooxygenase. In addition to its thrombotic actions it is also a powerful vasoconstrictor and contributes to vasospasm in atheromatous coronary arteries.

**Leukotrienes and platelet activation factors** Leukotrienes and platelet activating factors (PAF) are important vasoconstrictors released from leukocytes in response to inflammation. The leukotrienes mainly cause constriction of venules whereas platelet activating factors cause constriction and contribute to bronchoconstriction in asthmatics and vasospasm in atheromatous coronary arteries.
UTP Uridine triphosphate is an important signaling molecule and also has an autocrine or paracrine role (Lazarowski and Boucher, 2001). UTP is released from platelets during thrombus formation and also from endothelial and vascular smooth muscle cells during shear stress, high glucose and hypoxia (Lazarowski and Boucher, 2001, Lazarowski and Harden, 1999). Mechanical stress is one of the major reasons of UTP release from nonsecretory cells (Lazarowski and Boucher, 2001). UTP is believed to activate mainly P2Y receptors, preferentially P2Y_{2} and P2Y_{4}; whereas P2Y_{6} is more likely to be activated by its metabolite UDP (Lazarowski and Boucher, 2001). Some studies, however, suggest that P2X_{1} receptors can be activated by UTP (Lazarowski and Boucher, 2001).

1.4.3 The myogenic response of blood vessels
Arteries contract as a result of an increase in blood pressure and is called the myogenic response to blood pressure. Sir William Bayliss discovered the myogenic response and it is often termed the Bayliss myogenic response (Bayliss, 1902). The myogenic response is the contractile reaction of arteries and arterioles that occurs within seconds of mechanical distention in response to increased luminal pressure. The myogenic response contributes to basal tone and it is important that this tone is maintained to stabilize local blood flow and capillary filtration pressure. Little is known about the exact mechanism of the myogenic response but it involves smooth muscle depolarization and activation of Ca^{2+} channels (Omote and Mizusawa, 1996, Altura et al., 1987, Zhang et al., 2010). Myogenic constriction is reversed by increased shear stress of the blood flow which resulted in the increased production of vasodilators (e.g. nitric oxide) from endothelial cells (Levick, 2003).
1.5 **Smooth muscle**

Smooth muscle is the major component of several hollow organs including blood vessels (except capillaries) and represents about 2% of human body weight (Carsten, 1997). The term smooth muscle arises because of the lack of striations that are seen in skeletal and cardiac muscle. Each smooth muscle cell is 2 to 10 µm in diameter at the centre and is tapered at the end form a fusiform shape. In contrast to the T-tubules of striated muscle, smooth muscle cells have caveolae which are sac-like pockets within the membrane that associate with the sarcoplasmic reticulum of the cell. The caveolae, in conjunction with the sarcoplasmic reticulum, may be involved in the regulation of intracellular Ca\(^{2+}\) (Kamishima et al., 2007); caveolin1 and caveolin3 are reported to be involved in the regulation of Ca\(^{2+}\) homeostasis in smooth muscle cells of rat cerebral arteries (Kamishima et al., 2007). There is evidence that some ion channels such as K\(_{\text{ATP}}\) channels are also associated with the caveolae in smooth muscle cells (Das and Das, 2011, Adebiyi et al., 2010, Brainard et al., 2005, Cole, 2010, Sampson et al., 2007, Sampson et al., 2004). The sarcoplasmic reticulum is an intracellular store of Ca\(^{2+}\) from where Ca\(^{2+}\) can be released by IP\(_3\) or ryanodine receptor activation following a signaling cascade. The contractile machinery of smooth muscle comprises of myosin (thick) and actin (thin) filaments but their physical arrangement is not as structured as in striated muscle. Generally, vascular smooth muscle exhibits long-lasting tonic contraction where the cross-bridges enter a latch state where contraction is maintained whilst using very little energy. In many vessels this vascular tone is maintained throughout life.
1.5.1 Smooth muscle contraction

Smooth muscle contraction results from crossbridge activity of actin and myosin. A rise in global Ca$^{2+}$ concentration of the cytoplasm is required to activate the contractile machinery and hence it is the primary stimulus for contraction. A variety of stimuli trigger contraction by either increasing the intracellular Ca$^{2+}$ concentration or by increasing the sensitivity of the contractile machinery to Ca$^{2+}$. For contraction to occur, four Ca$^{2+}$ ions bind with one molecule of calmodulin which results in a conformational change enabling the Ca$^{2+}$-calmodulin complex to interact with and activate myosin light chain kinase (MLCK). The activated MLCK catalyzes the phosphorylation of a regulatory light chain on the myosin which is necessary for interaction with actin and cross-bridge cycling to occur (Kamm and Stull, 1985a, Kamm and Stull, 1985b, Sommerville and Hartshorne, 1986, Hai and Murphy, 1989a, Hai and Murphy, 1989b). The interaction between actin and myosin is stopped by de-phosphorylation of the myosin light chain by the enzyme myosin light chain phosphatase (MLCP) which is constitutively active. Thus the relative activity of these two enzymes regulates the contraction of smooth muscle (Hirano et al., 2004).
1.5.2 Regulation of contraction in vascular smooth muscle

An increase in intracellular free Ca$^{2+}$ stimulates smooth muscle contraction whereas a decrease in intracellular Ca$^{2+}$ leads to relaxation. The level of Ca$^{2+}$ in the cytosol is increased due to Ca$^{2+}$ release from intracellular stores or from Ca$^{2+}$ entry through Ca$^{2+}$ permeable ion channels. Vasoactive compounds initiate contraction by increasing the intracellular Ca$^{2+}$ concentration leading to the formation of the Ca$^{2+}$-calmodulin complex. Agonist induced vasoconstriction mainly comprises an initial phasic contraction due to Ca$^{2+}$ release from intracellular stores followed by a more prolonged tonic contraction maintained by Ca$^{2+}$ entry. Various agonists are known to induce a transient contraction in vascular smooth muscle by releasing Ca$^{2+}$ from the internal stores even in the absence of extracellular Ca$^{2+}$ (Kanashiro and Khalil, 1998, Khalil, 2010, Khalil and van Breemen, 1988, Salamanca and Khalil, 2005). Many
vasoconstrictors also increase the sensitivity of the contractile mechanism to Ca\textsuperscript{2+} by decreasing the activity of MLCP, thus for a given [Ca\textsuperscript{2+}] the balance towards the phosphorylated state of the myosin light chain will be favoured.

1.5.3 Smooth muscle membrane potential and its role in regulating contraction

Like all animal cells, smooth muscle cells are surrounded by a lipid bilayer membrane that is impermeable to ions. At rest, there is a negative charge inside the cell which is mainly due to the presence of large negatively charged protein molecules that are unable to diffuse out of the cell. A large difference in the ionic concentrations exists between the inside and outside of the membrane and typical values for smooth muscle cells are listed in table 1.1. Because of the charged nature of ions, and their separation by the membrane, an electrical force needs to be considered also. The potential at which the electrical force is equal but opposite to the diffusional (concentration gradient) force is termed the equilibrium potential which is calculated using the Nernst equation:

\[ E_{\text{ion}} = \frac{RT}{zF} \ln \left( \frac{[\text{ion}]_{\text{out}}}{[\text{ion}]_{\text{in}}} \right) \]

Where \( E_{\text{ion}} \) is the equilibrium potential for the ion, \( z \) the charge of the ion and \( R, T \) and \( F \) have their usual thermodynamic values. Values of the equilibrium potentials are also given in table 1.1. The combination of the diffusional force and the electrical force is termed the electrochemical gradient.

The hydrophobic lipid plasma membrane stops ions diffusing down their electrochemical gradient. However, the presence of trans-membrane pores, which are
structural components of ion channels, enables such movement. Ion channels exist in either an open state, where the pore is open and allows flow of selective ions down their electrochemical gradient, or a closed state when there is no flow of ions. From table 1.1 it is seen that the concentration of $\text{K}^+$ is higher inside than outside and the equilibrium potential for $\text{K}^+$ ($E_K$) is -89 mV. There is, therefore, a tendency for $\text{K}^+$ to move down its electrochemical gradient such that the membrane potential moves closer to $E_K$, a potential at which there would be no net movement of $\text{K}^+$. At the resting membrane potential the membrane is more permeable to $\text{K}^+$ than to other ions and hence $\text{K}^+$ ions move down their electrochemical gradient from the inside of the cell to the outside, resulting in an increase in the negative charge inside the cell. The resting membrane potential of smooth muscle in vivo is -40 to -55 mV (Harder, 1984, Nelson et al., 1990); however, in vitro the resting membrane potential ranges from -40 to -70 mV (Nelson et al., 1990, Neild and Keef, 1985). These potentials signify that the membrane potential of smooth muscle results from a relatively high permeability to $\text{K}^+$ ions at rest. A change in the relative permeability of the cell membrane, as a result of the alteration in the open probability of ion channels to one or more ionic species, eventually results in a change in the membrane potential because of the movement of ions down their respective electrochemical gradients.
### Table 1-1 Ionic composition of vascular smooth muscle

<table>
<thead>
<tr>
<th>Ion</th>
<th>Intracellular (mM)</th>
<th>Extracellular (mM)</th>
<th>Nernst equilibrium potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>165</td>
<td>5</td>
<td>-89</td>
</tr>
<tr>
<td>Na⁺</td>
<td>9</td>
<td>137</td>
<td>+69</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.0001</td>
<td>1.2</td>
<td>+124</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>54</td>
<td>134</td>
<td>-23</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>7.3</td>
<td>15.5</td>
<td>-19</td>
</tr>
<tr>
<td>pH</td>
<td>7.06</td>
<td>7.4</td>
<td>-20</td>
</tr>
</tbody>
</table>

(reproduced from (Levick, 2003))

1.5.4 **Ion channels and smooth muscle contraction**

The influx of Ca²⁺ from the extracellular space, either through voltage-dependent or receptor operated Ca²⁺ permeable channels, is one of the main regulators of smooth muscle contraction. Because of the presence of voltage-gated Ca²⁺ channels the amount of Ca²⁺ entry is tightly regulated by the membrane potential (Nelson et al., 1990). The following sections describe the main ion channels involved in regulating Ca²⁺ entry.

1.5.4.1 **Voltage gated Ca²⁺ channels**

Voltage gated Ca²⁺ channels are activated in response of membrane depolarization and mediate a subsequent Ca²⁺ influx. Voltage gated Ca²⁺ channels are composed of pore forming α subunit and many accessory subunits that regulate the pore formation and kinetic behavior of the voltage gated Ca²⁺ channels. The pore forming α subunits of voltage gated Ca²⁺ channels are large peptide molecules with four repeating segments, each with six membrane spanning domains (S1-S6) and a voltage sensing domain (S4). Because of the presence of a voltage sensor in the S4 domain of the α subunit, voltage gated Ca²⁺ channels are activated by depolarization of the plasma membrane (Catterall, 1995).
According to the electrophysiological characteristics of Ca\(^{2+}\) currents, Ca\(^{2+}\) channels can be divided into L-, T-, P/Q, R- and N-types (Horowitz et al., 1996). Among all these subclasses, L- and T- types are widely expressed in smooth muscles (Horowitz et al., 1996). L-type Ca\(^{2+}\) channels, also called long lasting Ca\(^{2+}\) channels, are widely distributed among smooth muscle cells with varying distribution between tissues. These channels are sensitive to dihydropyridines, an important class of antihypertensive drugs acting on L-type Ca\(^{2+}\) channels. The L-type Ca\(^{2+}\) channels are primarily regulated by the depolarization of smooth muscle resulting in L-type Ca\(^{2+}\) channel activation; however, maintained depolarization causes inactivation of these channels (Horowitz et al., 1996). A window current lies within the potential range where activation occurs but inactivation is incomplete. In human mesenteric arteries this window current starts at about -50 mV and peaks at -20 mV (Smirnov and Aaronson, 1992). In this physiologically important range a sustained non-inactivating Ca\(^{2+}\) influx occurs (Horowitz et al., 1996, Fleischmann et al., 1994). The important contribution of L-type Ca\(^{2+}\) channels to smooth muscle contraction has been demonstrated using dihydropyridine blockers such as nifedipine and nisoldipine which strongly inhibit depolarization induced contraction (Horowitz et al., 1996). Other voltage gated Ca\(^{2+}\) channel blockers such as verapamil and diltiazem belong to phenylalkyamine and bezothiazepine drug groups respectively (Perez-Reyes, 2003).

T-type Ca\(^{2+}\) channels, also termed low voltage activated (LVA) Ca\(^{2+}\) channels, are blocked by Ni\(^{2+}\) but are not significantly suppressed by L-type Ca\(^{2+}\) channel blockers such as nifedipine and nisoldipine (reviewed by (Horowitz et al., 1996). T-type channels possess distinct biophysical properties, such as their negative activation range, that are useful to isolate the T-type current from L-type (Horowitz et al., 1996). The T-type
current also inactivates more rapidly and at more negative potentials than the L-type Ca\(^{2+}\) current (Horowitz et al., 1996, Perez-Reyes, 2003).

1.5.4.2 TRP channels

The transient receptor potential channels or TRP channels are a large family of ion channels that are permeable to cations and many are permeable to Ca\(^{2+}\) as well as monovalent ions (Nilius et al., 2007). TRP channels, first discovered in *Drosophila* eye (Cosens and Manning, 1969) are also present on the membrane of smooth muscle cells (Chen et al., 2009, Hill et al., 2006, Minke and Cook, 2002, Tiruppathi et al., 2006). Most TRP channels are non-selective in nature and are permeable to Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\) ions (Soboloff et al., 2007). TRP channels are made up of six transmembrane domains (S1-S6), where the fifth and six domain forms the pore region. Four TRP subunits are required to form a functional channel (Clapham et al., 2001, Minke and Cook, 2002). The TRP channels are classified into three main groups: 1) TRPCx (short), 2) TRPVx (osm-9-like or vanilloid), and 3) TRPMx (long or melastatin). These groups can be divided further into subfamilies as shown in table 1-2.

As shown in the table 1-2, TRP channels are widely expressed in various tissues including the cardiovascular system (Clapham et al., 2001). TRP channels are often activated in conjunction with voltage gated Ca\(^{2+}\) channels and are therefore able to induce a sustained contraction in smooth muscle cells (Reading et al., 2005). Activation of TRP channels causes depolarization. Various TRP channels are involved in the regulation of important cellular functions such as Ca\(^{2+}\) influx. Dysfunction of TRP channels in cardiac myocytes is the potential cause of cardiac hypertrophy (Clapham et al., 2001). Studies on rat carotid arteries reveal that TRPC1/TRPC3 channels are
involved in depolarization induced by UTP in rat carotid arterial smooth muscle and hence are involved in maintaining the vascular tone (Chen et al., 2009).

Table 1-2 TRP channel subfamilies and their possible regulation

<table>
<thead>
<tr>
<th>TRP Family</th>
<th>TRP subfamilies</th>
<th>Tissue distribution</th>
<th>Possible regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC</td>
<td>TRPC1</td>
<td>Widely expressed in cells</td>
<td>Receptor operated, store depletion</td>
</tr>
<tr>
<td></td>
<td>TRPC2</td>
<td>VNO, testis, heart, brain, sperm</td>
<td>Store depletion</td>
</tr>
<tr>
<td></td>
<td>TRPC3</td>
<td>Brain, heart, muscle &amp; placenta</td>
<td>DAG, IP3R</td>
</tr>
<tr>
<td></td>
<td>TRPC4</td>
<td>Brain, testis, placenta, adrenal gland,</td>
<td>Receptor operated, store depletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>endothelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRPC5</td>
<td>Brain</td>
<td>Receptor operated, store depletion</td>
</tr>
<tr>
<td></td>
<td>TRPC6</td>
<td>Lung, brain, muscle</td>
<td>DAG</td>
</tr>
<tr>
<td></td>
<td>TRPC7</td>
<td>Heart, muscle, Lung, eye, brain</td>
<td>DAG</td>
</tr>
<tr>
<td>TRPV</td>
<td>TRPV1</td>
<td>Brain, spinal cord, peripheral sensory</td>
<td>Capsaicin, heat, PKC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRPV2</td>
<td>Brain, spinal cord, spleen, lung,</td>
<td>Heat, growth factors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>peripheral sensory neurons</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRPV4</td>
<td>Brain, liver, kidney, heart, fat, testis,</td>
<td>Osmolarity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>salivary gland, trachea</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRPV5</td>
<td>Kidney, placenta, intestine</td>
<td>Low intracellular Ca^{2+}</td>
</tr>
<tr>
<td></td>
<td>TRPV6</td>
<td>Kidney, intestine, placenta, prostate &amp;</td>
<td>Store depletion, low intracellular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>salivary gland</td>
<td>Ca^{2+}</td>
</tr>
<tr>
<td>TRPV</td>
<td>TRPM1</td>
<td>Eye</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TRPM2</td>
<td>Adult and fetal brain, placenta</td>
<td>ADP ribose</td>
</tr>
<tr>
<td></td>
<td>TRPM3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TRPM4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TRPM5</td>
<td>Widely expressed</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TRPM6</td>
<td>Heart, brain, kidney, liver, spleen,</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRPV7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TRPV8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table adapted from (Clapham et al., 2001)
1.5.4.3 $K^+$ channels
Small resistance arteries exist in a partially contracted state and respond to changes in transmural pressure by undergoing further dilation or constriction. This is called myogenic tone that in turn depends on the membrane potential of the smooth muscle cells of these arteries. The membrane potential, an important determinant of the myogenic tone, is regulated by the open probability of potassium channels and thereby controls the arterial tone and subsequently the diameter of the small resistance blood vessels. Upon opening of $K^+$ channels there is an efflux of $K^+$ causing membrane hyperpolarization resulting in a closure of voltage gated $Ca^{2+}$ channels and eventually vasodilation. Excessive activation of $K^+$ channels in conditions such as endotoxic shock lead to hypotension (Landry and Oliver, 1992); whereas inhibition of $K^+$ channels by vasoconstrictors lead to depolarization and vasoconstriction (Landry and Oliver, 1992, Betts and Kozlowski, 2000, Rainbow et al., 2009).

The four main types of potassium channels are voltage gated (Kv) channels; $Ca^{2+}$ activated (BK) channels; ATP-dependent ($K_{ATP}$) channels; and inward rectifier (Kir) channels. Potassium channel blockers have potential therapeutic benefits in conditions including arrhythmia, cancer, stroke and some neurological disorders (Tamargo et al., 2004, Surti and Jan, 2005).

1.5.4.4 Voltage-gated $K^+$ (Kv) channels
Voltage gated $K^+$ channels comprise a large number of closely related ion channels which share the basic property of being activated following membrane depolarization. Structurally they are composed of four similar or identical pore forming $\alpha$ subunits arranged together to form a tetramer. As shown in figure 1.4, each pore forming $\alpha$ subunit of the Kv channel is composed of six transmembrane domains (S1 to S6) with
an inner cytoplasmic N and C terminus. The S4 transmembrane domain acts as a voltage sensor. The H5 loop lies between the fifth and sixth transmembrane domain (S5-S6) of the Kvα subunits (Jan and Jan, 1992) and acts as a selectivity filter and is often the interaction site for extracellular ion channel blockers (Bett and Rasmusson, 2008). Kvα subunits may also coexist with the ancillary subunits of Kv channels. Examples of Kv ancillary subunits are KCNEs, KChIPs and Kvβs (Bett and Rasmusson, 2008).

Fig: 1.4 Schematic diagram of Kv channels and its ancillary subunits. (adapted from (Bett and Rasmusson, 2008, Cox, 2005)).

Among these ancillary subunits, the KCNEs are a family of single transmembrane domain with an intracellular C terminus and an extracellular N terminus. In contrast, to KCNEs, the KChIPs and Kvβs are cytoplasmic. KChIPs are believe to interact with the N terminus of the Kvα subunits whereas Kvβ is known to interact with N- and C-termini of Kvα subunits (Bett and Rasmusson, 2008). In most cases interaction of the
ancillary subunits with α subunits of the Kv channel modulate the activity of the ion channel.

Message and protein expression of various Kvα subunits have been reported in rat mesenteric arteries, including Kv1.1 to Kv1.5; Kv2.1 to Kv2.2; Kv3.2 to Kv3.4; Kv4.1 to Kv4.3; and Kv9.3 (Cox, 2005). Coexpression of the Kvα subunits with ancillary subunits can have a dramatic effect on the kinetics of channel gating, for example coexpression of Kvβ3 (cloned from rat brain) with Kvα1.1 introduced rapid N-type inactivation to the normally slowly inactivating Kv1.1 current (Bett and Rasmusson, 2008). Kvβ1, 2 and 3 subunits have been detected in smooth muscles (Cox, 2005). Among several Kvβ subunits Kvβ1.1, 1.2 and 2.1 expression has been detected in mesenteric arteries (Cox, 2005).

Kv channels are voltage dependent, and open upon membrane depolarization allowing efflux of $K^+$ and subsequent repolarization. In vascular smooth muscle at membrane potentials of -60 to -40 mV the activity of Kv channels is low, but when the membrane potential becomes more depolarized, activation of Kv channels results in $K^+$ efflux which limits depolarization (Nelson et al., 1990). However, a sustained depolarization will eventually result in inactivation of many Kv channels (Panaghie et al., 2008). Activation and steady-state inactivation of Kv currents have been measured and quantified using the Boltzmann equation:

$$G = 1/[1 + \exp((V_{1/2} - V)/k)]$$

1.3
Where $G$ is the relative conductance, $V$ the membrane potential, $k$ is the slope factor and $V_{1/2}$ is the voltage for half maximal conductance. Typical values obtained from rat mesenteric arterial smooth muscle cells for activation are -11 and 15 mV for $V_{1/2}$ and $k$ respectively, and for inactivation -40 and -14 mV for $V_{1/2}$ and $k$ respectively (Cox, 2005). The kinetic behaviour and pharmacological properties of smooth muscle Kv currents reveal the presence of several components in smooth muscle from different vascular beds (Cox, 2005).

Vasoconstrictors including ET-1, Ang-II and AVP have been shown to suppress Kv currents of mesenteric arterial smooth muscle cells following PKC activation (Rainbow et al., 2009, Mackie et al., 2008), and block of Kv channels by 4-aminopyridine increases myogenic tone of rat mesenteric arteries (Plane et al., 2005). Furthermore, impaired functions of Kv channels contribute to some vascular diseases such as hypertension, pulmonary hypoxia, and subarachnoid haemorrhage (Cox, 2005).

1.5.4.5 \(Ca^{2+}\) activated \(K^+\) channels

Ca\(^{2+}\) activated potassium channels can be divided into 3 main types depending on their size of their single channel conductance. Large conductance (BK) channels are voltage-dependent and Ca\(^{2+}\) dependent channels with a large single channel conductance of 100-250 pS; intermediate conductance (IK) channels have an intermediary single channel conductance of 18 to 50 pS; small conductance (SK) channels have a low single channel conductance of 2 to 25 pS and are voltage independent.

Like Kv channels the large conductance potassium channel (BK channel) is also expressed in vascular smooth muscle and comprises pore forming $\alpha$ subunits and regulatory $\beta$ subunits (Ko et al., 2008, Knaus et al., 1994, Tanaka et al., 2004). Four $\alpha$
subunits are closely arranged to form the tetramer where each α subunit contributes to the pore formation of the channel (Ko et al., 2008). Each α subunit is composed of 11 hydrophobic domains (S0 to S10) with an extracellular N terminus and long intracellular C terminus (Ledoux et al., 2006). The S1-S6 domains are similar to those in Kv channels. In addition to S1-S6 transmembrane domains the α subunit also contains an additional transmembrane domain (S0) and four cytosolic domains S7-S10 (Ledoux et al., 2006, Ko et al., 2008). The S4 domain contains several positive charges due to the abundance of lysine and arginine amino acids. The large C terminus also contains a Ca2+ sensing region termed the calcium bowl (Wei et al., 1994). The regulatory β subunits, which comprise 2 transmembrane domains, modulate the Ca2+ sensitivity of BK channels (Ko et al., 2008, Ko et al., 2010).

The cytosolic Ca2+ concentration is an important regulator of BK channel activity and an elevated level of Ca2+ and depolarization increases the open probability of these channels (Blatz and Magleby, 1986). BK channels play an important role in the regulation of tone in vascular smooth muscle. An increase in intracellular Ca2+ due to agonists activation causes release of Ca2+ from intracellular stores or Ca2+ influx through voltage gated Ca2+ channels. This elevated Ca2+ activates ryanodine receptors (RyR) leading to further local Ca2+ release termed Ca2+ sparks which causes opening of nearby BK channels leading to efflux of K+. The current recorded due to activation BK channel is known as spontaneous transient outward current (STOC) (Bolton and Imaizumi, 1996). BK channel activation limits depolarization and provides a negative feedback mechanism to regulate vasoconstriction (Ko et al., 2008).
1.5.4.6 ATP-dependent K\(^+\) (\(K_{\text{ATP}}\)) channels

These potassium channels, which are inhibited by intracellular ATP, were first described in cardiac myocytes (Noma, 1983) and later identified in a variety of other cells including vascular smooth muscle (Standen et al., 1989).

Structurally \(K_{\text{ATP}}\) channels are composed of four Kir 6.x subunits (Kir 6.1 or Kir 6.2) which are pore forming subunits and four sulphonylurea receptor (SUR1 and SUR2) subunits to form a hetero-octamer protein complex. Sulphonylurea receptors are the targets of sulphonylurea antidiabetic drugs. \(K_{\text{ATP}}\) channels expressed in smooth muscle are composed of SUR2B with either Kir6.1 or Kir6.2 subunits (Yamada and Sunaga, 1997, Koh et al., 1997, Standen and Quayle, 1998).

\(K_{\text{ATP}}\) channels are activated by an increase in the ADP: ATP ratio (Quayle and Standen, 1994) and are important regulators of membrane potential and hence control the myogenic tone of small resistance arteries (Nelson and Quayle, 1995). Many vasoconstrictors also inhibit \(K_{\text{ATP}}\) channel activity to inhibit the efflux of potassium ions resulting in depolarization, activation of L-type Ca\(^{2+}\) channels and vasoconstriction.

Based on the fact that \(K_{\text{ATP}}\) channels have important regulatory roles in various tissues their pharmacology has been studied extensively and several blockers and activators have been synthesized to date. Among these pharmacological agents pinacidil, cromakalim, levcromakalim, nicorandil, minoxidil, diazoxide, and BRL-55834 cause vasodilation (Quayle et al., 1997). However, glibenclamide, an antidiabetic agent, significantly inhibits \(K_{\text{ATP}}\) channels and causes vasoconstriction of hyperpolarized smooth muscle (Quayle et al., 1995, Quayle et al., 1997).
1.5.4.7 Inward rectifier $K^+$ (Kir) channels

Kir channels were first identified in skeletal muscle and have since been found in a variety of cells including neurons, cardiac myocytes, endothelial cells, pancreatic beta cells and kidney cells (Hibino et al., 2010). Kir channels are also expressed in smooth muscle cells, in particular those of small resistance arteries such as cerebral, coronary and mesenteric arteries (Ko et al., 2008, Park et al., 2005a, Park et al., 2006, Quayle et al., 1996, Smith et al., 2008, Standen and Quayle, 1998).

The Kir current, because of its strange behavior was first named the anomalous rectifier potassium current and later called the inward rectifier $K^+$ current. The activation of this channel is closely linked to the external $K^+$ concentration and hence on $E_K$. Kir channels are more active near to and at potentials negative to $E_K$.

The Kir channels are, like Kv channels, tetramers but each Kir subunit contains two transmembrane regions only called TM1 and TM2, a pore forming H5 loop and intracellular N and C termini (Hibino et al., 2010). Kir channel subunits do not have a voltage sensing domain and are therefore not a voltage-gated ion channel, though their activity does depend on the $K^+$ electrochemical gradient. Various (15 to date) Kir channel genes have been identified and have been grouped into families (Kir1.x - Kir7.x). According to their functional characteristics these Kir channels can be divided into four major groups: classical Kir channels (Kir2.x), G-protein gated Kir channels (Kir3.x) and ATP sensitive $K^+$ channels (Kir6.x) and $K^+$ transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x) (Hibino et al., 2010). The Kir channel subunits combine to form homomeric or heteromeric functional Kir channels where heteromerization occurs
by combination of the subunits from the same subfamily with the exception of Kir4.1 that combines with Kir5.1.

Several studies have confirmed that the mechanism of inward rectification occurs because of a block of the channel pore by intracellular polyamines and, to a lesser extent, Mg\(^+\) (Lopatin et al., 1994) and the membrane potential at which this block occurs is very dependent on the K\(^+\) concentration (Stanfield et al., 1994). In vascular smooth muscle, a modest increase in extracellular K\(^+\) concentration from 5 mM to 15 mM causes hyperpolarization of the membrane potential due to opening of Kir channels (Hibino et al., 2010).

Barium (Ba\(^{2+}\)) and Cesium (Cs\(^+\)) are the pharmacological agents most commonly used as Kir channel blockers. These blockers can be applied externally to block most Kir currents and the blocking effect is voltage dependent but the block can be relieved by raising external K\(^+\) concentration (Hibino et al., 2010).

1.5.5 **Receptors involved in regulating smooth muscle contraction**

Receptors are protein molecules, present either on the surface of the membrane or within the cytoplasm, that act as sensors to which ligands bind to initiate a signal. Some receptors termed ionotropic receptors are ligand gated ion channels containing a central pore that is opened or closed (rarely) on ligand binding. These ionotropic receptors are hence directly involved in mediating the signal of the binding ligand. However, other receptors, termed metabotropic receptors, work indirectly by transmitting the signal of the binding molecule to other enzymes or effectors.
Ligands, the molecules which bind to receptors, can be neurotransmitters, hormones, autacoids or an intracellular ligand. Ligand binding is required to induce a conformational change in the receptor which enables the receptor to exert its effects either directly by opening an intrinsic ion channels or indirectly by initiating a signaling cascade. A ligand that activates a receptor is called an agonist, whilst one that inhibits the receptor is an antagonist. Four main types of receptors and their effectors along with examples are enlisted in 1-3.

**Table 1-3 Types of receptor**

<table>
<thead>
<tr>
<th></th>
<th>Ligand gated ion channels</th>
<th>G-protein coupled receptors</th>
<th>Receptor kinase</th>
<th>Nuclear receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>Membrane</td>
<td>Membrane</td>
<td>Membrane</td>
<td>Intracellular</td>
</tr>
<tr>
<td><strong>Effectors</strong></td>
<td>Ion channel</td>
<td>Channel or Enzyme</td>
<td>Protein kinase</td>
<td>Gene transcription</td>
</tr>
<tr>
<td><strong>Coupling</strong></td>
<td>Direct</td>
<td>G protein</td>
<td>Direct</td>
<td>via DNA</td>
</tr>
<tr>
<td><strong>Examples</strong></td>
<td>nAChR, GABA receptor</td>
<td>Muscarinic acetylcholine receptors, adrenoceptors</td>
<td>Insulin, growth factors, cytokine receptors</td>
<td>Steroid receptors</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td>Oligomeric assembly of subunits with a central pore</td>
<td>Seven transmembrane helices making a monomeric or dimericor</td>
<td>Single transmembrane helix make a link between extracellular domain and intracellular kinase domain</td>
<td>Monomeric structure with separate receptor and DNA binding domains</td>
</tr>
</tbody>
</table>

Table adapted from (Rang, 2007)

**1.5.5.1 Ligand gated ion channels**

Ligand gated ion channels are integral membrane proteins with an intrinsic pore which, when open, allows flux of the permeant ions down their electrochemical gradient. These ion channels have an extracellular domain that contains a ligand binding site. Binding of
an agonist causes a conformational change in the receptor leading to opening of the pore. Examples of ligand gated ion channels are P2X receptors, which are found on smooth muscle and activated by ATP (Cho et al., 2010, Aronsson et al., 2010, Boland et al., 1992, Dobronyi et al., 1997, Zhao et al., 2004), fast acting neurotransmitter receptors such as the nicotinic acetylcholine receptor and the intracellular IP₃ and ryanodine receptors which are Ca²⁺ release channels.

1.5.5.2 G-protein coupled receptors (GPCRs)

The β adrenoceptor, the first G-protein coupled receptor (GPCR), was cloned in 1986 (Dixon et al., 1986). GPCRs constitute a family of more than 500 members and share some common features. G-protein coupled receptors have seven transmembrane α helices; each α helix comprises 22 to 28 hydrophobic amino acids. These receptors are named as G-protein coupled receptors because of their need for an intermediary protein, a G-protein (guanosine triphosphate binding protein), to mediate their signal to the effectors or other enzymes within the cell. GPCRs are also the therapeutic target of more than 30% of all drugs; therefore much attention is focused on the understanding of this rapidly emerging field of therapeutics. P2Y receptors, where UTP acts as a ligand, also belong to this important family of receptors (Abbracchio et al., 2003). G-protein coupled receptors are the target of several vasoconstrictors as well as neurotransmitters; therefore much attention has been focused on understanding the signaling cascade triggered by these receptors.
1.6 **Signaling pathways involved**

1.6.1 **G-protein coupled receptor (GPCR) signaling pathways**

Several vasoactive compounds, hormones and neurotransmitters are dependent on GPCR activation. These ligands bind to their specific GPCR present on the membrane. Ligand binding causes a conformational change in the receptor and enables the receptor to interact with the inactive heterotrimeric G protein. G proteins are membrane resident proteins and diffuse within the plane of the bilayer membrane to pass on the message of the receptor to the effector. The G-protein in its inactive state is a complex of α, β, and γ subunits and contains a GTP binding site in α subunit. This binding site is normally occupied by GDP in its inactive state; however, as soon as the G-protein trimer interacts with the activated GPCR a conformational change in the receptor decreases the binding affinity of the GDP to the G-protein so that GDP is displaced from its binding site and is replaced by GTP. The binding of GTP causes the G-protein to dissociate into Gα-GTP and dimer Gβγ. The activated free form of Gα-GTP complexes may activate or inhibit enzymes of various signaling cascades depending on the type of the α subunit (Gαs, Gαi/o, Gαq/11, Gα12/13) as summarized in the Table 1-4. In a similar way, the βγ-dimer also has the ability to activate certain ion channels or enzymes. However, the signaling pathways activated by βγ-dimers need more GPCRs to be activated (Rang, 2007).

G-protein signaling is terminated when the GTPase activity of the α-subunit hydrolyses GTP to GDP, resulting in the inactivation of the Gα subunit that subsequently binds to the βγ subunit to reform the inactive form of the G-protein (Sperelakis, 2001). It is also believed that GPCR of one type can interact with more than one type of G-protein and hence is able to initiates more than one signaling pathway. GPCRs are involved in regulating the following main signaling pathways:
1.6.1.1 Cyclic adenosine monophosphate (cAMP) signaling pathway

Cyclic AMP is an important intracellular mediator that is synthesized from ATP by the action of a membrane bound enzyme adenylate cyclase. Several drugs, hormones and neurotransmitters act on GPCRs to produce their effects by increasing or decreasing the activity of adenylyl cyclase and thereby affecting the production of cAMP. Two isoforms of G-protein α subunits modulate adenylate cyclase activity; Gαs (Gs) stimulates whereas Gαi inhibits adenylate cyclase.

Upon activation, the Gαs-GTP complex activates the catalytic domain of the adenylyl cyclase and enables the adenylyl cyclase to convert ATP to cAMP which then acts as a second messenger to mediate the signal of the GPCR to other components of the cell. In most animal cells cAMP activates cAMP dependent protein kinase (PKA). PKA catalyzes several important functions and in its inactive form consists of a complex of two catalytic subunits and two regulatory subunits. The binding of cAMP to the regulatory subunits of PKA causes a conformational change that liberates the catalytic subunits. The catalytic subunit of PKA phosphorylates specific target proteins, for example, in patches excised from mesenteric smooth muscle PKA causes an increase in the activity of Kv channels (Hayabuchi et al., 2001b).

1.6.1.2 Phosphoinositide or Phospholipase C signaling pathway

Activation of GPCRs coupled to Gq initiates a signaling cascade via activation of the enzyme phospholipase C. Phospholipase C is a plasma membrane bound enzyme that catalyzes the hydrolysis of the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Both of these are important signaling molecules. In general, IP3 causes release of Ca2+ by binding to IP3
receptors (IP₃R) present on the membrane of the endoplasmic reticulum. DAG activates the serine threonine protein kinase C (PKC) either alone or in combination with cytosolic free Ca²⁺, which in turn catalyzes the phosphorylation of many other proteins (Somlyo and Somlyo, 2000).

Protein kinase C was formerly described as a Ca²⁺ activated phospholipid-dependent protein kinase (Salamanca and Khalil, 2005). Protein kinase C (PKC) comprises a family of various isoforms with distinct characteristics and physiological functions (Salamanca and Khalil, 2005). PKC isoforms are classified into three sub groups (Nishizuka, 1992).

The conventional PKCs (cPKC): α, βI, βII, and γ. The conventional PKC isoforms comprise four conserved regions (C1–C4) and five variable regions (V1-V5). Cysteine-rich zinc finger-like motifs are present in the C1 region of the regulatory domain. It also contains an autoinhibitory pseudosubstrate sequence. A recognition site for phosphatidylserine, DAG and phorbol ester is also present within the C1 region (Salamanca and Khalil, 2005). The C2 region contains the binding site for Ca²⁺. The C3 and C4 regions act as catalytic domains and form the ATP- and substrate-binding sites (Salamanca and Khalil, 2005).
Fig: 1.5 Structure of PKC isoforms
adapted from (Salamanca and Khalil, 2005).

The novel PKCs (nPKC): δ, ε, η and θ The C2 region is not present in the novel PKC isoforms and hence these do not require Ca^{2+} for their activation (Salamanca and Khalil, 2005).

The atypical PKCs (aPKC): ζ and λ/ι The atypical PKC isoforms also lack a C2 region in their structure and also only one cysteine-rich zinc finger-like motif is present and therefore they are dependent on phosphatidylserine, but are unaffected by DAG, phorbol esters or Ca^{2+} (Salamanca and Khalil, 2005).
The PKD family comprises three structurally related isozymes i.e. PKD1 (also called PKC µ), PKD2, PKD3 (formerly called PKCv) that also belongs to the family of serine threonine kinases. However these are classified as a subfamily of the Ca^{2+}- calmodulin dependent kinases (CaMK) due to their close structural homology to CaMK and MLCK at their catalytic domain. The PKD is a DAG receptor that possesses high mobility and communicates the signal from DAG and PKC (Wang, 2006).

In mesenteric artery smooth muscle cells PKCα, γ, δ, ε, and ζ isoforms are expressed (Salamanca and Khalil, 2005). PKC phosphorylates important regulatory proteins in vascular smooth muscle many of which are important in the regulation of contraction (Lee and Severson, 1994, Salamanca and Khalil, 2005). Many vasoconstrictors induce contraction via activation of specific PKC isoforms, for example Ang-II and ET-1 induce contraction in rat mesenteric arteries by activating PKCε and PKCα respectively (Rainbow et al., 2009). It is generally believed that most of the PKC isoforms including conventional, novel and atypical are activated via the PLC signaling pathway. Several agonists act at GPCRs and activate PLC via G_{q/11} to produce IP₃ and DAG as second messengers. The DAG, either alone or in combination with Ca^{2+} activates PKC.

PKC phosphorylates various regulatory proteins such as CPI-17 and MARCKS (myristoylated alanine-rich C-kinase substrate) (Salamanca and Khalil, 2005). PKC phosphorylates CPI-17 which interacts with myosin light chain phosphatase to decrease its activity effectively increasing the Ca^{2+} sensitivity of the contractile machinery (Mueed et al., 2005, Xie et al., 2006). It is also reported that PKC affects plasma membrane ion channels including L-type Ca^{2+}, Kv, Kir; and K_ATP channels and pumps including the Na⁺-H⁺ exchanger (Maddali et al., 2005, Park et al., 2006, Rainbow et al., 2009, Ratz and Miner, 2009, Sampson et al., 2007, Endoh et al., 1998,
Hayabuchi et al., 2001a). PKC inhibits L-type voltage gated Ca\textsuperscript{2+} channels (Navedo et al., 2005, Schuhmann and Groschner, 1994) and vasoconstrictors inhibit several types of potassium channels via PKC mediated pathways (Salamanca and Khalil, 2005). Among smooth muscle K\textsuperscript{+} channels PKC has been reported to modulate are Kv channels (Fish et al., 1988, Hayabuchi et al., 2001b), inward rectifier potassium channels (Park et al., 2007, Park et al., 2005b, Wu et al., 2007) and ATP sensitive potassium channels (Standen and Quayle, 1998, Kubo et al., 1997). PKC also suppresses the BK channel activity in pulmonary arterial smooth muscle (Barman et al., 2004). Vasoconstrictors also activate non selective cation channels through PKC dependent or independent pathways (Soboloff et al., 2007).

1.6.1.3 RhoA/ RhoA- kinase signaling pathway

Vasoconstrictors are also able to sensitize the contractile machinery by employing Rho kinase signaling pathway (Somlyo and Somlyo, 2000). Agonists binding to GPCRs recruit G\textsubscript{a12/13} which activates guanosine nucleotide exchange factor (RhoGEFs) which changes RhoA from its inactive GDP bound form (RhoA-GDP) to its active RhoA-GTP form by the exchange of GDP with GTP (Bishop and Hall, 2000). Subsequently, RhoA-GTP activates RhoA-kinase (ROCK) which, in smooth muscle, can increase the activity of MLCP (Kimura et al., 1996, Hirano et al., 2004). G\textsubscript{a12/13} coupled GPCRs also tend to couple to other subclasses of GPCRs, such as G\textsubscript{q/11}(Somlyo and Somlyo, 2000).

Rho kinase is a serine threonine kinase and among its various isoforms, ROCK-1 and ROCK-2 are expressed in vascular smooth muscle (Khalil, 2010). In addition to sensitizing the contractile machinery, ROCK is also employed in various other cellular and metabolic processes of the vascular smooth muscle including cell proliferation, cell migration and gene transcription (Khalil, 2010). Luykenaar et al provide evidence for a
role of Rhokinase in Kv current modulation by UTP (Luykenaar et al., 2004) and demonstrated that the ROCK inhibitor (Y-27632) significantly modulated the Kv current inhibition and depolarization by UTP in rat cerebral arteries.

Fig: 1.6 GPCR mediated IP3 and RhoKinase signaling pathway.
Figure modified from (Hirano et al., 2004)

1.6.1.4 Ion channels

Some ion channels can be modulated by direct interaction with the free Gβγ subunits released on activation of GPCRs. An example of this modulation follows ACh activation of M2 receptors in sinoatrial node cells of the heart which leads to the activation of G-protein regulated inwardly rectifying K⁺ channels (GIRKs); these channels are activated by the Gβγ released from the Gᵢ-protein (Roche and Treistman,
1998, Vorobiov et al., 1998). Some voltage-gated Ca$^{2+}$ Channels (P/Q- and N-type) are also modulated by direct Gβγ interaction (Tedford and Zamponi, 2006, Hernandez-Ochoa et al., 2007, Yoon et al., 2008).

Table 1-4 G-protein subtypes and their functions

<table>
<thead>
<tr>
<th>G-protein subunit</th>
<th>GPCR</th>
<th>Effector</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha s}$</td>
<td>Amines &amp; other receptors like: histamine, catecholamine, and serotonin</td>
<td>Activates adenylyl cyclase [↑] cAMP</td>
<td>Activated by cholera toxin</td>
</tr>
<tr>
<td>$G_{\alpha i}$</td>
<td>Same as $G_{\alpha s}$; also opioid and cannabinoid receptors</td>
<td>Inhibits adenylyl cyclase [↓] cAMP</td>
<td>Blocked by pertussis toxin</td>
</tr>
<tr>
<td>$G_{\alpha o}$</td>
<td>Same as $G_{\alpha s}$; also opioid and cannabinoid receptors</td>
<td>Effects mainly due to Gβγ subunits</td>
<td>Blocked by pertussis toxin</td>
</tr>
<tr>
<td>$G_{\alpha q}$</td>
<td>Amine, peptide and prostanoid receptors</td>
<td>Activates PLC, [↑] DAG and IP3</td>
<td>-</td>
</tr>
<tr>
<td>Gβγ</td>
<td>All GPCR</td>
<td>As for Ga, also: Activate K+ channel; inhibits VGCC; activate GPCR kinases and other kinases</td>
<td>Higher levels of GPCR activation required</td>
</tr>
</tbody>
</table>

Table adapted from (Rang, 2007)

1.6.2 Ca$^{2+}$ regulation

The cytosolic Ca$^{2+}$ concentration is in the nanomolar range (50-150 nM) compared to an external concentration of 1.8 mM. However, the Ca$^{2+}$ concentration rises to approximately 600-800 nM in response to depolarization or the action of vasoconstrictors (Horowitz et al., 1996). This small rise in Ca$^{2+}$ concentration is sufficient to initiate contraction and comes from two main sources; external Ca$^{2+}$ influx and Ca$^{2+}$ release from the sarcoplasmic reticulum. Several vasoconstrictors induce a biphasic response in the smooth muscles. The initial rise in intracellular Ca$^{2+}$
concentration in response to vasoconstrictor occurs due to the release of Ca\(^{2+}\) from the internal stores following activation of IP\(_3\) receptors on the sarcoplasmic reticulum (Berridge, 2009). A more sustained phase of the contraction is maintained by Ca\(^{2+}\) entry across the cell membrane through Ca\(^{2+}\) permeable channels (Khalil and van Breemen, 1988, Horowitz et al., 1996). The increased Ca\(^{2+}\) levels of the cytosol is decreased to resting levels by Ca\(^{2+}\) extrusion from the smooth muscle cell by plasma membrane Ca\(^{2+}\) ATPase and the Na\(^{+}\)/Ca\(^{2+}\) exchanger in addition to being taken up into the sarcoplasmic reticulum via Ca\(^{2+}\) ATPase (Khalil, 2010).

1.7 **Purinergic signaling**

1.7.1 **Introduction to purinergic signaling**

The work done in this thesis concentrates on uridine 5’ triphosphate, which is a pyrimidine nucleotide. UTP is a uracil nucleotide containing a uracil base which is linked to a ribose sugar that in turn contains three phosphates at its fifth carbon atom.

![UTP structure](image)

Among purine and pyrimidine nucleotides, adenosine compounds have been studied extensively since the discovery of a physiological role of ATP in the heart more than 80 years ago by Drury and colleagues (Drury and Szent-Gyorgyi, 1929). It is now known that nucleotides are released from platelets, endothelial cells and some other secretory
cells under normal as well as under stress conditions (Anderson and Parkinson, 1997); however, their release from non-secretory cells is as a result of tissue injury, inflammation or hypoxia. Nucleotides are released in the blood vessels from nerve terminals and from blood platelets (Boarder and Hourani, 1998). The signaling mechanism of ATP was studied in guinea pig cerebral cortex by Sattin and Rall in 1970 (Sattin and Rall, 1970) and later, in 1972, Burstock defined ATP as a non-adrenergic and non-cholinergic neurotransmitter (Burnstock, 1976, Burnstock et al., 1978). Burnstock and coworkers classified purine receptors into P1 and P2. According to the current classification adenosine receptors are distinguished from other nucleotides and these are classified into P1 receptors and there are four adenosine receptors namely, A1, A2A, A2B, and A3. However, P2 receptors are classified into two broad categories of ionotropic P2X and metabotropic P2Y receptors (Abbracchio and Burnstock, 1994). Currently, seven subtypes of P2X and eight subtypes of P2Y receptors are recognized so far (Ralevic and Burnstock, 1998, North, 2002).

The classification was widely adopted when new receptors were identified (Ralevic and Burnstock, 1998). Within P2Y receptors, mammalian and non mammalian receptors could also be distinguished by writing the P2Y in lower case letters (p2y). The first P2Y1 and P2Y2 receptors were formerly named as P2U receptors. Later research based on cloning and identification discovered several P2U receptors and hence P2Y followed by a subscript number sequentially list proteins in their chronological order of cDNA cloning with the exception of P2Y1 and P2Y2 that were formerly classified on the pharmacological basis (Abbracchio et al., 2006). To date eight human P2Y receptors have been cloned and identified as P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (Abbracchio et al., 2003, Abbracchio et al., 2006). However, the missing
numbers are those receptors that are either non mammalian receptors and were initially grouped into this classification; or some receptors that are irresponsive to the purine or pyrimidine nucleotides. Since the pyrimidine nucleotide UTP is the focal point of this thesis the discussion will be limited to those receptors activated by pyrimidine nucleotides with especial reference to P2Y receptors.

1.7.2 Purinergic signaling

The ionotropic P2X receptor are widely stimulated by adenosine compounds with different rank orders of potency (table 1-5). UTP has also been shown to activate P2X$_1$ receptors at a higher concentration compared to ATP in rat tail artery (McLaren et al., 1998).

P2Y receptors, on the other hand, are G protein coupled receptors with seven transmembrane domains, an extracellular N- and intracellular C-terminus like other GPCRs and are activated upon binding of some purine and pyrimidine nucleotides. The P2Y$_1$ receptor, isolated from chick brain was the first in the series to be cloned and characterized as a G protein coupled receptor (Abbracchio et al., 2006). Several other P2Y receptors were isolated, cloned and characterized thereafter.

On the basis of pertussis toxin sensitivity and measurements of Ca$^{2+}$, IP$_3$ and cAMP levels, P2Y receptors are believed to couple with G proteins (Abbracchio and Burnstock, 1994, Abbracchio et al., 2006). However, direct measurements of the coupling of nucleotides with G protein were done in vesicles reconstituted with various isoforms of G proteins and purified P2Y receptors. In these heterologous expression systems, G protein coupling was confirmed with P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors (Abbracchio et al., 2006) and the signaling was via the activation of phospholipase C.
and Ca\textsuperscript{2+} mobilization through IP\textsubscript{3} (Boarder and Hourani, 1998, Abbracchio et al., 2006). Furthermore, the same P2Y receptor coupling to distinct G proteins was demonstrated in studies on HEL cells and gastric smooth muscle cells (Baltensperger and Porzig, 1997, Murthy and Makhlouf, 1998). The studies on isolated gastric smooth muscle cells demonstrated that P2Y\textsubscript{2} receptors couple to PLC\textbeta\textsubscript{1} via G\textalpha\textsubscript{q/11} and to PLC\textbeta\textsubscript{3} via the \beta\gamma subunit of G\textalpha\textsubscript{i3}. However, P2Y\textsubscript{2} receptors also interact with integrins in astrocytoma cells to mediate the signaling cascade via G\textalpha\textsubscript{o}, not via G\textsubscript{q} (Bagchi et al., 2005). UTP and ATP equipotently activates P2Y\textsubscript{2} receptors (Lazarowski et al., 1995), however, human as well as rat P2Y\textsubscript{4} receptors are potently activated by UTP (Harper et al., 1998) as well as other uracil nucleotides. UTP along with other tri and diphosphonucleotides were detected by Goetz et al (1971) in blood platelets of man, rabbit, guinea pig and cattle by chromatographic and spectrophotometric techniques (Goetz et al., 1971). Under normal physiological conditions, these purine (ATP and ADP) and pyrimidine nucleotides (UTP in small amount) are released from platelets which then act on the endothelium of the blood vessels and it is speculated that P2Y receptor activation of the endothelium induces release of prostacyclin and nitric oxide resulting in vasodilation (Boarder and Hourani, 1998). Endothelium under normal physiological conditions acts as a barrier and nucleotides have access to the vascular smooth muscle only under various stress conditions such as subarachnoid haemorrhage and migraine (Boarder and Hourani, 1998, White et al., 2000).

It was reported that in rat mesenteric arteries UTP as well as ATP showed dual effects i.e. with intact endothelium, both nucleotides relaxed the noradrenaline induced vasoconstriction whereas in endothelium denuded arteries both of these nucleotides constricted the vessels (Ralevic and Burnstock, 1991).
Table 1-5 P2 receptor, agonists, antagonists, tissue distribution and the downstream signaling pathways

<table>
<thead>
<tr>
<th>P2</th>
<th>Ligand</th>
<th>Antagonist</th>
<th>Tissue distribution (human)</th>
<th>Signaling pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>ADP &gt; ATP</td>
<td>Suramin, PPADS</td>
<td>Endothelium, platelets, red blood cells (turkey), perivascular sensory nerves (low), perivascular sympathetic nerves (medium)</td>
<td>Gq11; PLCβ activation</td>
</tr>
<tr>
<td>P2Y2</td>
<td>ATP = UTP</td>
<td>Suramin, not PPADS</td>
<td>Endothelium, VSMC, heart, inflammatory cells, perivascular sensory nerves (low), perivascular sympathetic nerves (possible)</td>
<td>Gq11; PLCβ activation</td>
</tr>
<tr>
<td>P2Y4</td>
<td>UTP &gt; ATP</td>
<td>Not PPADS or suramin</td>
<td>VSMC</td>
<td>Gq11; PLCβ activation</td>
</tr>
<tr>
<td>P2Y6</td>
<td>UDP &gt; ADP</td>
<td>Endothelium, VSMC, heart</td>
<td>Gq11; PLCβ activation</td>
<td></td>
</tr>
<tr>
<td>P2Y11</td>
<td>ATP &gt;&gt; ADP</td>
<td>-</td>
<td>Endothelium (possible), heart, inflammatory cells</td>
<td>Gq11; PLCβ activation</td>
</tr>
<tr>
<td>P2Y12</td>
<td>ADP</td>
<td>-</td>
<td>VSMC, platelets, inflammatory cells</td>
<td>Gi/o; adenylate cyclase ↓</td>
</tr>
<tr>
<td>P2Y13</td>
<td>ADP</td>
<td>-</td>
<td>Red blood cells, inflammatory cells</td>
<td>Gi/o; adenylate cyclase ↓</td>
</tr>
<tr>
<td>P2Y14</td>
<td>UDP-glucose,</td>
<td>UDP-glucose, UDP-galactose</td>
<td>-</td>
<td>Gi/o; adenylate cyclase ↓</td>
</tr>
<tr>
<td></td>
<td>UDP-galactose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X1</td>
<td>ATP &gt; ADP &gt; UTP</td>
<td>Suramin, PPADS</td>
<td>VSMC, heart, platelets, inflammatory cells</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X2</td>
<td>ATP &gt;&gt; ADP, UTP</td>
<td>Suramin, PPADS</td>
<td>Perivascular sensory nerves, perivascular sympathetic nerves</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X3</td>
<td>ATP &gt; ADP &gt; UTP</td>
<td>Suramin, PPADS</td>
<td>Perivascular sensory nerves</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X4</td>
<td>ATP</td>
<td>Suramin, PPADS</td>
<td>Endothelium, heart, inflammatory cells</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X5</td>
<td>ATP &gt; ADP</td>
<td>Suramin, PPADS</td>
<td>-</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X6</td>
<td>ATP &gt; ADP</td>
<td>Suramin, PPADS</td>
<td>-</td>
<td>Positive ion channel</td>
</tr>
<tr>
<td>P2X7</td>
<td>ATP &gt; ADP</td>
<td>Suramin, PPADS</td>
<td>VSMC (possible), red blood cells, inflammatory cells</td>
<td>Positive ion channel</td>
</tr>
</tbody>
</table>

Table adapted from (Boarder and Hourani, 1998, Erlinge and Burnstock, 2008, Abbracchio et al., 2006)
1.8 **Aims and Overview of the thesis**

The aim of the present study is to determine the mechanism by which UTP induces contraction of rat mesenteric arteries. The work described in this thesis focuses on two aspects: (i) measurements of contraction of rat small mesenteric arterial segments; and (ii) measurements of ionic currents in rat isolated mesenteric artery smooth muscle cells.

Chapter 3 describes the experiments done to investigate the UTP induced contraction of mesenteric arteries. The role of extracellular calcium and the downstream signaling pathways in response to UTP application were studied in this chapter. UTP induced sustained contractions of rat mesenteric arteries and my experiments were designed to investigate possible signalling mechanisms using various pharmacological agents. One aim was to use these pharmacological tools to dissect out the relative role that voltage gated \( \text{Ca}^{2+} \) channels and non-selective cation channels have in mediating the UTP induced contraction. Specific peptide blockers of selected PKC isoforms were linked to the Tat-peptide, making them highly membrane permeable, with the aim of examining possible specific roles that the PKC isoforms PKC\( \alpha \), \( \beta I \), \( \beta II \), \( \delta \) and \( \varepsilon \) have in mediating the UTP response.

Chapter 4 describes the experiments done to determine the ionic basis of the UTP induced contraction. Using the whole cell patch clamp technique various currents were recorded in the absence and presence of UTP. The currents examined were the \( \text{Kv} \) current, the \( \text{K}_{\text{ATP}} \) current and a non-selective cation current. Many vasoconstrictors I, including Ang II and ET-1, have been shown to modulate these currents through specific PKC isoforms. The aim of the work presented here was to ascertain whether
UTP modulated these currents using specific PKC isoforms, again utilizing the specific Tat-linked PKC isoform inhibitors. An additional objective was to assess whether voltage-gated Ca\textsuperscript{2+} currents could be reduced directly by UTP induced signaling events. Although in my preliminary experiments I did manage to record whole-cell inward Ba\textsuperscript{2+} currents these were too small and labile to enable the effect of UTP to be tested on these currents.
Chapter 2  Material and Methods
2.1 **Dissection of rat mesenteric arteries**

Mesenteric arteries are small resistance arteries that supply blood to the small intestine. They are subdivided into branches and form the vascular mesenteric bed to provide a rich blood supply to the walls of the small intestine.

Mesenteric arteries of adult male Wistar rats (weight range 200-250 g) were used. The rats were killed by stunning followed by cervical dislocation and the mesenteric arteries were dissected out as described below.

The abdomen was cut carefully using forceps and a pair of scissors. The skin around the abdomen was lifted with the help of forceps and a small piece of the skin was cut for a short distance to expose the peritoneum. A piece of skin was cut away from the level of the pelvic region towards the bottom of the diaphragm. The peritoneal membrane was then picked up with help of forceps and carefully cut in a vertical direction from the lower abdomen towards the diaphragm. Furthermore, two small horizontal cuts at the abdominal region allowed easy access to the gut. To prevent drying of the exposed gut, zero Ca\(^{2+}\) solution was poured on to the exposed tissues. The mesenteric arteries lie in the middle of the gut and can be identified as a fat covered group of blood vessels connected with the intestine. The mesenteric arterial bed was exposed by a gentle movement of the small intestine.

The vascular bed of mesenteric arteries, along with the primary branch of the superior mesenteric artery, were dissected out carefully without stretching the arteries by cutting the mesenteric arteries away from the small intestine using a pair of micro scissors (Fig: 2.1). The arteries were then transferred to zero Ca\(^{2+}\) PSS. The dissection was
carried out under a microscope at room temperature. As shown in the Fig: 2.1 the vascular bed was transferred and pinned onto a Sylgard-based petri dish containing zero Ca$^{2+}$ PSS such that the most visible blood vessels (mesenteric veins) were uppermost. Mesenteric veins could easily be distinguished from the mesenteric arteries due to their dark colour and fragile nature. The mesenteric veins were separated from the mesenteric arteries forceps and micro scissors. The mesenteric arteries were then cleaned of fat and adipose tissue Fig: 2.1. Following which they were clearly visualized as primary branches of the superior mesenteric artery which was branched into second, third and fourth order branches till the distal end. After dissection mesenteric arteries were either placed into some fresh ice cold normal PSS and maintained on ice for myography or were immediately enzymatically digested for cell isolation.

For enzymatic isolation of the cells the residual blood was gently squeezed out from the mesenteric bed without stretching the blood vessels; however for myography experiments stretching of the vascular bed was avoided as much as possible.
Fig: 2.1 Dissection of rat mesenteric artery

A-B) The mesenteric arterial bed from adult male Wistar rats were dissected out immediately after stunning and cervical dislocation; C) Mesenteric arteries were kept in a Sylgard petri dish containing ice cold PSS (0 Ca²⁺) while removing adipose tissues and fat; D-E) Mesenteric arteries were cleaned of fat under microscope using forceps and micro scissors; F) The mesenteric artery with its lateral branches is seen clearly.
2.2 Myography

2.2.1 Principles of small vessel myography
Small vessel wire myography enables the force developed during isometric contraction of arterial segments to be measured. Several other parameters including the thickness of a vessel and its internal diameter could also be determined by this technique.

Before the 1980s information about the pharmacological and mechanical properties of the small resistance arteries were limited to histological examination and perfusion experiments. The myograph technique was developed by Bevan and Osher in 1972 and later adapted by Mulvany and Halpern (1976) (Bevan et al., 1972) for making measurements in small vessels. The myograph mounting procedure does not rely on the direct attachment of a wire to the vessel segment; instead a mounting wire is threaded through the middle of the vessel lumen and secured under tension on both sides therefore allowing isometric contraction to occur. The arterial ring segments are stretched horizontally; hence the sheer force is the same along the length of the vessel wall.

In my myographic experiments I used a Myo-interface model 500A myograph (Fig: 2.2). This allows simultaneous measurement of two vessel segments and incorporates a system for adjusting the vessel extension, thereby enabling automatic normalization, calibration and data acquisition. Using this myograph the isometric contractile forces of mesenteric arterial segments during the application of various pharmacological agents were measured.
Fig: 2.2 Small vessel myograph.
The dual channel wire myograph (top); An illustrated diagram (bottom) of the wire myograph chambers, where small pieces of vessel are hanged using pieces of wires.
2.2.2 **Mounting of mesenteric arterial segment**

The myograph chamber was filled with ice cold physiological saline solution (PSS). One end of a 40 µm thick stainless steel wire was secured with fixing screws to the far end of the mounting jaw. The dissected mesenteric arteries were transferred into the myograph chamber and a suitable piece of third order mesenteric artery was selectively cut. This small piece of vessel was pushed gently into the near end of the screwed wire. The vessel was pulled along the wire and set at the central position of the mounting jaws Fig: 2.3A. The jaws were screwed together, the near end of the wire fixed under the fixing screws, and the jaws were moved apart slightly. Another wire was gently fed through the lumen of the vessel Fig: 2.3B and the jaws screwed together. Both ends of the wire were then fixed under fixing screws. In the wire myograph one of the jaws is movable and is attached to micropositioner whereas the other jaw is attached to a sensitive isometric transducer.
A) One end of the small piece of wire was secured under fixing screw in one of the jaw; the lumen of the mesenteric artery was passed along the wire.

B) The vessel was positioned in the middle of the mounting jaw; the free end of the wire was fixed under screw.

C) A second wire was also passed through the lumen of the same vessel and fixed under screw.

D) The free end of the second wire was also fixed under screw.

Fig: 2.3 Various steps of mounting mesenteric arterial segment in a wire myograph.
2.2.3 Normalization

Since the size of the vessel and its response to various agonists depends on the degree of stretch a normalization procedure was carried out to define the internal circumference as that when the vessel was fully relaxed and under a transmural tension equivalent to a pressure of 100 mmHg. Normalization was routinely performed after mounting the mesenteric arteries by distending the arterial segments in a step wise manner and measuring sets of micrometer readings and force readings on chart recorder or digital display. From these measurements various parameters can be calculated.

i) The internal circumference of the vessel is calculated from the known diameter of the wire and the distance between the wires.

ii) The wall length of the vessel is also determined in ocular divisions with the help of the eye piece of the microscope. It should also be noted that the wall length is the sum of the upper and the lower wall and is twice the segment length.

iii) The wall tension is another parameter and is the measured force divided by the wall length.

iv) The effective pressure, \( P_i \), is an estimate of the pressure that would be necessary to extend the vessel to the measured internal circumference and is given by the Laplace equation:

\[
P_i = \frac{\text{Wall tension}}{[\text{internal circumference}/(2\pi)]}
\]

The effective pressure corresponding to vessel distension is therefore calculated by entering each pair of readings and the relevant calibration factors into the myograph controller. The stepwise distension of the vessel is stopped when the effective pressure exceeds 100 mmHg; where 100 mmHg = 13.3 kPa. The internal circumference is set to \( IC_1 = 0.9 \times IC_{100} \), since at this internal circumference the force production of the vessel would be...
(with special reference to rat mesenteric arteries) is maximal. To the internal circumference data an exponential curve was fitted and the internal diameter of each vessel corresponding to a transmural pressure of 100 mmHg i.e. \( l_{100} = \frac{l_C_{100}}{\pi} \) was determined.

**Output:**

\[ r^2 = \text{regression coefficient for fit of } (x_i, y_i) \text{ to an exponential curve; where } x_i \text{ and } y_i \text{ are micrometer reading (} \mu \text{m} \text{) and force reading (chart recorder division) respectively.} \]

\[ l_{100} = \text{internal diameter corresponding to a transmural pressure of 100 mmHg} \]

\[ x_1 = \text{micrometer screw setting for which internal circumference corresponds to an internal diameter of } l_1 \text{ (} l_1 = 0.9 \ast l_{100} \). \]

**2.2.4 Measurement of contractile responses**

After normalization the vessels were equilibrated with 6 mM K\(^+\) normal PSS for 15 to 20 minutes to get a steady base line at 37°C. Since the endothelium was intact within the mesenteric arteries, 20 mM L-NAME was added to all solutions to inhibit the production of nitric oxide which might interfere with the contractile responses of mesenteric arteries. The solutions (maintained at 37°C in a water bath) were added or changed using a 1 ml transferring pipette in to the static bath of the myograph chamber. Contractile responses were digitized using an ADC interface (Digidata 1332A or Minidigi 1A, Axon Instruments), and stored on computer for subsequent analysis using pClamp 9 (Axon Instruments).

After obtaining a steady baseline with 6 mM K\(^+\) PSS, the mounted mesenteric arteries were challenged with 60 mM K\(^+\) as indicated in Fig: 2.4. A contraction on addition of 60 mM K\(^+\) was used as a measure of the likely ability of the arterial segment to respond
to various vasoactive compounds. The contractile response of 60 mM K$^+$ was washed
by normal PSS as indicated by the immediate decline of the sustained phase to the base
line as shown in the Fig: 2.4B. UTP (100 µM in normal PSS) was applied twice to
measure the contractile response of the mesenteric arteries to UTP and to ascertain any
possible desensitization of the receptor. The involvement of various ion channels or
second messengers was studied using a range of pharmacological agents which were
added 20 minutes prior to the third addition of UTP. Any unspecific effects were
assessed by measuring the contractile response to 60 mM K$^+$ in the presence of the
compound at the end of the experiment. The dual channel wire myograph allows
simultaneous measurements of the contractile responses of two arterial segments treated
separately using a removable separator. Hence it was possible to treat one segment as a
control and add the compound under test to the other segment only.
Fig: 2.4 Protocol for the measurement of contractile response

Representative traces of contractile responses of third order mesenteric arteries to the 60 mM $K^+$ and 100 $\mu$M UTP in the absence and presence of antagonist.

A) The mesenteric arteries were allowed to equilibrate for about 15 minutes in normal salt Solution (PSS) after mounting in the wire myograph until steady base line was established at 37°C. To inhibit the production of NO all experiments were performed in the presence of L-NAME, an inhibitor of eNOS. The mesenteric arteries were washed with normal PSS after getting contractile response to the vasoconstrictors. The contractile response to 60 mM $K^+$ assured the viability of the mesenteric artery. The UTP was applied twice to assess the consistent contractile response evoked by UTP under control conditions. Mesenteric arteries were incubated for 20 minutes in antagonist; a third UTP induced contractile response was recorded and analyzed by normalizing it with the second UTP induced contraction.

B) The contractile responses of the mesenteric arteries to the vasoconstrictors were measured from the steady base line to the maximum steady contractile response to the vasoconstrictors.
2.2.5 Analysis

The contractile response to various pharmacological agents was measured relative to the nearest base line. The contraction evoked by UTP in presence of a blocker was normalized with the equivalent UTP induced contractile response in the absence of blocker (control, considered as 1 or 100 %) from the control portion of the same arterial segment. The contractile responses of UTP in the presence and absence of the blocker (control) were plotted as means ± SEM. For analysis Student’s paired t test was used; P< 0.05 was considered as significant.

The response to 60 mM K⁺ in the presence of blocker was also measured in a similar way and normalized to the equivalent 60 mM K⁺ evoked response in the absence of the blocker (control) from the same arterial segment and plotted as a histogram.

ET-1 induced contractions of mesenteric arteries were measured in a similar way by taking the maximal peak response relative to the nearest base line response. The ET-1 induced responses of each arterial segment were normalized to their respective 60 mM K⁺ induced contractile responses. ET-1 induced contractile responses in the presence of the blocker were compared and plotted as a histogram against the contractile responses induced by ET-1 in absence of the blocker.

2.2.6 Experimental Solutions:

All chemicals were bought from Sigma-Aldrich (unless otherwise stated). Solutions (Table: 2.1) were made up in Elga deionized water and chemicals were added in solid form except for calcium chloride and magnesium chloride which were added from 1M stock solutions (both from BDH laboratory supplies). The solutions were adjusted to pH
7.4 with NaOH or HCl. The freshly made solutions were stored at 5° C in the refrigerator and used within one week. The pH of each solution was checked every day before experiments.

Table 2.1 Solutions for myography

<table>
<thead>
<tr>
<th>Chemical</th>
<th>0 Ca(^2+) PSS</th>
<th>6 mM K(^+) Normal PSS</th>
<th>60 mM K(^+) PSS</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>135 mM</td>
<td>135 mM</td>
<td>81.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>6.0 mM</td>
<td>6.0 mM</td>
<td>60.0 mM</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.2 mM</td>
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<td>CaCl(_2)</td>
<td>___</td>
<td>1.8 mM</td>
<td>1.8 mM</td>
</tr>
<tr>
<td>Glucose</td>
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<td>4.0 mM</td>
<td>4.0 mM</td>
</tr>
<tr>
<td>Mannitol</td>
<td>6.0 mM</td>
<td>6.0 mM</td>
<td>6.0 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0 mM</td>
<td>10.0 mM</td>
<td>10.0 mM</td>
</tr>
</tbody>
</table>
2.3 Patch clamp electrophysiology

2.3.1 Cell isolation:

Mesenteric arteries were dissected out and placed in Ca\(^{2+}\) free solution (4ºC) containing (in mM): 137 NaCl, 5.4 KCl, 0.42 Na\(_2\)HPO\(_4\), 0.44 NaH\(_2\)PO\(_4\), 4 glucose, 6 mannitol, 10 HEPES, 1 MgCl\(_2\) adjusted to pH 7.4 with NaOH. The mesenteric arteries were cleaned of fat and connective tissues and transferred to fresh Ca\(^{2+}\) free solution as described in section 2.1. The enzymes were added to a low Ca\(^{2+}\) solution containing bovine serum albumin (BSA, 0.9mg/ml). The composition of the low Ca\(^{2+}\) solution was the same as the Ca\(^{2+}\) free solution except that it contained 0.1 mM CaCl\(_2\). Two enzyme solutions were used for isolation of mesenteric arterial smooth muscle cells. The first contained (in mg/ml): 1.05 papain and 0.9 dithioerythritol and the second contained (in mg/ml): 0.45 collagenase and 0.6 hyaluronidase. The tissue was incubated in the first and second enzyme solutions (pre warmed for 10 min at 35° C) for 31 and 12.5 min respectively in a water bath at 35°C. At the end of tissue digestion, the enzyme solution was carefully drained out without disrupting the tissue and washed with low Ca\(^{2+}\) BSA to prevent further digestion and following this the tissue was washed with 2-3 ml of BSA free low Ca\(^{2+}\) solution. The tissue was then placed into a fresh bottle containing 3–4 ml of low Ca\(^{2+}\) solution and was tritutrated for a few min with a wide-bore pipette (glass or plastic) until the solution appeared cloudy due to the presence of isolated cells. The residual tissue was then removed and placed in a separate container and the cells were stored on ice. Healthy cells were long, thin and bright and were ready for experiments 20 to 30 minutes after isolation.
2.3.2 Principles of patch clamp electrophysiology

E. Neher and B. Sakmann first used the patch clamp technique to measure current in cell attached patches of membrane of frog skeletal muscle (Neher et al., 1976a; Neher et al., 1976b). The technique was subsequently refined by Hamill et al (Hamill et al., 1981) to enable various recording configurations including the measurement of whole cell currents in cells normally too small for conventional microelectrode recording. For this purpose a glass pipette containing suitable electrolyte solution is pressed against the membrane of the cell so that a high seal resistance is achieved between the tip of a clean glass electrode and the surface of the cell membrane. A high seal resistance of the order of $>10^9 \, \Omega$ (termed a gigaseal) is required for the complete isolation of the membrane patch and to ensure that the total ionic current flows from the cell membrane to the pipette which is then measured and amplified by a current sensitive headstage amplifier. With a gigaseal the distance between the pipette electrode and the cell membrane is estimated to be around 1Å, so that small molecules are unable to diffuse through the seal (Walz, 2002). The high seal resistance is therefore effective in reducing the noise level of the recording by allowing current flow into the glass electrode rather than under the seal as shown in the Fig: 2.5.
The gigaseal formation allowed Hamill and colleagues to develop several different configuration of the patch clamp technique as shown in Fig: 2.6. The various configurations are described briefly:

The cell-attached mode is the first step of the patch clamp technique where a high seal resistance is achieved between the tip of the electrode and the patch of the membrane. In general the seal resistance increases as the tip of the pipette electrode is pressed against the surface of the membrane and a light suction to the interior of the pipette increases the seal resistance gradually to achieve a high seal resistance (Fig: 2.6). The cell attached configuration allows single channel recording from a variety of cells. From
the cell-attached mode experimenter can establish various other configurations such as inside out, whole cell, outside out and perforated patch.

The inside out configuration is used to measure single channel currents where the cytoplasmic side of the membrane patch is in contact with the bath solution. This configuration is obtained by pulling the electrode away from the cell in the cell-attached configuration so that the patch of membrane covered by the tip of the electrode is excised from the rest of the cell. The cytoplasmic side of the membrane patch is in contact with the bath solution, whereas the extracellular side of the membrane faces towards the patch pipette.

In contrast to inside out patch whole cell patch clamp configuration is used to measure the currents flowing through the ion channels of the membrane of the entire cell. Once a gigaseal is obtained, the whole-cell configuration is achieved by applying gentle suction, or a brief 1 V pulse, to rupture the membrane patch while retaining the gigaseal (Fig: 2.6). The patch pipette solution is in contact with the intracellular solution and ionic currents can be recorded from the whole cell.

The perforated patch configuration (Fig: 2.6) is an alternative method of recording from the whole cell where instead of rupturing the membrane patch a pore forming antibiotic such as amphotericin or nystatin is used in the internal pipette solution which acts on the membrane of the cell to make it permeable to monovalent ions in the cell-attached configuration. In this method the cytoplasmic contents remain intact and do not diffuse out of the cell into the patch pipette.
Fig: 2.6 Patch clamp configurations

Fig: 2.6 also illustrates the outside out configuration which can be used to study single channels. In the outside out configuration a neck of the membrane is pulled away after establishing the whole cell which then reseals so that the extracellular surface of the patch is bathed in the external solution and the cytoplasmic surface in contact with the pipette solution.

Among the configurations described above, and illustrated in Fig: 2.6, I used the whole cell patch clamp configuration with the aim of studying any modulation of ionic currents by UTP which may be linked to its contractile mechanisms.
2.3.3 **Whole cell patch clamp configuration**

A whole cell voltage clamp measurement allows recording of membrane current under constant voltage so that current is directly proportional to the conductance of permeable ions which in turn depends on channel activity.

![Diagram of patch pipette and headstage circuit](image)

**Fig: 2.7 Schematic diagram of the headstage circuit of current / voltage amplifier.**

2.3.4 **Practical aspects of the whole cell patch clamp technique**

A) **Patch clamp recording setup**

The setup I used to record whole-cell currents included an inverted microscope (Nikon), a motorized manipulator (Siskiyou), an Axopatch 200A patch-clamp amplifier, an oscilloscope, a Digidata 1322A interface and computer.

The microscope was placed on an anti-vibration table (Technical manufacturing corporation USA) which was surrounded by a Faraday cage used to minimize electrical
noise. The anti-vibration table, Faraday cage and microscope were connected to the single high quality earth of the patch-clamp amplifier to minimize the introduction of earth loops and all cables were of the shielded BNC type wherever possible. All of my patch-clamp experiments were performed at 30 ± 1°C.

B) Choice of glass for the construction of electrode

For any patch clamp measurements obtaining a high resistance seal between the patch pipette and cell membrane is a necessity and as such patch pipettes should be constructed carefully. Patch pipettes are made by drawing out glass capillaries with an electrode puller to produce a tip diameter of 1-2 µm (Aidley, 1996). The type of glass selected for making electrodes varies depending on the recording mode e.g. single channel recordings requires lower noise as compared to whole cell recordings and thick walled glass gives lower noise and is therefore preferably used for single channel recording. However, thin walled glass capillaries give a lower access resistance and are suitable for whole cell recordings. For whole cell recordings blunt and low resistance electrodes facilitate the seal formation therefore I used standard thick walled borosilicate glass tubing (GC150F-7.5) by Harvard Instruments). Several general properties of glasses must be considered for constructing patch clamp electrodes such as thermal and optical properties but one of the most important is the electrical properties of the glass which determines the background noise level. The electrical properties also determine the size of the capacity transients following a step change in potential. Thermal properties determines the optimal temperature for the fabrication of the tip, the melting point of quartz, for example, is above 1600° C and therefore cannot be pulled with ordinary electrode pullers. The chemical composition of the glass is also an
important consideration since it may lead to leaching of the chemical constituents of the glass into the pipette solution that can ultimately interfere with the currents measured.

B) **Pulling electrodes**

I used a vertical puller (Narishige; Tokyo, Japan) to pull electrodes from standard-walled borosilicate glass using two steps pulling setting. The vertical pullers work under gravitational force. The Narishige puller used a two stage process with two different heating steps to pull the electrodes. During the first step, the middle of the capillary was heated and pulled under gravitational force to around 7 mm with automatic settings. The second pull was used to pull the capillary apart to yield two electrodes of optimal geometry.

C) **Fire polishing**

Fire polishing is an additional step used often to blunt the electrode tip which aids the formation of a gigaseal. To fire polish a pipette its tip was placed near (several micrometers) a heated, glass coated, platinum wire for approximately 5 seconds. I used fire polishing in my initial experiments but later I was able to seal onto the smooth muscle cells without fire polishing and found it unnecessary.

D) **Obtaining a whole-cell recording**

Cells were placed in the recording chamber containing 6 mM K⁺ external solution and left to settle for approximately 10 minutes. A patch electrode was back filled with the appropriate solution (see below) and tapped gently to remove air bubbles before being inserted into the holder of the headstage. After placing the patch pipette into the holder, the electrode was moved closer to the recording chamber using coarse control of the
micromanipulators. The liquid junction potential that was generated when the electrode tip was immersed into the bath was offset using the adjustment on the amplifier. The concentration and ionic composition of the internal pipette and the external bath solution are responsible for the magnitude of the junction potential. Electrode resistances, which were monitored by observing currents resulting from the application of repetitive 5 mV pulses, were in the range of 4 – 6 MΩ. As the tip of an electrode was brought near a cell, the resistance of the electrode was monitored carefully, and contact with the cell was detected as an increase of 40-50% of this resistance. At this point gentle suction using a 10 ml syringe was applied to aid seal formation. To improve the seal quality a holding potential of about -40 mV was applied; success was deemed when the resistance rose above 1 GΩ (gigaseal). During and after the process of sealing the external solution was continuously perfused at a rate of 2 ml / min.

Once a gigaseal was obtained the electrode capacitance spikes were cancelled using the fast capacitance compensation controller of the amplifier and the output gain was increased. To rupture the seal and to get the access to the whole cell further suction was applied with a 1 ml syringe. Access to the whole cell was indicated by the increased capacitance transient spikes arising from the added membrane capacitance. At this stage the membrane capacitance and series resistance were adjusted to minimize these transients. The value of the membrane capacitance after this adjustment was used as an indicator of membrane surface area and was used to give a measure of current density (pA pF⁻¹). As soon as the whole cell configuration was achieved the contents of the intracellular pipette solution equilibrated with the cell contents.
2.3.5 Data collection and analysis

2.3.5.1 Recording of Kv current – pulse protocol

Whole-cell Kv currents were recorded with 6 mM external K\(^+\) and 140 mM internal K\(^+\) in the pipette and Penitrem A (100 nM) was added to the external solution to block BK channels. Kv currents were induced by applying 400 ms depolarizing voltage pulses ranging from -40 to +60 mV from a holding potential -65 mV. Prior to these test pulses a P/6 leak subtraction protocol was used. Currents were filtered at 2 kHz, digitized at 10 kHz and stored on the hard drive of a computer for subsequent analysis.

To plot current-voltage relationships the mean current over 50 ms (between 320-370 ms) was calculated and plotted against test potential. Data are expressed as means ± SEM; and differences between currents measured under control conditions and those measured during UTP application were analyzed by ANOVA with Bonferroni’s test or Student’s paired or unpaired t-test as appropriate and a value of P< 0.05 was considered significant.

2.3.5.2 Recording of current from K\(_{\text{ATP}}\) channels

The initial establishment of the whole-cell configuration was done in 6 mM external K\(^+\). Following this, steady state whole cell currents were recorded at a holding potential -65 mV and the external solution changed to one containing 140 mM K\(^+\). Pinacidil (10 μM) was used to open K\(_{\text{ATP}}\) channels further and reversal of the current by glibenclamide (10 μM) was used to assess the amount of K\(_{\text{ATP}}\) current present. Currents were filtered at 2 kHz and digitized at 10 kHz.
Total $K_{ATP}$ current, which included the steady-state plus pinacidil activated current, was measured from the baseline current with 6 mM $K^+$ (adjusted manually to zero) to the maximum amplitude of the pinacidil activated current (see Fig. 4.11). UTP inhibition was measured as the current remaining in the presence of UTP normalized to the maximum total $K_{ATP}$ current. Data were plotted as means ± SEM and intergroup differences were analyzed by ANOVA or Students paired or unpaired $t$-test as appropriate. $P < 0.05$ was considered as significant.

2.3.5.3 Recording of current from non selective cation channels (NSCC)

1. **Steady state current**

When recording non-selective cation currents $K^+$ was replaced by $Cs^+$ in the patch pipette to minimize contamination from $K^+$ currents. Steady-state currents were recorded at -65 mV, and the current was filtered at 2 kHz and digitized at 10 kHz.

2. **Ramps**

Whole cell current ramps were recorded by applying symmetrical ramps, first from -100 to +30 mV in 400 ms and back to -100 mV again in 400 ms. Ramps were applied every 5 seconds from a holding potential of -65 mV. Currents were filtered at 2 kHz and sampled at 10 kHz. The ramps were used to generate instantaneous current-voltage relationships of the non-specific cation current (see section 4.5).

2.3.5.4 Recording of current from voltage gated Calcium channels

To record $Ca^{2+}$ currents the pipette solution contained 140 mM $Cs^+$ instead of $K^+$ to minimize contamination by $K^+$ currents. Cells were clamped at -65 mV and currents
were elicited by applying 150 ms voltage pulses ranging from -40 to +50 mV. Currents were filtered at 2 kHz and sampled at 10 kHz.

2.3.6 Chemicals

Uridine 5´ triphosphate, cadmium chloride, (+)-cis-diltiazem hydrochloride, Gö6983, myristoylated PKC20-28-IP, gadolinium chloride, pinacidil, glibenclamide, penitrem A, endothelin-1, angiotensin-II, chelerythrine chloride and L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) were bought from Sigma-Aldrich. Stock concentrations of all chemicals were made by dissolving them in Elga / MilliQ water (double distilled deionized water) except chelerythrine and U73122 which were dissolved in DMSO. Aliquots of all stock solutions were kept at -20°C in a freezer (except L-NAME that was kept in a refrigerator and used within few weeks), thawed on the day of an experiment and diluted to the desired concentration in 6 mM physiological salt solution. BQ123 and myristoylated PKC20-28-IP were bought from Calbiochem and dissolved in Elga / MilliQ water to make the stock concentration. Small aliquots were kept in a freezer for further use.

The general and isoform-specific PKC inhibitor peptides were also linked to a HIV derived Tat peptide (Tat-PKC20-28-IP, Tat-PKCα-IP, Tat-PKĆδ-IP, Tat-PKCβIV5-3-IP and Tat-PKCβII-IP) rendering them membrane permeable. Linking these inhibitory peptides to the Tat peptide was done by Dr. R.I. Norman of the Department of Cardiovascular Sciences, University of Leicester.
2.3.7 PKC inhibitor peptides:

To date many PKC inhibitors have been developed that compete at different binding sites of PKCs. PKC inhibitors such as chelyrethrine, Gö6983, staurosporine and H-7 compete with ATP at the C3 region of the catalytic domain of PKC and are non specific in their action. Chelyrethrine is known as a competitive inhibitor with histone IIIS but is less competitive with ATP (Herbert et al., 1990) whereas Gö6983 is a non-selective PKC inhibitor that competitively inhibits various PKC isoforms at different IC\textsubscript{50} values. Some PKC inhibitors, for example calphostin C and sphingosine, specifically compete with the DAG, phorbol ester or phosphatidylserine binding sites of PKC and hence are more specific in their action.

The amino acid sequence of the regulatory domain of PKCs between amino acid residues 19 and 36 resembles the PKC substrate and is bound to the substrate binding site when the enzyme is in its inactive conformation and hence are useful targets for inhibiting the PKC–substrate phosphorylation. Therefore, investigators have developed the peptides (19-36) and (19-31) based on pseudosubstrate sequences which possess the capability of inhibiting the PKC by binding at the PKC–substrate binding site to specifically inhibit the PKC-substrate phosphorylation. This inhibition occurs without interfering with the ATP binding site (House et al., 1987). Since these peptide inhibitors are not permeable through the hydrophobic lipid bilayer various approaches have been employed to make permeable PKC inhibitor peptides.

Introduction of a fatty acid (myristoyl group) makes the PKC pseudosubstrate peptide inhibitor more permeable. Myristoylated PKC20-28 inhibitor peptide is such an example where a myristoylated nonapeptide sequence, based on the pseudosubstrate...
domain of PKC-α and PKC-β, was shown to inhibit TPA (12-O-tetradecanoyl-phorbol-13 acetate) induced phosphorylation of MARCKS (myristoylated alanine rich C kinase substrate protein) (Eichholtz et al., 1993). To understand the role of different PKC isoymes another approach has been adopted to develop isoform specific peptide inhibitors.

![Schematic diagram representing the mechanism of PKC binding to its RACK binding protein and PKC isoform inhibition (e.g. RACK1 (β)).](image)

As mentioned before in chapter 1, several PKC isoymes exist and many of these may be present within a single cell type. In their inactive state these PKC isoymes are differentially localized within sub cellular compartments, and upon activation translocate either to the plasma membrane or to the various intracellular organelles,
depending on their corresponding anchoring proteins (Mochly-Rosen, 1995). Several protein molecules have been shown to act as anchoring proteins, being involved in translocating the PKC isoforms either in their inactive or active state (Mochly-Rosen et al., 1998; Mochly-Rosen et al., 1991). Examples of these anchoring proteins are RACKs (receptor for activated C kinase) and RICKs (receptor for inactive C kinase) (Mochly-Rosen et al., 1998). To achieve PKC isoform selective inhibition, peptide fragments have been developed, based on these RACK binding sites (Mochly-Rosen et al., 1998), to compete with the PKC isoform for its particular membrane bound RACK binding protein (e.g. RACK1 (β) as shown in the figure: 2.8). The peptide-RACK binding is of such a high affinity that it mimics the PKC docking with its specific RACK protein (Mochly-Rosen et al., 1998).

In 1994, Fawell and colleagues first demonstrated the ability of HIV derived Tat peptide 37-72 to deliver large protein molecules such as RNAarse, β-galactosidase and peroxidase into cells from the extracellular solution (Fawell et al., 1994). Mochly-Rosen’s group utilized this approach to deliver PKC isoform selective peptide inhibitors by linking them with Tat peptides (Begley et al., 2004) and recently results from our lab demonstrated the ability of Tat linked PKCα-IP and Tat linked PKCε-IP to inhibit selectively ET-1 and Ang-II modulation of Kv currents respectively in rat mesenteric arterial smooth muscle cells (Rainbow et al., 2009). The HIV derived Tat peptide, which is able to deliver several large molecules across the membrane, contains arginine and lysine in abundance and is highly cationic. This high polarity of Tat peptides is known to be responsible for their ability to deliver large molecules across the membrane (Brooks et al., 2005).
Table: 2.2 lists the Tat PKC inhibitor peptides that I have used in my thesis, along with their corresponding amino acid sequences. Tat peptide 47-57 is a 11 amino acid peptide which was utilized to deliver the PKC peptide inhibitors. Tat peptide 47-57 and the various PKC peptide inhibitors (listed in table: 2.2) were obtained from Pepceuticals Ltd. Each peptide inhibitor was reversibly coupled to the Tat peptide by a disulphide bond through N-terminal cysteine residues with a coupling efficiency of 40%. This was done by Dr Bob Norman, Department of Cardiovascular Science, University of Leicester, to whom I am very grateful. The resulting cell permeable Tat linked peptides enter the cell where the Tat carrier peptide is released by the splitting of the disulphide bond due to the reducing intracellular environment of the cell (Begley et al., 2004; Brooks et al., 2005; Hallbrink et al., 2001)

**Table 2.2 Tat PKC inhibitor peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat 47-57</td>
<td>YGRKKRRQRRR Cys at N-terminal</td>
<td>PKC antagonist</td>
<td>(Begley et al., 2004; Vives et al., 1997)</td>
</tr>
<tr>
<td>αC2-4</td>
<td>SLNPQWNET</td>
<td>αPKC antagonist</td>
<td>Xiao et al., 2003</td>
</tr>
<tr>
<td>βI V5-3</td>
<td>KLFIMN</td>
<td>βIPKC antagonist</td>
<td>Xiao et al., 2003</td>
</tr>
<tr>
<td>βII V5-3</td>
<td>QEVIRN</td>
<td>βIIPKC antagonist</td>
<td>Xiao et al., 2003</td>
</tr>
<tr>
<td>δ V1-1</td>
<td>8-17 SFNSYELGSL RACK binding site PKCδ V1/C2 domain</td>
<td>PKCδ antagonist</td>
<td>Chen et al., 2001</td>
</tr>
<tr>
<td>ε V1-2</td>
<td>14-21 EAVSLKPT RACK binding site V1/C2</td>
<td>PKCe antagonist</td>
<td>Johnson et al., 1996</td>
</tr>
<tr>
<td>PKC20-28</td>
<td>N-Myr-FARKGALRQ-NH₂ pseudosubstrate</td>
<td>PKC inhibitor</td>
<td>Eichholtz et al., 1993; Ward et al., 1993</td>
</tr>
</tbody>
</table>
Experimental solutions for electrophysiology

All solutions listed in tables 2.3, 2.4 and 2.5 were made using doubled distilled deionized water either from Elga or MilliQ deionizer from chemicals bought from Sigma-Aldrich except CaCl\(_2\) and MgCl\(_2\), both of which were from BDH laboratory supplies in the form of 1 M stock solutions. Solutions were made and stored in refrigerator for further use except internal pipette solutions listed in table 2.5; that were stored in freezer in small bijou bottles for further use. As shown in the table 2.5, internal pipette solutions contain 3.9 mM Ca\(^{2+}\) which was buffered with 10 mM EGTA (as listed in table 2.5) giving a total free \([\text{Ca}^{2+}]\) of 100 nM as calculated using [http://www.stanford.edu/~cpatton/webmaxcS.htm](http://www.stanford.edu/~cpatton/webmaxcS.htm). For 20 nM free Ca\(^{2+}\) the internal pipette solution contained 1 mM CaCl\(_2\) along with 10 mM EGTA.

Table 2.3 Solutions for dissection and isolation of smooth muscle cells

<table>
<thead>
<tr>
<th>Chemical (mM)</th>
<th>Zero Ca(^{2+}) solution</th>
<th>Low Ca(^{2+}) solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>NaCl</td>
<td>137.0</td>
<td>137.0</td>
</tr>
<tr>
<td>Na(_2)HPO(_4)</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>NaH(_2)PO(_4)</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.4 with NaOH/HCl.
### Table 2.4 External solutions for whole cell patch clamp recording

<table>
<thead>
<tr>
<th>Chemicals (mM)</th>
<th>6 mM K(^+) (K(<em>V) / K(</em>{ATP}) / NSCC)</th>
<th>140 mM K(^+) (K(_{ATP}))</th>
<th>I(_{Ba}^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>135</td>
<td>-</td>
<td>135</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>BaCl(_2)</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>KCl</td>
<td>6.0</td>
<td>140</td>
<td>6.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.4 with NaOH / HCl every day.

### Table 2.5 Internal pipette solution

<table>
<thead>
<tr>
<th>Chemicals (mM)</th>
<th>K(_V)</th>
<th>K(_{ATP})</th>
<th>NSCC</th>
<th>I(_{Ca}^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>110</td>
<td>110</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KOH</td>
<td>30</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CsCl</td>
<td>-</td>
<td>-</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>CsOH</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>†CaCl(_2)</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>†EGTA</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Na(_2)ATP*</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>GTP*</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>ADP*</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Added (solid) on the day of experiment and pH was adjusted to 7.2 with NaOH/HCl.

† The free [Ca\(^{2+}\)] was 100 nM as calculated from [http://www.stanford.edu/~cpatton/webmaxcS.htm](http://www.stanford.edu/~cpatton/webmaxcS.htm); for 20 nM free Ca\(^{2+}\), 1 mM CaCl\(_2\) and 10 mM EGTA was used in some experiments (also calculated from [http://www.stanford.edu/~cpatton/webmaxcS.htm](http://www.stanford.edu/~cpatton/webmaxcS.htm)).
Chapter 3  UTP induced contractile response of rat mesenteric arteries
3.1 Introduction

3.1.1 Uridine 5’ triphosphate

Uridine 5’ triphosphate (UTP) is a pyrimidine nucleotide which shares the property, along with several other nucleotides, of being a potent vasoconstrictor. UTP has been implicated in several physiological and pathological situations; for example it has been shown to have a role in regulating vascular tone of cerebral arteries under conditions such as subarachnoid hemorrhage or migraine (Ralevic and Burnstock, 1998, Debdi et al., 1993). The role of UTP in cardiomyocytes under stress conditions was studied by Yitzhaki (2005) who found that UTP protects cultured cardiomyocytes of newborn rat against hypoxic damage (Yitzhaki et al., 2005).

To exert its biological effects in resistance arteries, UTP activates membrane bound P2Y receptors (Erlinge and Burnstock, 2008). However, the constriction or relaxation of resistance arteries by pyrimidine nucleotides depends on the type of P2Y receptor activated (Horiuchi et al., 2001, Miyagi et al., 1996). Among various subtypes, P2Y\textsubscript{2} and P2Y\textsubscript{4} receptors are potently activated by UTP and blocked by suramin and PPADS respectively. UTP stimulates two different subtypes of P2Y receptors in cerebral arteries; activation of P2Y\textsubscript{6} receptors causes vasoconstriction, whereas endothelial P2Y\textsubscript{2} receptor activation is responsible for vasodilation of cerebral arteries (Horiuchi et al., 2001). The P2Y\textsubscript{6} receptors are distributed widely within the heart, brain and blood vessels; it is preferentially activated by UDP rather than UTP.

UTP is thought to exert its contractile effects by the activation of membrane bound P2Y\textsubscript{2} and P2Y\textsubscript{4} receptors that are G-protein coupled receptors (G\textsubscript{q/11} and possibly G\textsubscript{i}). Activation of G\textsubscript{q} linked receptors causes activation of phospholipase C leading to
hydrolysis of plasma membrane phosphatidylinositol 4, 5 bisphosphate (PIP\(_2\)) to yield inositol 1, 4, 5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG) (Kanashiro and Khalil, 1998, Nishizuka, 1992). IP\(_3\) stimulates Ca\(^{2+}\) release from the sarcoplasmic reticulum and initiates contraction in smooth muscle (Sima et al., 1997, Strobaek et al., 1996). UTP induces contraction of rat basilar arteries by increasing the frequency of Ca\(^{2+}\) waves and inhibiting Ca\(^{2+}\) spark frequency (Jaggar and Nelson, 2000) that occurs due to Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) through IP\(_3\)R activation (Syyong et al., 2009). IP\(_3\)R1 activation has been shown to be involved in generating a UTP induced cation current in cerebral arteries, leading to elevated intracellular global Ca\(^{2+}\) concentration and vasoconstriction (Zhao et al., 2008). UTP also activates TRPC3 and TRPC7 in rat cardiomyocytes via activation of DAG (Alvarez et al., 2008).

Several studies have been undertaken on UTP signaling mechanism in rat aorta, cerebral and basilar arteries; however little is known about the mechanism underlying the UTP induced contractile response of mesenteric arteries. The objective of the present study was to understand the underlying mechanisms involved in the UTP induced contraction of mesenteric arterial smooth muscle with the aim of dissecting out the role of protein kinase C in this response. The contractile responses of mesenteric arteries to UTP were measured using the dual channel wire myograph technique.
3.2 General conditions

3.2.1 Contractile response

Contractions of rat mesenteric arterial segments to 60 mM K\(^+\) and UTP were measured in the absence and presence of antagonists (as described in the methods chapter) at 37°C and in the presence of L-NAME, an eNOS inhibitor, to inhibit the production of nitric oxide from the intact endothelium of the mesenteric artery. At the start of the experiment a steady baseline with 6 mM K\(^+\) PSS was established followed by addition of 60 mM K\(^+\) containing saline (see methods) to ascertain the viability of the mesenteric arteries. Subsequently, mesenteric arterial segments were challenged twice with UTP (100 µM) and then washed. Following these initial procedures the arteries were exposed to the test compound for 20 minutes (unless stated otherwise) followed by addition of 100 µM UTP in the continued presence of the test compounds. At the end of the experiments the response to 60 mM K\(^+\) was determined to assess whether the experimental procedure had affected the ability of the artery to contract to depolarization.

3.2.2 Analysis

Contractile responses were measured as the difference between the baseline and the peak response of the contraction induced by the vasoconstrictors (e.g. 60 mM K\(^+\), UTP). The change in UTP induced contraction following pre-treatment of the artery with a compound was recorded and expressed as a percentage of the contraction induced by the second application of UTP (UTP2) under control conditions (see Fig: 2.4).
The normalized data are expressed as means ± SEM. Student’s paired t- test was used to assess statistical significance of the data. Student’s t- test was performed on non normalized data. A value of P<0.05 was considered significant.

3.3 Results

3.3.1 UTP induced contraction was sustained and reproducible

Application of 100 µM UTP to mesenteric arteries resulted in a rapid and maintained contraction that returned readily to base line on washing. The sustained nature of the UTP induced constriction is indicative of P2Y receptor activation that is in contrast to P2X receptor activation, where ATP is known to elicit transient constrictions.

To assess whether UTP induced contractions were consistent and reproducible mesenteric arteries were challenged with repeated applications of 100 µM UTP. UTP was applied directly into the bath for 5 minutes in the presence of L-NAME, followed by a 10 minute wash before the next application as shown in Fig. 3.1A. Such repeated applications of UTP produced robust and consistent contractions. Mean normalized contractions (to the first UTP response) from 4 arterial segments plotted against time of application is shown in Fig. 3.1B. The consistent contractile responses of UTP under control conditions suggested that there was little or no desensitization of the receptors activated by UTP under these conditions. Therefore, subsequent myograph experiments were designed such that responses to UTP in the absence and presence of a pharmacological agent were observed in the same arterial segment.
Fig: 3.1 Time course of UTP induced contraction

A) Representative traces showing the contractile response of a mesenteric artery segment to repeated applications of UTP. 100 µM UTP was applied each time for 5 min and washed off with 6 mM PSS. The time interval between each UTP application was 10 min. All solutions contained L-NAME.

B) UTP induced contractile responses were measured and normalized with their first UTP induced response and mean normalized responses were plotted against time of application. The responses to repeated applications of UTP were consistent over time.
3.3.2 **Cumulative dose response curve for UTP**

The contractile responses of mesenteric arterial segments following applications of a range of concentrations of UTP were measured to ascertain an appropriate concentration of UTP to use in my experiments. After obtaining a contractile response to 60 mM K\(^+\), mesenteric arteries were challenged with increasing concentrations of UTP (1 µM to 300 µM). UTP was added in a cumulative manner without washing between applications. The response to each concentration of UTP was measured from the plateau response relative to the baseline (before first application of UTP). The cumulative addition of UTP constricts rat mesenteric arteries in a dose dependent manner. To quantify these results equation 3.1 was fitted to the data obtained from each arterial segment.

\[
R = \frac{R_{\text{max}} [UTP]^n}{([UTP]^n + EC_{50}^n)}
\]  

where \(R\) is the contractile response to UTP, \(R_{\text{max}}\) the maximum contractile response, \(n\) the Hill coefficient and \(EC_{50}\) the excitatory concentration needed to produce 50% of the maximal response. The value for \(R_{\text{max}}\) returned by the fitting routine for each arterial segment was then used to normalize the data for that particular segment. The normalized data for 3 segments were then averaged and equation 3.1 was fitted to this data with \(R_{\text{max}}\) constrained to 1. The fit returned a value of 52 µM for \(EC_{50}\) and \(n\) of 2.10. A sub-maximal excitatory concentration of 100 µM was found to induce a consistent response and was therefore used in all the experiments in this thesis.
Fig: 3.2 Cumulative dose response curve

A) Representative recording of the effects of various concentrations of UTP on the contractile response of a mesenteric artery. Following a 60 mM K evoked contraction, the mesenteric artery was challenged with UTP ranging from 1 µM to 300 µM (cumulative addition) to contract the mesenteric artery in a dose dependent manner.

B) UTP induced responses from 3 arterial segments were normalized as explained in the text and a cumulative dose response curve was constructed. The curve shows the fit with equation 3.1, giving values of 52 µM for EC$_{50}$ and 2.1 for n.
3.3.3 Role of extracellular Ca\textsuperscript{2+} influx in UTP induced contraction

To investigate the signaling pathway and to examine the role of extracellular Ca\textsuperscript{2+} influx in evoking UTP induced contractions, responses to UTP were measured in Ca\textsuperscript{2+} free solution. Following 20 minutes incubation in Ca\textsuperscript{2+} free solution the response to UTP was measured. As shown in Fig. 3.3, removal of extracellular Ca\textsuperscript{2+} prevented UTP induced contraction (P< 0.001; n=4). However, the contractile response to UTP recovered when 1.8 mM Ca\textsuperscript{2+} was returned to the bath. These results indicate that Ca\textsuperscript{2+} influx plays a large contribution to the UTP induced contraction of mesenteric artery.

In addition to its effect on UTP induced contraction, removing extracellular Ca\textsuperscript{2+} also abolished the contraction resulting from application of 60 mM K\textsuperscript{+} Fig. 3.3. As was the case with UTP, the 60 mM K\textsuperscript{+} induced contraction recovered upon addition of Ca\textsuperscript{2+} to the extracellular solution.
Fig: 3.3 Effect of extracellular Ca$^{2+}$ on UTP induced contraction

A) Representative traces of UTP induced contraction in the presence and absence of extracellular Ca$^{2+}$. In the absence of extracellular Ca$^{2+}$, neither UTP nor 60 mM K$^+$ induced a contraction. These contractions recovered on addition of Ca$^{2+}$.

B) Mean normalized UTP (100 µM) induced contraction in the absence of extracellular Ca$^{2+}$ as indicated. Removal of extracellular Ca$^{2+}$ reduced the UTP induced contraction to $4.1 \pm 2.4\%$ (P< 0.001; n=4).

C) Mean normalized 60 mM K$^+$ induced contraction in the absence of extracellular Ca$^{2+}$ as indicated (n=4).
3.3.4 Involvement of voltage gated Ca$^{2+}$ channels

As indicated in the previous section UTP evoked contraction was mainly dependent on Ca$^{2+}$ influx from the extracellular space. To investigate this further I studied the involvement of Ca$^{2+}$ entry pathways using a variety of Ca$^{2+}$ channel blockers. Initial experiments were done using Cd$^{2+}$, a non selective Ca$^{2+}$ channel blocker. As expected, application of 100 µM Cd$^{2+}$ markedly reduced the contractile response to 60 mM K$^+$ (Fig. 3.4). As shown in Fig: 3.4, pretreatment of mesenteric arteries with 100 µM Cd$^{2+}$ reduced UTP induced contractions to 19.3 ± 3.7% (n = 4); suggesting the involvement of Ca$^{2+}$ channels. Because of the non selectivity of Cd$^{2+}$ further studies were needed to dissect out the role of voltage gated Ca$^{2+}$ channels in UTP induced contractions. Among various subtypes of voltage gated Ca$^{2+}$ channels, the L- and T- types are highly expressed and heterogeneously distributed in smooth muscle cells, both of which are blocked by Cd$^{2+}$.

To test whether L-type voltage gated Ca$^{2+}$ channels are involved the effect of d-cis-diltiazem, a blocker of these channels, on UTP induced contractions was examined. Pretreatment of mesenteric arteries with 50 µM diltiazem inhibited UTP induced contractions by 41 ± 6% (n = 15, P < 0.001, see Fig.3-5). In contrast, contractions induced by 60 mM K$^+$, in the same arterial segments, were blocked substantially by diltiazem (Fig. 3.5C).
Fig: 3.4 Effect of Cd\(^{2+}\) on UTP induced contractile response.
A) A representative trace showing the effect of Cd\(^{2+}\) on the contractile response of a mesenteric artery to UTP.
B) The response to UTP in the presence of Cd\(^{2+}\) was normalized with that in its absence (control) and plotted as a percentage of the control contraction.
C) Contraction to 60 mM K\(^{+}\) in the absence and presence of Cd\(^{2+}\) was also measured in a parallel experiment and the response were normalized and plotted as a percentage of the response to 60 mM K\(^{+}\) in the absence of Cd\(^{2+}\).
Fig: 3.5 Effect of diltiazem on UTP and 60 mM K⁺ induced contractions.

A) Trace showing the effect of 50 µM diltiazem on contractions induced by 100 µM UTP and 60 mM K⁺ as indicated. UTP induced contractions were only partly inhibited by diltiazem; whereas the contractile response to 60 mM K⁺ was abolished.

B) Mean normalized data showing that UTP induced contractions were inhibited by 41 ± 6% (n = 15; **P < 0.001) in the presence of 50 µM diltiazem.

C) Mean normalized data showing that 60 mM K⁺ induced contractions were inhibited to 1.1 ± 0.5%; **P< 0.001) in the presence of 50 µM diltiazem.
3.3.5 UTP induced contractions and activation of PLC

Among several P2Y receptors expressed in vascular smooth muscle P2Y\textsubscript{2} and P2Y\textsubscript{4} potently respond to UTP (Morris et al., 2010, Luykenaar et al., 2004). Since P2Y receptors are G protein coupled receptors it was therefore postulated that UTP induced responses are mediated by activation of phospholipase C.

To assess the possible role of PLC in mediating UTP induced contractions in mesenteric arteries I used the PLC inhibitor U73122 (Helliwell and Large, 1997, Smith et al., 1990) to abolish any signaling downstream of PLC. Pre-treatment of mesenteric arterial rings for 20 minutes with 10 µM U73122 caused a significant block of the UTP induced response to 0.8 ± 1.8% of control values (n= 5; P < 0.01), consistent with the hypothesis that UTP acts via a G\textsubscript{q} coupled receptor pathway. However, pre-treatment with U73122 also irreversibly abolished the contractile response to 60 mM K\textsuperscript{+}. 
Fig: 3.6 Effect of U73122 on UTP induced contractions.
A) Typical traces showing the effects of the PLC inhibitor U73122 on UTP induced contraction of a mesenteric arterial ring segment. 20 minutes incubation with 10 µM U73122 prevented UTP from evoking a contraction 60 mM K+ induced contraction was also observed in the presence and absence of U73122 within same arterial segment. U73122 irreversibly blocked the 60 mM K+ to induce contraction (0.8 ± 1.8%; n= 5; P < 0.01).
3.3.6 PKC involvement in the contractile response of mesenteric arteries to UTP

Protein kinase C (PKC) is a family of serine/threonine kinase and is involved in the mechanism of agonist induced contraction. PKC, by altering the phosphorylation state of key proteins of the contractile machinery, performs distinct role in the Ca$^{2+}$ signaling that leading to contraction. Because of the potential role of PKC in receptor mediated constriction, its contribution was examined by using a selection of non peptide and peptide PKC inhibitors.

Chelerythrine is a naturally occurring plant alkaloid isolated from Chelidonium majus L (family Papaveraceae) and is a non selective PKC inhibitor that also exerts a wide range of biological activities from antimicrobial, antifungal, anti-inflammatory, sympatholytic, adrenolytic and local anesthetic (Colombo and Bosisio, 1996). As shown in Fig. 3.7A & B, pre-treatment of arterial segments with 5 µM chelerythrine for 20 minutes attenuated the contractile response to UTP by 85.7 ± 1.9% of the control (n=3; P<0.05). Chelerythrine also showed unspecific blocking effects on 60 mM K$^+$ induced contraction within the same arteries as shown in Fig. 3.7A.

In view of the unspecific blocking effects of chelerythrine, the effects of a more selective inhibitor, Gö6983, that inhibits the Ca$^{2+}$ dependent PKC isoforms PKCα (IC$_{50}$ =7 nM), PKCβ (IC$_{50}$=6 nM) and PKCγ (IC$_{50}$=6 nM). Gö6983 also inhibits the Ca$^{2+}$ or DAG independent PKC isoenzyme PKCζ (IC$_{50}$=10 nM); however, it poorly suppressed the PKCµ activity at slightly higher concentration (Gschwendt et al., 1996).
Fig: 3.7 Effect of chelerythrine and Gö6983 on UTP induced contraction.

A) Representative trace showing mesenteric arterial response to 60 mM K⁺ and 100 µM UTP in the absence and presence of 5 µM chelerythrine.

B) Mean normalized contraction to UTP (100 µM) under control conditions and after treatment with chelerythrine (5 µM). Chelerythrine reduced UTP induced contraction by 85.7 ± 1.9% of control. (n = 3; P<0.05).

C) Mean normalized contraction to UTP (100 µM) under control conditions and after treatment with Gö6983 (1 µM). Gö6983 reduced UTP induced contraction by 54.3±5.8% of control. (n = 3; P<0.005).
Since at a concentration of 1 µM Gö6983 inhibits almost all Ca\(^{2+}\) dependent PKC isoforms, therefore 1 µM Gö6983 was used to dissect out the involvement of Ca\(^{2+}\) dependent PKC isoforms in UTP induced contraction. As shown in the in Fig. 3.7C Gö6983 partly inhibited the contractile response to 100 µM UTP by 22.4 ± 3.1% (P < 0.005, n = 5).

To investigate the role of PKC in more detail I used several PKC inhibitor peptides (PKC-IP), including the general PKC inhibitor PKC20-28-IP, and peptide inhibitors specific for PKC isoforms α, β, δ and ε. PKC inhibitors are less permeable, therefore, in order to make them membrane permeable, they were linked with HIV tat-derived peptide. The HIV tat-derived peptide is a small peptide i.e. 86 amino acid peptide which has been shown to successively deliver a large variety of molecules, from small particles to proteins (Brooks et al., 2005, Vives et al., 1997). In addition to these Tat-linked peptides the membrane permeable myristoylated PKC20-28–IP was used.

Pre-treatment of mesenteric arteries for 20 min with 40 µM myristoylated PKC20-28-IP reduced the UTP evoked contraction by only 21% in two arterial ring segments. The tat linked PKC20-28-IP (Tat-PKC20-28-IP), applied at 100nM for 20 minutes which has been shown to be effective at this concentration (Rainbow et al., 2009), reduced the UTP induced contraction by a similar amount, 17.1±6.8%; (P<0.05; n=7). In a parallel series of experiments, Tat-PKC20-28-IP reduced ET-1 evoked contractions by 40% in four arterial ring segments (P<0.05).
Fig: 3.8 Effect of PKC20-28-IP on UTP induced contraction

A) An example trace showing the contractions induced by 60 mM K⁺ and 100 µM UTP before and after the addition of 100 nM Tat-PKC20-28-IP as indicated.

B) Mean normalized data of the UTP induced contractions in the absence and presence of 100 nM Tat-PKC20-28-IP. The response to UTP was reduced by 17.1±6.8% (P<0.05; n=7).

C) Mean normalized data of the UTP induced contractions in the absence and presence of 40 µM myristoylated-PKC20-28-IP. The response to UTP was reduced by 28.8% (n=2).
Fig: 3.9 Effect of Tat-PKC20-28-IP on ET-1 induced contraction.

A) Traces showing the contractions induced by 60 mM K⁺ and 10 nM ET-1 in control (black trace) and in 100 nM Tat-PKC20-28-IP (red trace) as indicated.

B) Mean contraction to ET-1 (10 nM) as percentage of 60 mM K⁺ induced contraction under control conditions and in the presence of Tat-PKC20-28-IP (100 nM). ET-1 induced contraction was reduced from 120% to 80% (P<0.05; n=4).
Since the effect of the general PKC20-28-IP was not clear more selective PKC isoform inhibitors were used in an attempt to dissect out the role of specific PKC isoforms in UTP induced contractions. Pre-treatment of arterial segments with 100nM Tat PKCαC2-4-IP for 20 minutes did not affect UTP induced contractions as shown in Fig. 3.10. However, a parallel experiment was designed to check the effectiveness of Tat PKCαC2-4-IP (100 nM) to UTP (30 µM) as well as ET-1 (3 nM) induced contractions.

Tat PKCαC2-4-IP effectively blocked the contraction induced by ET-1, previously reported by (Rainbow et al., 2009).

Using similar experimental conditions Tat-PKCδ-IP (50 nM), Tat-PKCε-IP (100 nM), Tat-PKCβI-IP (100 nM) and Tat-PKCβII-IP (100 nM) were all unable to reduce UTP induced contractions significantly, see Fig. 3.14 and a summary of the effects of PKC inhibitors is shown in Fig. 3.16. Rainbow et al. (2009) reported the Ang-II induced contraction was significantly inhibited by Tat PKCε-IP under experimental conditions similar to those where I found no significant reduction in UTP induced contraction of mesenteric arteries pretreated for 20 minutes with Tat PKCε-IP. Therefore a parallel experiment was performed, using the Ang II induced contraction, to check the effectiveness of Tat PKCε-IP in my experiments. As shown in Fig. 3.15, the Ang-II induced contraction was reduced by 45% (n=1) after pretreatment for 20 minutes with100 nM Tat-PKCε-IP (bottom traces).
**Fig: 3.10 Effect of Tat-PKCaC2-4-IP on contractile response of mesenteric arteries.**

A) Representative traces showing the effect of Tat-PKCaC2-4-IP (100 nM) on UTP (100 nM) induced contraction. Following 60 mM K⁺ induced contraction arterial segment was challenged with repeated applications of UTP under control conditions and subsequently UTP induced contraction was observed after pretreatment of the arterial segment with Tat PKCaC2-4-IP (100 nM). Tat-PKCaC2-4-IP was unable to prevent the UTP to induce contraction.

B) Mean normalized data of UTP induced contraction is expressed as percentage (± SEM). No significant reduction in the UTP induced contraction was obtained (n=3).
Fig: 3.11 Effect of Tat-PKCα-IP on UTP (30 μM) and ET-1 (3 nM) induced contraction

A) Typical tracings of a myograph experiment showing the effect of Tat PKCα-IP (100 nM) on ET-I (3 nM) induced contraction. Upper control traces (black) shows the contraction of a mesenteric arterial ring to 60 mM K⁺ and subsequently to repeated applications of UTP (30 μM) and later to ET-1 (3 nM); Bottom traces (red) indicate the recording from a paired ring as in the upper trace, but with 20 min exposure to 3 nM Tat-PKCα-IP (100 nM) before application of ET-I (3 nM).

B) Plot of mean contraction to ET-1 (3 nM) as percentage of 60 mM K⁺ induced contraction under control conditions and after treatment of Tat-PKCα-IP (100 nM). Tat-PKCαC2-4-IP reduced the ET-1 response by 69% (n=3).
**Fig: 3.12 Contractile response of UTP in the presence of Tat PKCδ-IP**

A) Myograph recording of contraction of mesenteric arterial ring segment to 60 mM K⁺ and subsequently to 100 µM UTP in the absence and presence of 50 nM Tat PKCδ-IP. Response to third UTP after pretreatment of Tat PKCδ-IP was normalized to second UTP induced response (in the absence of Tat PKCδ-IP) and mean normalized data was plotted as percentage of the control.

B) Histogram plotted from the above experiment showed no profound reduction in UTP induced contraction in six arterial ring segments.
Fig: 3.13 Effect of Tat-PKCβ-IP on UTP induced contraction.
A) Typical traces of a myograph experiment showing the effect of Tat-PKCβI-IP (100 nM) on UTP (100 µM) induced contraction. No reduction in the contraction was observed.
B) Mean normalized data plotted as histogram shows no reduction in UTP induced contraction when arterial ring segment was pretreated for 20 minutes in Tat-PKCβI-IP (n=4).
C) Mean normalized data plotted as histogram shows no reduction in UTP induced contraction when arterial ring segment was pretreated for 20 minutes in Tat-PKCβII-IP (n=3).
Fig: 3.14 Response of mesenteric artery to UTP in the presence of Tat PKCε-IP.

A) Typical tracings of a myograph experiment showing the effect of Tat PKCε-IP (100 nM) on UTP induced contraction. Response of mesenteric arterial segment to 60 mM K⁺, UTP was assessed prior to addition of Tat-PKCε-IP (100 nM). After 20 minutes of pretreatment of Tat PKCε-IP, UTP (100 µM) induced contraction was determined.

B) There was no profound reduction of UTP induced contraction was seen in four mesenteric arterial ring segments.
Fig: 3.15 Effect of Tat-PKCε-IP on Ang-II induced contraction.

A) Typical tracings of a myograph experiment showing the effect of Tat PKCε-IP (100 nM) on Ang-II induced contraction. Upper traces (control) shows the contraction of a third order mesenteric arterial ring to 60 mM K⁺ and subsequently to Ang-II; Bottom traces indicate the recording from a paired ring as in the upper trace, but with 20 min pretreatment to 100 nM Tat-PKCε-IP before application of Ang-II.

B) Ang-II induced contraction was expressed as percentage of the 60 mM K⁺ induced contraction (n=1).
Fig: 3.16 Summary of the effects of various PKC inhibitors on UTP induced contraction.

Plot of mean normalized data of UTP induced contractions in the absence (control) and presence of various PKC inhibitors.

<table>
<thead>
<tr>
<th>PKC Inhibitor</th>
<th>Mean normalized contraction (%)</th>
<th>SEM</th>
<th>P-value</th>
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<tr>
<td>Gö6983 (1µM)</td>
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<td>Chelerythrine (5 µM)</td>
<td>14.3</td>
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<td>P&lt;0.05</td>
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<td>-</td>
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<td>Tat PKCε-IP (100 nM)</td>
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<td>5.1</td>
<td>-</td>
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</table>
3.3.7 Are non-selective cation channels involved in UTP induced contraction?

To examine whether Ca$^{2+}$ influx via non-selective cation channels is involved in UTP induced contraction the effect of Gd$^{3+}$, a non-selective cation channel blocker, was examined. Following normal contractile responses to 60 mM K$^+$ and UTP, mesenteric arterial segments were pretreated with various concentrations of Gd$^{3+}$ for 20 minutes and contractile responses to UTP were recorded in the presence of Gd$^{3+}$. The UTP induced contraction was virtually abolished in the presence of 100 µM Gd$^{3+}$ and reduced by 85% in 10 µM Gd$^{3+}$ (Fig.3.17). The effect of Gd$^{3+}$ was concentration dependent as observed in some parallel experiments with varying concentrations of Gd$^{3+}$. Normalized contractile responses of mesenteric arteries to 100 µM UTP in the presence of 1, 10, 30 and 100 µM Gd$^{3+}$ are plotted in Fig. 3.18. Maximum inhibition of UTP induced contraction was observed with 100 µM Gd$^{3+}$; however at this concentration it also inhibited the 60 mM K$^+$ induced contraction of mesenteric arteries.

During the time course of these recordings the inhibition of UTP induced contractions by Gd$^{3+}$ did not recover. However, a recovery of 60 mM K$^+$ induced contractions was observed. These results suggest a possible involvement of non-selective cation channels in eliciting the contraction of UTP.
Fig: 3.17 Effect of Gd$^{3+}$ on UTP induced contraction.

A) Recording of the response of a mesenteric arterial segment challenged with 60 mM K$^+$ and 100 µM UTP in the absence and presence of 100 µM Gd$^{3+}$ as indicated.

B) Recording of the response of a mesenteric arterial segment challenged with 60 mM K$^+$ and 100 µM UTP in the absence and presence of 10 µM Gd$^{3+}$ as indicated. At a concentration of 10 µM Gd$^{3+}$ still blocked the UTP induced contraction, but was much less effective at inhibiting the contraction evoked by 60 mM K$^+$. 

117
Fig: 3.18 Effect of different concentrations of Gd$^{3+}$ on UTP induced contraction.

The effect of various concentrations (1, 10, 30 and 100 µM) of Gd$^{3+}$ on UTP (100 µM) induced contractions. Maximum reduction in UTP induced contraction was observed when arterial segments were pretreated with 100 µM Gd$^{3+}$. 
3.4 Discussion

3.4.1 UTP induced sustained contraction depends on extracellular Ca^{2+} influx

The results of the myograph experiments presented here show that the UTP induced contraction of rat mesenteric arteries is sustained in nature and is likely to result from activation of P2Y receptors (Gitterman and Evans, 2000). However, more recently Sugihara and coworkers reported a P2X_{1}-like receptor activation in addition to P2Y receptor activation in response to UTP application in rat aorta (Sugihara et al., 2011). In my experiments, UTP elicited a concentration dependent contraction with an EC_{50} of 52 µM and a Hill coefficient of 2.1 in three arterial ring segments. A similar EC_{50} value (28 µM) for mesenteric arteries was found by Morris et al. (2011). A comparison of the effect of UTP on rat mesenteric artery segments of different diameters yielded variable results which depended not only on the diameter but also on the measurement technique (Gitterman and Evans, 2000). Using wire myography the EC_{50} and Hill coefficient values for different diameter segments were 1.6 mM and 0.9 for medium (131-342 µm) and 1.4 mM and 1.3 for large (386-806 µm) diameter segments respectively. However, UTP was more potent when vessel diameter was measured using the diamtrak technique, with EC_{50} values of 15 µM and 88 µM for small and medium arteries and corresponding Hill coefficient values of 1.1 and 2.2 respectively (Gitterman and Evans, 2000). The vessel diameters (265±4 µm; n=86) in my experiments were within the medium range defined by Gitterman and Evans but I found a greater potency in the UTP induced response (EC_{50}: 52 µM and Hill coefficient: 2.1). This variation could result from the inclusion of L-NAME in my solutions to inhibit endothelial nitric oxide synthase thus limiting the production of nitric oxide. It is interesting to note that the Hill coefficient for UTP I found (2.1) agrees closely with the 2.2 reported by Gitterman and
Evans (2000) using the diamtrak technique in medium sized arteries. UTP potency is higher in coronary and cerebral arteries with EC₅₀ values of 3.2 and 3.1 µM respectively (Welsh and Brayden, 2001; Luykenaar et al., 2004). The sensitivity of the response of rat mesenteric arteries to UTP and suramin indicates the presence of P₂Y₂ receptors in this tissue (Gitterman and Evans, 2000).

Furthermore, UTP induced contractions were reproducible even with several repeated applications of UTP. The contractions were largely dependent on extracellular Ca²⁺ influx. The potential sources of Ca²⁺ that contribute to agonist induced contractions in arterial smooth muscle cells are: Ca²⁺ influx from the extracellular space and Ca²⁺ release from internal stores. UTP was unable to induce contraction of mesenteric arteries in a Ca²⁺ free solution. My results are consistent with Syyong’s findings who reported that in cerebral arteries only one minute incubation period in Ca²⁺ free external solution significantly suppressed UTP induced Ca²⁺ waves (Syyong et al., 2009). Furthermore, I also found that UTP induced contractions in rat mesenteric arteries returned as soon as the extracellular Ca²⁺ was replenished. In contrast to my findings, Lopez et al (2000) found that the contraction induced by UTP was not affected even 30 min after depleting extracellular Ca²⁺ and in the presence of EGTA (Lopez et al., 2000). This difference in the sensitivity of UTP induced contractions to external Ca²⁺ may be due to variation between aortic and mesenteric arterial smooth muscle, possibly reflecting the size and the amount of Ca²⁺ present in the sarcoplasmic reticulum.
3.4.2 Voltage gated L-type Ca\textsuperscript{2+} channels and UTP induced vasoconstriction

Because of the sustained contractile behavior and the role of extracellular Ca\textsuperscript{2+} in UTP induced contraction, it was expected that the Ca\textsuperscript{2+} influx occurred via voltage gated Ca\textsuperscript{2+} channels. Voltage gated Ca\textsuperscript{2+} channels are highly expressed in smooth muscle (Horowitz et al., 1996). UTP induced contractions were significantly reduced but not abolished by diltiazem, suggesting that Ca\textsuperscript{2+} entry was at least partially dependent on L-type Ca\textsuperscript{2+} channel activity. This is consistent with the results reported by Luykenaar (2004) in rat cerebral arteries where UTP induced contraction was partly reduced by diltiazem (Luykenaar et al., 2004). The more potent L-type Ca\textsuperscript{2+} channel blocker, nifedipine, also reduced, but did not abolish, the UTP induced Ca\textsuperscript{2+} wave frequency as well as tonic contractile responses in rat basilar arteries (Syyong et al., 2009).

3.4.3 UTP activates PLC to initiate a signaling cascade leading to contraction.

UTP has been shown to exert its effects by activating G-protein coupled P2Y receptors (Abbracchio et al., 2006, Burnstock, 1997, Burnstock, 2007, Burnstock, 1976, Erlinge and Burnstock, 2008, Gitterman and Evans, 2000). In general, GPCRs activate PLC via G\textsubscript{q} which in turn hydrolyzes PIP\textsubscript{2} to DAG and IP\textsubscript{3}. IP\textsubscript{3} causes activation of IP\textsubscript{3}R and Ca\textsuperscript{2+} release from the sarcoplasmic reticulum. Ca\textsuperscript{2+} binds to calmodulin and the resulting Ca\textsuperscript{2+}-calmodulin complex activates myosin light chain kinase which phosphorylates MLC, leading to actin-myosin interaction and contraction. DAG, either alone or in combination with calcium, activates several protein kinase C isoforms. Various PKC isoforms have been shown to have roles in agonists induced contractile processes for example causing Ca\textsuperscript{2+} sensitization of the contractile machinery and inhibition of K\textsuperscript{+} channels (Rainbow et al., 2009). The signaling molecules of the
phosphoinositide pathway have important roles in the activation of non-selective cation channels. DAG plays a central role in the noradrenalin activated cation current which involves TRPC6 via a protein kinase C-independent mechanism (Helliwell and Large, 1997). Whereas, stimulation of ET(A) and ET(B) receptors activate PKC-dependent TRPC1 channels in rabbit coronary artery (Saleh et al., 2009). Stimulation of IP3R1 contributes to UTP-induced vasoconstriction and non-selective cation current activation in rat cerebral arteries (Zhao et al., 2008). The complete inhibition of the UTP induced contraction I observed with the PLC inhibitor U73122 indicates that UTP induced contraction is mediated by a receptor mediated pathway, possibly P2Y2, which is a Gq linked receptor that activates PLC/DAG/IP3 signaling. However, U73122 also abolished the contractile response to 60 mM K+. Nonspecific blocking effects of this compound has been reported by other groups. For instance a nonspecific blocking effects of higher concentrations (10 µM) of U73122 on contractions evoked by 50 mM K+ in rat mesenteric arteries have been reported during an attempt to titrate the minimal effective concentration of U73122 needed to inhibit noradrenaline and endothelin 1 induced contractions of rat mesenteric arteries (Clarke et al., 2008). Other authors have also reported nonspecific effects of U73122 including its interference with adenosine A1 receptor signaling (Walker et al., 1998).

3.4.4 Contribution of PKC to UTP induced vasoconstriction
Vasoconstrictors such as ET-1 and Ang II, activate serine-threonine protein kinase C (PKC) to exert their contractile response. PKC comprises a family of isoenzymes; which are classified into three groups. The conventional PKC isoforms are: α, βI, βII and γ; all these require both Ca2+ and DAG for their activation. However, the novel isoenzymes δ, ε, η and θ do not need Ca2+ for their activation; they are activated by
DAG alone, while the atypical isoenzymes \( \zeta \) and \( \lambda/\theta \) need neither \( \text{Ca}^{2+} \) nor DAG phorbol esters for their activation. Among various PKC isoforms \( \alpha, \beta, \gamma, \delta, \epsilon, \) and \( \zeta \) have been reported in vascular smooth muscle (Salamanca and Khalil, 2005). The PKC isoforms may be activated differentially in response to various agonists (Rainbow et al., 2009). Vasoconstrictors can cause activation of non-selective cation channels through PKC dependent or PKC independent pathways (Saleh et al., 2009, Soboloff et al., 2007). The inhibition of various types of \( \text{K}^+ \) channels via PKC in vascular smooth muscle have also been reported (Kubo et al., 1997, Hayabuchi et al., 2001, Park et al., 2006, Jaggar and Nelson, 2000, Fujita et al., 2007, Park et al., 2005a, Rainbow et al., 2009, Rainbow et al., 2006, Park et al., 2007, Standen and Quayle, 1998, Ko et al., 2008, Xiao et al., 2003, Park et al., 2005b). PKC by means of phosphorylation of CPI-17 can cause \( \text{Ca}^{2+} \) sensitization of the contractile machinery (Mueed et al., 2005, Erlinge and Burnstock, 2008); this in turn inhibits myosin light chain phosphatase resulting in the increase in light chain phosphorylation.

Because of the potential role of PKC in the contractile action of UTP in mesenteric arteries I used a selection of peptide as well as non-peptide PKC inhibitors. The UTP induced contractions of mesenteric arterial segments were attenuated significantly by the general PKC inhibitors Gö6983 and chelerythrine. UTP induced contraction was partly inhibited by the cell permeable myristoylated PKC20-28-IP. To confirm these results I used Tat-PKC20-28-IP (synthesized and kindly provided by Dr R.I. Norman) which is actively accumulated in the cell by a, to date, undetermined transport process. The involvement of various PKC isoforms were also investigated using Tat-linked PKC inhibitor peptides. Rat mesenteric arteries express PKC \( \alpha, \beta, \delta, \) and \( \epsilon \) and the particulate levels of these isoforms were increased by ET-1 (Mueed et al., 2005). Tat-linked PKC
inhibitor peptides against the α and ε isoforms differentially inhibit the contractile responses of mesenteric arteries to ET-1 and Ang-II respectively (Rainbow et al., 2009). ET-1 was used as a pharmacological tool for comparison purpose; therefore I have also observed ET-1 induced contractile response of mesenteric artery. The ET-1 response was significantly blocked (~40%, n=4) by Tat linked PKC20-28-IP. However, similar experiments showing a comparatively bigger block of ET-1 induced contraction by Tat-PKCα-IP has been reported previously (Rainbow et al., 2009). Moreover, in my experiments UTP induced contractions were not inhibited by Tat PKCι α, βI, βII or δ isoforms. Since it has been shown that the effects of two vasoconstrictors are mediated by different PKC isoforms, it may be possible that several PKC isoforms, with different effects on the contractile machinery, are activated in response to agonist stimulation.

3.4.5 Are non-selective cation channels involved in UTP induced contractions?

Canonical transient receptor potential protein (TRP) is a family of seven (TRPC1–TRPC7) non-selective cation channels. TRPCs are expressed widely in mammalian tissues and are activated by G protein (Gq/11) coupled receptors. TRPCs are involved in several cardiovascular functions and diseases. The TRPC group can be divided into four subfamilies (TRPC1, TRPC4, 5, TRPC3, 6, 7 and TRPC2) on the basis of sequence homology and functional similarities. The TRPC1 and TRPC6 expression were detected in mesenteric arterial smooth muscle cells and found to be involved in responses of vascular smooth muscle to α1-adrenoceptor (Hill et al., 2006). In cerebral arteries UTP stimulates TRPC3 channels via IP3R activation, leading to membrane depolarization by Na+ influx, opening of voltage-gated Ca2+ channel and vasoconstriction (Zhao et al., 2008). Because of the potential for activation of these non selective cation channels via PLC, IP3 or DAG activation, during UTP induced contraction I investigated the possible
role of these channels in the UTP induced contraction by using the non selective cation channel blocker Gd$^{3+}$. A significant block of non selective cation channels by 10 μM Gd$^{3+}$ prevented the UTP induced vasoconstriction. The trivalent Gd$^{3+}$ also had an inhibitory effect at higher concentrations on the contraction of mesenteric arteries by 60 mM K, which may reflect a blocking effect of Gd$^{3+}$ on voltage gated Ca$^{2+}$ channels. Although Gd$^{3+}$ is a blocker of non selective cation channels, I observed its unspecific blocking effects at 100 μM concentration on 60 K$^+$ induced contractile response of mesenteric artery.

### 3.5 Summary

UTP induced a sustained dose dependent and reproducible contraction of mesenteric arteries. The contraction induced by UTP depends on the extracellular Ca$^{2+}$ influx. UTP induced contraction was abolished by Cd$^{2+}$, however partly inhibited by L-type voltage gated Ca$^{2+}$ channels blocker, diltiazem. L-type voltage gated Ca$^{2+}$ seems to be partly involved in the UTP induced contraction in mesenteric arteries. The non selective cation channels were also studied using Gd$^{3+}$. Nonselective cation channels are involved in Ca$^{2+}$ influx and contraction of mesenteric arteries. The involvement of G$_q$ coupled receptor signaling pathway was studied by blocking one of the signaling molecule i.e. PLC$_{β}$. The inhibition of phospholipase C$_{β}$ blocked the UTP induced contractile response and therefore it can be concluded that UTP activates signaling cascade of G$_q$ coupled receptor pathway leading to the production of IP3 and DAG. The involvement of Protein kinase C was also studied using a range of PKC blockers. It was found that PKC is only partly involved in UTP induced vasoconstriction in mesenteric arteries.
Chapter 4 Investigation of the modulation of ionic currents by UTP
4.1 Introduction

Ion channels play an important role in the regulation of membrane potential and are hence involved in the mechanism of inducing contraction in the vasculature. Potassium ion efflux from smooth muscle cells in response to opening of various potassium channels including Kv, K<sub>ATP</sub>, BK<sub>Ca</sub> and K<sub>ir</sub> channels, hyperpolarize the membrane potential resulting in smooth muscle relaxation. However, inhibition of these potassium channels by vasoconstrictors or modulators ultimately result in contraction of smooth muscle. These potassium channels are in turn activated or inhibited by several mechanisms including phosphorylation by PKA or PKC (Cole et al., 1996, Hayabuchi et al., 2001b, Kubo et al., 1997, Park et al., 2007). In contrast to potassium channels, non-selective cation channels or voltage gated Ca<sup>2+</sup> channels are involved in smooth muscle contraction.

In this chapter the effects of UTP on several ionic currents in rat mesenteric arterial smooth muscle cells were studied using the whole cell patch clamp technique. The currents investigated were Kv, K<sub>ATP</sub>, I<sub>NSCC</sub> and, to a lesser extent, I<sub>Ca</sub>. The involvement of PLC and PKC in the modulation of Kv and K<sub>ATP</sub> current by UTP were studied using the pharmacological inhibitors U73122 (PLC inhibitor) and Tat-linked PKC inhibitory peptides. Data from my contractile experiments inferred that UTP induced contraction also depends on Ca<sup>2+</sup> influx through non selective cation channels. Therefore non selective cation channels were examined on the basis of the functional myography experiments.
4.2 **Kv current and UTP**

4.2.1 **Isolation of Kv current**
Voltage-gated K⁺ currents were recorded from acutely isolated rat mesenteric arterial smooth muscle cells with 140 mM intracellular K⁺ and 6 mM extracellular K⁺. Outward currents were evoked by 400 ms depolarizing pulses ranging from -40 to +60 mV in 10 mV increments from a holding potential of -65 mV. The contribution of other ionic currents, mainly resulting from BK channel activity, was minimized by several approaches. A P/6 leak subtraction protocol was used to remove any linear leak components. BK channel activity was seen at positive potentials despite the relatively low intracellular Ca²⁺ concentration in the pipette (100 nM free Ca²⁺ with 10 mM EGTA). In order to completely remove BK channel activity at more depolarizing voltages 100 nM penitrem A, a selective BK channel blocker, was added to the external solution. Under these experimental conditions the time-course of the currents had the characteristics expected for Kv currents.

The average cell capacitance of the mesenteric arterial smooth muscle cells was 13.9 ± 0.7 pF and the average current recorded from these cells in 6 mM K⁺ control solution was 290.7 ± 52.3 pA at + 60 mV.

4.2.2 **Modulation of Kv current by UTP**
Voltage gated potassium channels are widely expressed in vascular smooth muscle cells including rat mesenteric arteries and it is reported that vasoconstrictors induced contraction is partly due to Kv current inhibition in a variety of tissues (Luykenaar et al., 2004).
**Fig: 4.1 Kv-I protocol**

A) Protocol used for whole cell recording from mesenteric arterial smooth muscle cells. Cells were clamped at -65 mV and currents were evoked by 400 ms voltage pulses from -40 to +60 mV using 10 mV increments. A brief voltage step to -40 mV was applied after each 400 ms voltage step to repolarize the cell. The current was measured at 2 kHz and sampled at 10 kHz; B) Voltage protocol designed to measure the stability of the Kv current over time. The cells were held at -65 mV and 400 ms voltage pulses were applied to +20 mV every 20 seconds; C) Example traces of Kv current recording from mesenteric arterial smooth muscle cells in control. Mesenteric arterial smooth muscle cells with an average membrane capacitance 13.4 ± 0.8 were clamped at -65 mV and currents were recorded as described in protocol (A).

D) Current voltage plot from the cell shown in part (C). The current value for each pulse was taken as the mean current between 320-370 ms. The average Kv current recorded from mesenteric arterial smooth muscle cells in 6 mM K⁺ was 290.7 ± 52.3 pA at +60 mV.
In the present study the effect of UTP on Kv channel activity was studied. Initially, whole-cell Kv currents were recorded under control conditions using the protocol shown in Fig. 4.1. Following this, the cells were exposed to external solution containing 100 µM UTP and Kv currents were recorded after 3, 6, 9 and 12, and occasionally 15, 18 and 21 minutes after UTP application. A comparison of the whole cell current recorded under control conditions and after application of UTP revealed that UTP inhibited the Kv current, especially at more depolarized voltages, as shown in figure 4.2A. To generate a current-voltage (I-V) curve the mean currents over 50 ms towards the end of the 400 ms voltage pulses (320 - 370 ms) were measured. An example of an I-V curve is shown in Fig. 4.1. To compare the inhibition produced by UTP over many cells I-V curves were normalized to the maximum control current at +60 mV. Plotting the normalized I-V curves revealed that 100 µM UTP inhibited Kv currents, especially at more depolarized voltages. As shown in figure 4.3A, the mean Kv current in the presence of UTP at +60 mV was reduced to 59.8 ± 7.3% of the control value (n= 9, ***P< 0.001).

UTP reduced the Kv current further to 55.9 ± 2.3% (n=4) within 12 minutes of addition of UTP as shown by the I-V curve of figure 4.3B. To test whether the reduction in current observed following UTP application was not simply a result of run-down the stability of the Kv current over time was assessed by applying a 400 ms depolarizing step to +20 mV every 20 sec from a holding potential of -65 mV (protocol as shown in the 4.1B). A plot of the current amplitude, normalized to that of the first pulse, against time is shown in figure 4.2B.
Fig: 4.2 modulation of Kv current by UTP

Currents were recorded from mesenteric arterial smooth muscle cells using conventional whole-cell recording with an external [K⁺] of 6 mM and a pipette solution containing a free [Ca²⁺] of 100 nM (using 10 mM EGTA as a chelator) and 140 mM K⁺. Voltage pulses were applied from -40 to +60 mV in 10 mV increments from a holding potential -65 mV. Currents were recorded in the presence of Penitrem A to block BK channels.

A) Example traces of Kv current recordings in control (6 mM K⁺) and after addition of 100 µM UTP.

B) Mean Kv current amplitudes at +20 mV, normalized to the amplitude of the first pulse, plotted against time in control (●, n=6). Pulses were applied every 20 s from a holding potential of -65 mV. The values in the presence of 100µM UTP are also shown (○, n=5) where the current at +20 were measured as part of an I-V protocol given every 3 minutes and are normalized to the corresponding first pulse in control conditions.
**Fig: 4.3 Effects of UTP on Kv current recording.**

Mean current-voltage curves, normalized to the current measured at +60 mV in control conditions (●) and in the presence of 100 µM UTP (○) for 9 minutes (n=9) and 12 minutes (C, n=3). The voltage protocols used to generate these I-V curves were as shown in Fig 4.1A and the current for each pulse was taken as the mean current between 320 and 370 ms. Significant difference from control is expressed as *P<0.05, **P< 0.01 and ***P<0.001.
It can be seen that the Kv current amplitude was quite stable up to 12-15 minutes of recording; however, current rundown was observed from 15 minutes onwards. Figure 4.2B also shows the time course of the reduction in Kv current induced by 100 µM UTP (protocol as in figure 4.1A). As can be seen the Kv current was suppressed within 3 to 9 minutes of UTP application with maximum reduction occurring after 12 to 18 minutes (figure 4.2B). In view of the possible rundown of Kv currents due to dialysis of cellular contents with the passage of time after going to whole cell, unless stated otherwise the effect of UTP on Kv currents were measured after 9 minutes of UTP application.

To study the effect of UTP on Kv channel activation parameters, activation curves were constructed by measuring tail current amplitudes on repolarization to -40 mV after 400 ms voltage pulses (from -40 to +60 mV). Activation curves give information about the relative channel open probability at a given voltage. The tail current after each depolarizing pulse was fitted with an exponential function and the value of this, extrapolated to the beginning of the tail current, was used as the magnitude of the tail current (see Fig.4.4A, figure with the tail current fits). This method of obtaining the amplitude of the tail currents minimizes possible measurement artifacts resulting from current noise and any capacitative transients remaining. The tail current amplitudes thus obtained were normalized with the maximum tail current amplitude (\( I_{tail} / \max I_{tail} \)) and plotted against test pulse potential. The activation plots were fitted with Boltzmann distributions of the form:

\[
\frac{I_{tail}}{\max I_{tail}} = \frac{1}{(1 + \exp (V_{1/2} - V)/k)}
\]  

4.1
Where, $I_{\text{tail}}$ is the magnitude of the tail current; $V$ is the membrane potential; $\max I_{\text{tail}}$ is the magnitude of the maximum tail current; $V_{1/2}$ is the membrane potential required for half maximal activation and $k$ determines the voltage dependence of activation.

Activation plots of the Kv current in control conditions and in the presence of UTP are shown in Fig. 4.4B. The lines are drawn to the Boltzmann equation (4.1) with values for $V_{1/2}$ and $k$ of -14.8 and 9.36 in control and -26.3 and 5.27 in the presence of 100 µM UTP respectively.

Since UTP clearly reduced the Kv current at more depolarized voltages the activation curve in the presence of UTP was re-plotted whereby the tail current amplitudes in the presence of UTP were normalized with the maximum tail current amplitude in control conditions (Fig.4.4A). This plot was fitted with a modification of equation 4.1:

$$\frac{I_{\text{tail}}(\text{UTP})}{\max I_{\text{tail}} \text{ (control)}} = \frac{f}{(1 + \exp (V_{1/2} - V)/k))}$$  \hspace{1cm} 4.2

Where $I_{\text{tail}}(\text{UTP})$ is the magnitude of the tail current in the presence of UTP, $\max I_{\text{tail}} \text{ (control)}$ is the maximum tail current in the absence of UTP and $f$ is the fractional reduction in the tail currents caused by UTP. The control line of Fig.4.4C shows the fit to equation 4.1 where $V_{1/2}$ and $k$ are -15.4 and 8.05 mV respectively, while the UTP line shows the fit to equation (4.2); where $V_{1/2}$, $k$ and $f$ are -26.0, 6.42, and 0.69 respectively.
**Fig: 4.4 Effect of UTP on Kv current activation curve.**

Example traces of Kv currents recorded in control solution to measure the tail current. The tail current after each depolarizing pulse was fitted with an exponential function and the value of this, extrapolated to the beginning of the tail current, was used as the magnitude of the tail current.

Activation curves for the Kv current recorded under control conditions (●) and after 9 minutes in the presence of 100 µM UTP (○). Tail currents were measured as described in the text and in Fig. 4.4 A). Mean tail currents were normalized with the maximum tail current in either control or UTP (B) or with the maximum tail current in control for both control and UTP (C) before averaging. Data are expressed as means ± SEM. In part (B) the lines are drawn to equation (4.1) with \( V_{1/2} \) and \( k = -14.8 \) and 9.36 mV in control and -26.3 and 5.27 mV in UTP; and in part (C) the lines are drawn to equation (4.2) with \( V_{1/2} \), \( k \) and \( f \) being -15.4 mV, 8.05 mV and 1 in control and -26.0 mV, 6.42 mV, and 0.69 in UTP.
4.2.3 Effect of low intracellular Ca$^{2+}$ on Kv current modulation by UTP

Whole cell Kv currents were recorded from single cells held at -65 mV with an intracellular pipette solution containing 0 Ca$^{2+}$ and 10 mM EGTA. Recording protocol was same as in figure 4.1A i.e. currents were recorded by applying 400 ms pulses between -40 to +60 mV ($V_h$ = -65) in control (6 mM K$^+$ solution) and after addition of 100 μM UTP as shown in figure 4.5A. 100 nM penitrem A was also included in the external solution to block currents from BK$\text{Ca}$ channels. Net I-V relationship was plotted in the absence and presence of UTP (after 9 min) by measuring the average current from 320 to 370 ms of the voltage steps (-40 to 60 mV) and normalizing them with maximum control current at +60 mV (figure 4.5B). Current-voltage relationship (I-V curve) revealed that when the pipette solution contained 10 mM EGTA and no added Ca$^{2+}$ UTP was unable to modulate the Kv current (at 60 mV Kv current in response to UTP at 9 minute was 95%).

Figure 4.6A shows examples of Kv currents in control and 9 minutes after the application of 100 μM UTP in a cell where the intracellular free Ca$^{2+}$ was calculated to be 20 nM (1 mM Ca$^{2+}$ and 10 mM EGTA) using the pulse protocol described in figure 4.1A. As can be seen, UTP was less effective at inhibiting the current. Mean normalized I-V curves (figure 4.6B) revealed similar results to those obtained with zero internal Ca$^{2+}$. UTP was unable to suppress the Kv current with low intracellular Ca$^{2+}$ (20 nM free Ca$^{2+}$). Using 20 nM free Ca$^{2+}$ mean normalized current in the presence of UTP was $93.9 \pm 11.2$ % of control at 60 mV.
Fig: 4.5 Effect of zero internal Ca$^{2+}$ on Kv current suppression by UTP

Currents recorded from mesenteric arterial smooth muscle cell using conventional whole-cell recording with an external K$^+$ of 6 mM and a pipette solution containing no added Ca$^{2+}$ and 10 mM EGTA and 140 mM K$^+$. Voltage protocol is same as in figure 4.1A; pulses were applied from -40 to +60 mV with a 10 mV increment to the whole cells held at a holding potential -65 mV. To block BK channel activity penitrem A was included in the external solution. Kv current was recorded in the absence and presence of UTP; A) Trace showing the whole cell current recording in a nominally zero Ca$^{2+}$ internal pipette solution in control (n=3) and after 9 min addition of 100 µM UTP (n=1). For simplicity only 20 mV increments of 400 ms voltage pulses are shown in the trace.

B) Net current voltage relationship (I-V curve) was constructed from mean currents obtained from last 50 ms voltage pulses (i.e. 320 to 370 ms) in control (●) and after 9 min of UTP (○). In each case, currents were normalized with the maximum control current at +60 mV.
Fig: 4.6 Effect of low intracellular Ca$^{2+}$ (20 nM) on Kv current suppression by UTP

(A) Whole cell currents recorded from a cell with an external K$^+$ of 6 mM and a pipette solution containing 20 nM free Ca$^{2+}$ and 140 mM K$^+$. The voltage protocol is same as in figure 4.1A; pulses were applied from -40 to +60 mV with a 10 mV increment to the cells from a holding potential -65 mV (currents are displayed at 20 mV increments). To block BK channel activity penitrem A (100 nM) was included in the external solution. Kv currents were recorded in the absence and presence of UTP as indicated.

B) Mean normalized I-V curves in control (●) and after 9 min of UTP (○). In each case, currents were normalized with the maximum control current at +60 mV.
4.2.4 **Effect of PLC on Kv current modulation by UTP**

To determine the role of PLC in Kv current suppression by UTP, the effect of the PLC inhibitor, U73122, on the ability of UTP to inhibit Kv currents was assessed. Currents were recorded in the absence (control) and presence of 100 µM UTP while cells were dialyzed with a 140 mM K⁺ internal pipette solution containing 5 µM U73122. Because of the unspecific blocking effects of U73122 (10 µM), observed in functional myograph experiments, a slightly lower concentration of this inhibitor was used in these experiments. Furthermore, the potential contamination and toxicity of U73122 in the perfusion system was avoided by including U73122 in the pipette solution. The addition of U73122 was not practically favorable due to its oily nature which would make the surface of the cells slippery and difficult to seal.

Representative traces showing the effect of U73122 on Kv current in control and 9 minutes after UTP (100 µM) application are shown in Fig. 4.7. Inclusion of 5 µM U73122 in the pipette solution reduced the ability of UTP to suppress the Kv current. Normalized I-V curves obtained with U73122 in the pipette in the absence and presence of 100 µM UTP are shown in figure 4.7B. The currents were normalized to that measured at +60 mV in the absence of UTP. At this potential, in the presence of U71322, 100 µM UTP reduced the Kv current to 90 ± 4.2% (n=6, P < 0.01). However, U73122 also inhibited the Kv current as shown in the bar chart (figure 4.7C).
Fig: 4.7 Effect of U73122 on UTP inhibited Kv current

Currents recorded from a cell with an external K\(^+\) of 6 mM and a pipette solution containing 5 \(\mu\)M U73122 (PLC inhibitor) in addition to 100 nM free Ca\(^{2+}\) and 140 mM K\(^+\). Pulses were applied from -40 to +60 mV in 10 mV increments from a holding potential -65 mV. To block BK channel activity penitrem A was included in the external solution. Kv current was recorded in the absence and presence of UTP.

A) Example traces showing the currents recorded at -40 to +60 mV in 20 mV increments in the absence (control) and 9 minutes after the addition of 100 \(\mu\)M UTP as indicated; B) Normalized I-V curves in control (●) and 9 minutes after the application of 100 \(\mu\)M UTP (○). In each case, currents were obtained as the mean current measured from 320 to 370 ms of each pulse and normalized with the maximum control current observed for each cell at +60 mV; C) Currents normalized to their cell capacitance and plotted as a histogram. U73122 also reduced the Kv current.
4.2.5 Does UTP modulate Kv current via PKC?

Vasoconstrictors such as Ang-II and ET-1 constrict smooth muscle by the activation of protein kinase C which in turn regulates the Kv channels (Hayabuchi et al., 2001b, Cole et al., 1996, Ko et al., 2008, Park et al., 2005, Rainbow et al., 2006, Rainbow et al., 2009, Standen and Quayle, 1998, Xiao et al., 2003). To study whether PKC has a role in Kv current modulation by UTP, whole cell currents were recorded from arterial smooth muscle cells pretreated with 100 nM Tat-PKC20-28-IP. The cells, which were initially bathed in 6 mM K+ external solution to settle, were pretreated with 100 nM Tat PKC20-28-IP for at least 10 minutes before Kv current recording. The voltage protocol (400 ms steps to -40 to +60 mV from a holding potential of -65 mV) used to generate the Kv currents was as described earlier 4.2.1 and Fig: 4.1, using an intracellular pipette solution containing 140 mM K+ and 100 nM free Ca2+. The currents were recorded after pretreatment with Tat PKC20-28-IP and 9 minutes following the addition of UTP to the bath (Fig: 4.8).

To examine the role of different PKC isoforms in the mediation of Kv current suppression by UTP, currents were recorded from cells pretreated with either 50 nM Tat PKCe-IP or 100 nM Tat PKCα-IP for at least 10 minutes before the Kv current was recorded (Fig: 4.9A, B & C). The voltage protocol (400 ms steps to -40 to +60 mV from a holding potential of -65 mV) used to activate Kv currents was as described earlier in Fig: 4.1 and using an intracellular pipette solution containing 140 mM K+ and 100 nM free Ca2+. The currents were recorded after pretreatment with Tat PKCe-IP and 9 minutes following the addition of UTP to the bath (Fig: 4.9A & B). Similarly currents were also recorded from cells pretreated with Tat PKCα-IP for at least 10 minutes using the same voltage protocol (Fig: 4.9C).
Whole cell currents recorded from a cell bathed in an external K⁺ of 6 mM containing 100 nM Tat PKC20-28-IP and a pipette solution containing 100 nM free Ca²⁺ and 140 mM K⁺. Pulses were applied in 10 mV increments from -40 to +60 mV from a holding potential -65 mV. To block BK channel activity penitrem A was included in the external solution and Kv currents were recorded in the absence and presence of UTP.

A) Example traces showing the currents recorded at -40 to +60 mV in 20 mV increments in the absence (control) and 9 minutes after the addition of 100 µM UTP as indicated in cells pretreated with 100 nM Tat PKC20-28-IP.

B) Normalized I-V curves in control (●, cells pretreated with Tat-PKC20-28-IP) and 9 minutes after the application of 100 µM UTP (○). In each case, currents were obtained as the mean current measured from 320 to 370 ms of each pulse and normalized with the maximum control current observed for each cell at +60 mV. Data are the mean ± SEM from 7 cells.

Fig: 4.8 Effect of Tat PKC-20-28-IP on UTP modulated Kv current
Fig: 4.9 Effect of Tat PKC-IP isoforms on UTP modulated Kv current.

Whole cell currents recorded from single cells pretreated with Tat PKC-IP for <10 min in the absence and presence of UTP (after 9 min). Voltage protocol is same as in figure 4.1A. For plotting I-V curve, mean currents obtained from 320 to 370 ms voltage pulses from -40 to 60 mV in Tat PKC-IP (●) and after 9 min perfusion of UTP (○). In each case, currents were normalized with the maximum control current at +60 mV.

A) Representative traces showing the effect of 50 nM Tat PKCε-IP on Kv currents in the absence and presence of 100 µM UTP (after 9 min);
B) Net I-V curve of Tat PKC-ε-IP (●) and after 9 min perfusion of UTP (○) (n=1);
C) Net I-V plot Tat PKCα-IP (●) and after 9 min perfusion of UTP (○). Data are the mean from 2 cells.
4.2.6 $\text{K}_{\text{ATP}}$ current and UTP

$\text{K}_{\text{ATP}}$ channels are widely distributed among smooth muscle cells and play an important role in vasodilation and regulation of vascular tone. Many vasodilators activate $\text{K}_{\text{ATP}}$ channels whereas inhibition of $\text{K}_{\text{ATP}}$ channels by vasoconstrictors causes constriction of the vasculature. The effect of UTP on $\text{K}_{\text{ATP}}$ channels was investigated using whole-cell recording of steady-state $\text{K}_{\text{ATP}}$ current.

$\text{K}_{\text{ATP}}$ channel activity depends on the metabolic state of the cell and is generally increased by a fall in the intracellular concentration of ATP and a rise in ADP (Beech et al., 1993, Quayle et al., 1995, Nelson and Quayle, 1995). Therefore, to record $\text{K}_{\text{ATP}}$ current from mesenteric arterial smooth muscle an intracellular pipette solution containing suitable proportions of intracellular metabolites was used. The pipette solution contained (mM): 110 KCl, 30 KOH, 1 MgCl$_2$, $^{*}$3.9 CaCl$_2$, 10 EGTA, 10 HEPES, $^\dagger$1 Na$_2$ATP, $^\ddagger$0.5 GTP, $^\ddagger$0.1 ADP ($^*$free Ca$^{2+}$ was 100 nM; $^\dagger$added on the day of experiment and pH was adjusted to 7.2). Recordings were made at 29 to 30$^\circ$C.

Steady-state $\text{K}_{\text{ATP}}$ currents were recorded at -60 mV with symmetrical 140 mM K$^+$ containing solutions, with 100 nM free Ca$^{2+}$ in the pipette. These conditions minimized contamination by voltage-gated currents whilst giving a large, inwardly directed, electrochemical gradient for K$^+$. Initially, sealing was done in 6 mM K$^+$ external solution which was exchanged to 140 mM K$^+$ solution soon after a whole-cell configuration was achieved. When the external K$^+$ concentration was raised from 6 to 140 mM there was an increase in the inward current that reflected the increased driving force for K$^+$ and the basal activity of $\text{K}_{\text{ATP}}$ channels. Pinacidil (10 μM), a synthetic activator of $\text{K}_{\text{ATP}}$ channels, was applied to increase the $\text{K}_{\text{ATP}}$ current. Pinacidil activated current (e.g. 258 pA in figure 4.10) was quite stable for up to 10 minutes and thereafter...
declined slowly. Glibenclamide, a sulphonylurea antidiabetic drug, is often used to isolate and characterize $K_{ATP}$ currents because of its ability to selectively inhibit $K_{ATP}$ channels. In my experiments I added 10 μM glibenclamide to check that the current observed was the $K_{ATP}$ current. Currents were measured after subtracting the baseline current observed in glibenclamide. Currents were normalized with the maximum pinacidil activated current.

Fig: 4.10 Pinacidil activated $K_{ATP}$ current.
Whole cell currents recorded from acutely isolated mesenteric arterial smooth muscle cell voltage clamped at -60 mV with 140 mM internal $K^+$. The extracellular $K^+$ was raised from 6 to 140 mM at the point indicated by the arrow. Pinacidil (10 μM) and glibenclamide (10 μM) were applied as indicated by the bars. Pinacidil activated an inward current which was reversed by the addition glibenclamide. The bars indicate the estimated time the drugs reach the bath after subtracting the dead space. The top dashed line (blue) indicates the zero current level and the bottom dashed line indicates the pinacidil activated current.
4.3  **Modulation of $K_{ATP}$ current by UTP**

The effect of UTP on the $K_{ATP}$ current was assessed at -60 mV in symmetrical 140 mM $K^+$ with 100 nM free $Ca^{2+}$ in the pipette; under these conditions ($E_K = 0$) the $K_{ATP}$ current is inward. $K_{ATP}$ channels were activated with 10 µM pinacidil, as describe in section 4.3.6 above and 100 µM UTP was then applied. As shown in figure 4.11, pinacidil (10 µM) caused a significant activation of the $K_{ATP}$ current, which reached a plateau (230 pA in the example traces shown in figure: 4.11A) within 30 - 60 seconds of application. The average current density at -60 mV in the presence of pinacidil was $204 \pm 37$ pA ($n=7$). Once the $K_{ATP}$ current had remained stable for about 1 minute 100 µM UTP was added. UTP caused a substantial inhibition of the pinacidil activated current and application of 10 µM glibenclamide reduced the remaining current further (Fig. 4.11A). To quantify these effects the baseline current in glibenclamide was subtracted and all currents were then normalized to the maximum pinacidil induced current. Mean normalized currents are plotted in figure 4.11B. Pinacidil significantly activated the $K_{ATP}$ current as expected, whereas UTP significantly reduced the $K_{ATP}$ current to $15 \pm 7\%$ of the pinacidil value ($n=8; **P<0.0001$).

4.3.1  **Effect of $Ca^{2+}$ on $K_{ATP}$ current modulation by UTP**

$K_{ATP}$ currents were recorded as described in section 4.2.6 and 4.3 with a slight change in the pipette solution. The free $[Ca^{2+}]_i$ was lowered from 100 nM to 20 nM (1mM $Ca^{2+}$ and 10 mM EGTA). As shown in figure 4.12, the activation of the $K_{ATP}$ current by pinacidil was relatively slow with 20 nM internal $Ca^{2+}$ compared to that with 100 nM $Ca^{2+}$ in the pipette solution. The effect of 100 µM UTP was much less pronounced, reducing the pinacidil activated current to $77 \pm 2.9\%$ ($n=4$; not significant). In low
internal $\text{Ca}^{2+}$, the effect of UTP was quite slow and maximum inhibition of the $K_{\text{ATP}}$ current was only obtained several minutes after UTP application.
Fig: 4.11 $K_{ATP}$ current modulation by UTP

UTP inhibits pinacidil (10 μM) activated $K_{ATP}$ currents in rat mesenteric arterial smooth muscle cells. Currents were recorded at -60 mV with a pipette solution containing 100 nM free Ca$^{2+}$; A) An example trace showing the pinacidil activation of glibenclamide sensitive $K^+$ currents in a mesenteric arterial smooth muscle cell. External 6 mM K$^+$ was exchanged for 140 mM K$^+$ at the time indicated by the arrow; all other drug applications are indicated by the bars. All currents were measured from the baseline current, taken as that in the presence of glibenclamide as indicated by the dashed horizontal red line. B) Currents normalized to those in the presence of pinacidil and plotted as a histogram. The fractional pinacidil induced current remaining in UTP was 0.15 ± 0.07 (n=8; ***P<0.0001).
Fig: 4.12 $K_{ATP}$ current modulation by UTP in low internal Ca$^{2+}$ solution

Currents were recorded at -60 mV with a pipette solution containing 20 nM free Ca$^{2+}$.

A) An example trace showing the $K_{ATP}$ current recording from mesenteric arterial smooth muscle cell. External 6 mM K$^+$ was exchanged for 140 mM K$^+$ at the time indicated by the arrow; all other drug applications are indicated by the bars. The dashed line shows the zero current level. All currents were measured from the baseline current measured in glibenclamide. B) Currents normalized to those in the presence of pinacidil and plotted as a histogram. The fractional amount of the pinacidil induced current remaining in 100 µM UTP was 0.77 ± 0.29 ($n= 4$). The effect of UTP was not significant.
4.3.2 Does UTP modulate $K_{ATP}$ current via PKC

To evaluate the role of PKC in $K_{ATP}$ current modulation by UTP, mesenteric arterial smooth muscle cells were pretreated in 100 nM Tat PKC20-28-IP for more than 10 minutes. Figure 4.13A shows an example of a current recorded from a cell pretreated with Tat PKC20-28-IP (100 nM) at a holding potential of -60 mV and a pipette solution containing 100 nM free Ca$^{2+}$. After switching to an external 140 mM K$^+$ solution, pinacidil (10 µM) was added to activate the $K_{ATP}$ current further. It can be seen that addition of 100 µM UTP was still able to suppress the $K_{ATP}$ current and the remaining current was inhibited further by 10 µM glibenclamide. As described previously, the currents in response to various drugs were measured and the basal current (with glibenclamide) was subtracted. These values are plotted as a fraction of the current in pinacidil current. As shown in figure 4.13B, there was no significant difference in UTP inhibition of $K_{ATP}$ current between Tat-PKC20-28-IP treated and untreated cells (n=8 in each case).

To test whether the effect of Tat PKC20-28-IP on the inhibition of $K_{ATP}$ current by UTP was dependent on intracellular [Ca$^{2+}$] a similar set of experiments were done with a lower free [Ca$^{2+}$] (20 nM) in the pipette. Although cells were pretreated with Tat-PKC20-28-IP, it was also included in the solutions during recording. As shown in figure 4.14, the inhibition of the $K_{ATP}$ current by UTP was not significant in 20 nM Ca$^{2+}$ and inclusion of Tat-PKC20-28-IP did not alter the effect of UTP.
**100 nM free Ca\(^{2+}\)**

A) 

Tat PKC20-28-IP

![Graph showing K\(_{\text{ATP}}\) current with Tat PKC20-28-IP](image)

B) 

Tat PKC20-28-IP (100 nM)

![Bar graph showing normalized current](image)

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**Fig: 4.13 Effect of Tat PKC20-28-IP on UTP modulated K\(_{\text{ATP}}\) current.**

A) Example traces showing K\(_{\text{ATP}}\) current recorded from a cell pre-treated with Tat PKC20-28-IP (100 nM) for more than 10 minutes. The pipette solution contained 100 nM free Ca\(^{2+}\). All drugs were applied as indicated by the bars and the arrow indicates the exchange of 6 mM K\(^+\) with 140 mM K\(^+\). B) Histogram of the currents normalized to the maximum current in pinacidil in response to various drugs in the absence and after pre-treatment with Tat PKC20-28-IP (n = 8 in each case). No significant difference was observed in the suppression of K\(_{\text{ATP}}\) current by UTP before and after treatment of Tat PKC20-28-IP.
Fig: 4.14 Effect of Tat PKC20-28-IP on UTP inhibited $\text{K}_{\text{ATP}}$ current.

A) Example traces showing $\text{K}_{\text{ATP}}$ current recorded from a cell pre-treated with Tat PKC20-28-IP (100 nM) for more than 10 minutes. The pipette solution contained 20 nM free $\text{Ca}^{2+}$. All drugs were applied as indicated by the bars and the arrow indicates the exchange of 6 mM $\text{K}^+$ with 140 mM $\text{K}^+$. The holding potential was -60 mV.

B) Histogram showing the effect of Tat PKC20-28-IP (n=3) on UTP inhibited $\text{K}_{\text{ATP}}$ current in the absence (n=5) and presence of Tat PKC20-28-IP. There was no significant difference between Tat PKC20-28-IP treated (n=3) and untreated cells (n=4).
4.4 Nonselective cation current evoked by UTP

The isometric contraction of mesenteric arteries in response to UTP was effectively blocked by the trivalent cation Gd$^{3+}$ (see section 3.3.7), which is a widely used blocker of non-selective cation channels (Hill et al., 2006). Whole-cell recordings were made to evaluate whether UTP activated non-selective cation channels in isolated mesenteric artery smooth muscle cells.

Whole cell steady state currents were recorded from mesenteric arterial smooth muscle cells voltage clamped at -65 mV with an external [K$^+$] of 6 mM and a pipette solution containing 140 mM Cs$^+$ instead of K$^+$ and a free [Ca$^{2+}$] of 100 nM. Cs$^+$ was used internally because it will minimize K$^+$ currents yet is able to pass through the non-selective channel (Hill et al., 2006). As shown in Fig. 4.15, at a holding potential of -65 mV UTP (100 μM) induced a noisy inward current that was blocked on addition of Gd$^{3+}$ (10 μM, Fig 4.15A & B).

To evaluate the characteristics of this inward current further, 400 ms ramps from -100 to +30 mV and back were applied at regular intervals from a holding potential of -60 mV (figure 4.16A). The cells were bathed in 6 mM K$^+$ for control recordings and 100 μM UTP was applied after several control ramps were recorded. The UTP induced current was obtained by subtracting the mean ramp current in the absence of UTP from that in its presence, as shown in Fig.4.16. The current was normalized to cell capacitance and the resulting I-V relationship was that of the UTP induced current. The current reversed around 0 mV and showed inwardly rectifying properties similar to that reported previously for this type of current (Hill et al., 2006, Helliwell and Large, 1997, Alvarez et al., 2008).
The effects of gadolinium on ion channels other than non selective cation channels were also studied to ascertain the blocking effects of gadolinium to Kv and K\textsubscript{ATP} channels. To study the effects of gadolinium on K\textsubscript{ATP} channels steady state currents were recorded from mesenteric arterial smooth muscle cell at -65 mV using 20 nM free Ca\textsuperscript{2+} in the pipette solution. Penitrem A was also included in the external solution to block BK channels. As described before, when measuring K\textsubscript{ATP} current pipette sealing was done in 6 mM [K\textsuperscript{+}] and this was raised to 140 mM [K\textsuperscript{+}] once the whole-cell configuration was established. Following activation of K\textsubscript{ATP} currents by 10 µM pinacidil application of 10 µM Gd\textsuperscript{3+} significantly inhibited this pinacidil activated current to 62 ± 7% of control (n = 3; *P< 0.05) as shown in Fig. 4.17.

The effect of Gd\textsuperscript{3+} on the Kv current in these cells was also assessed. Kv currents were induced by 400 ms depolarizing steps from -40 to +60 mV in 10 mV increments from a holding potential of -65 mV. Kv currents were recorded in control (6 mM K\textsuperscript{+}) and in the presence of 10 µM Gd\textsuperscript{3+} (Fig 4.18A). Mean currents measured from -60 to +40 mV were normalized to their cell capacitance and plotted to give an I-V relation. A comparison of the I-V relations revealed no significant difference in control and in presence of Gd\textsuperscript{3+}. 

154
Fig: 4.15 Effect of UTP on nonselective cation channels

Mesenteric arterial smooth muscle cells were clamped at -65 mV and whole-cell recordings were obtained with an external [K⁺] of 6 mM and a pipette solution containing 140 mM Cs⁺ instead of K⁺ and a free [Ca²⁺] of 100 nM (using 10 mM EGTA as a chelator). Penitrem A was added to the external bath solution to prevent activation of BK channels. A) Steady-state current recorded at -65 mV; application of UTP (100 µM) induced an inward current that was reversed by Gd³⁺ (10 µM); B) Another trace showing an inward current induced by UTP (100 µM) that was reversed by Gd³⁺ (10 µM); C) Trace showing repeated applications of UTP induced inward currents.
**Fig: 4.16 UTP induced non selective cation current**

a) Whole-cell currents elicited in response to voltage ramps from -100 to +30 mV and back as indicated by the protocol (top). Control currents and those in the presence of 100 µM UTP are shown in A and B respectively. The current activated by UTP was given by B-A as indicated.

b) Net I-V relationship of the mean UTP activated current from two cells obtained by averaging the current produced by 0.4 s ramps from -100 to +30 mV after subtracting the mean ramp current in the absence of UTP from that obtained during the UTP induced current.
Fig: 4.17 Effect of gadolinium on \( K_{\text{ATP}} \) channels

The effect of gadolinium on \( K_{\text{ATP}} \) current was recorded from mesenteric arterial smooth muscle cells held at -65 mV; A) Whole cell steady state currents were recorded from cell with a pipette solution containing 140 mM K\(^+\) and a free [Ca\(^{2+}\)] of 20 nM. Penitrem A was added to the external bath solution to prevent activation of BK channels. The current was recorded with 6 mM K followed by addition of 140 mM K as indicated by the arrow. Gadolinium (10 μM) reduced the pinacidil activated current and glibenclamide reversed the residual current to baseline. The glibenclamide sensitive current was taken as the baseline current and all the currents were measured from this baseline and normalized to the pinacidil activated current; B) Histograms showing the effect of gadolinium (10 μM) on the current in the presence of pinacidil. The mean fractional current remaining in 10 μM gadolinium was 0.62 ± 0.07 (n = 3; *P< 0.05).
Fig: 4.18 Effect of gadolinium on Kv currents.

A) Kv currents recorded from a single cell held at -65 mV in control and after addition of gadolinium (10 μM). Currents were recorded from by applying 400 ms voltage pulses between -40 to +60 mV. The external solution contained 6 mM K⁺ and the pipette solution contained 100 nM free Ca²⁺. Penitrem A was included in the external solution to inhibit BK currents.

B) Mean I-V curves were constructed from currents measured between 320 and 370 ms of each pulse and normalized to cell capacitance (n = 3).
4.5 **Isolation of voltage gated Ca$^{2+}$ current**

An attempt was made to measure voltage gated calcium currents from single smooth muscle cells enzymatically isolated as described in the methods chapter. The smooth muscle cells were voltage clamped at -60 mV and recordings were done in external solution containing 10 BaCl$_2$ and internal solution containing 140 mM Cs$^+$ and 100 nM free Ca$^{2+}$. The internal Cs$^+$ virtually abolished all outward K$^+$ currents and using Ba$^{2+}$ as a charge carrier maximized any currents flowing through Ca$^{2+}$ channels.

Whole cell currents were recorded from cells by applying 150 ms voltage pulses from -40 to +50 mV from a holding potential -65 mV. Most cells investigated had no detectable Ba$^{2+}$ current and in the few cells where I observed a current it was very small (see Fig. 4.19). For this reason I did not pursue this any further.
Fig: 4.19 Voltage gated Ca\(^{2+}\) channel activation.

A) Trace showing whole cell Ba\(^{2+}\) currents recorded from a mesenteric arterial smooth muscle cell. Depolarizing voltage steps were given from -40 to +50 mV from a holding potential of -65 mV.

B) Plot of the I-V relationship for the cell shown in part (A). A maximum inward current of -30 pA was obtained at +20 mV.
4.6 Discussion

4.6.1 Kv current and effect of UTP

Whole cell voltage gated potassium (Kv) currents recorded from mesenteric arterial smooth muscle cells were suppressed by UTP at depolarizing voltages. The outward potassium current in control recordings was quite stable over time with a slow current rundown occurring after 12-15 minutes. Because of the presence of a BK channel blocker there was no-contamination of the Kv currents with BK channel activity so the resulting current modulated by UTP under these conditions was the Kv current only. In rat cerebral arteries, Luykenaar reported a similar inhibition of smooth muscle Kv currents by UTP (Luykenaar et al., 2004). The amount of inhibition of the Kv current by UTP was reduced by the PLC blocker, U73122, suggesting a role for PLC activation, although it must be stressed that U73122 also unexpectedly reduced the contractile response to 60 mM K⁺ observed in myography experiments. I also observed that U73122 inhibited the Kv current itself to some extent.

It was an interesting observation that in the virtual absence of free Ca²⁺ in the pipette (10 mM EGTA, no added Ca²⁺) UTP was unable to inhibit the Kv current. A similarly small reduction in the Kv current by UTP persisted when the free Ca²⁺ concentration in the pipette solution was increased to 20 nM. However, raising free [Ca²⁺] in the pipette to 100 nM resulted in pronounced inhibition of Kv currents by UTP. This Ca²⁺ dependence of modulation of Kv channels by UTP indicates that UTP is likely to be more effective at inhibiting K⁺ currents when the intracellular [Ca²⁺] is raised, such as during a high state of myogenic tone.
Kv channels contribute to the regulation of membrane potential in several arterial beds as shown by an increase in vessel tone following application of Kv channel blockers such as 4-AP (Cole et al., 1996, Prior et al., 1998, Shimoda et al., 1998, Cox, 2005). These channels open as a result of depolarization and their role could be to limit the extent of depolarization thereby limiting contraction. Many vasoconstrictors such as Ang-II and ET-1 have been shown to inhibit potassium channels, including Kv channels, via PKC dependent mechanisms (Rainbow et al., 2009, Xiao et al., 2003). However, UTP induced contraction was partially dependent on the inhibition of Kv current via a PKC independent Rho kinase pathway in rat cerebral arteries (Luykenaar et al., 2004). Luykenaar et al (2004) demonstrated that the PKC inhibitors calphostin C and bisindolylmaleimide I, failed to inhibit the Kv current modulation by UTP whereas a Rho kinase inhibitor (Y-27632) inhibited this modulation in rat cerebral arterial smooth muscle cells. They, therefore, argued that instead of PKC the small GTP RhoA / Rhokinase is involved in UTP modulation of Kv current in rat cerebral arteries.

In this study the role of PKC in mediating the inhibition of mesenteric artery Kv currents by UTP was investigated using several cell permeable Tat-linked PKC inhibitor peptides. These Tat linked peptide inhibitors are more selective and isoform specific, and due to their linkage with Tat peptide 47-57 possessed the ability to cross the hydrophobic lipid bilayer. Within the cell the internal reducing environment causes the cleavage of the bonding between the Tat peptide and its cargo PKC inhibitor protein. Our lab demonstrated the effectiveness of these Tat linked PKC peptide inhibitors at distinguishing between the PKC isoforms involved in the modulation of Kv current by ET-1 and Ang-II (Rainbow et al., 2009). Pretreatment of cells with Tat linked PKC peptide inhibitors for more that 10 minutes was shown to be sufficient to enable
effective accumulation of PKC inhibitor peptides within the cells (Rainbow et al., 2009). Based on the observation that UTP inhibition of Kv current appeared unaffected by these PKC inhibitors it is unlikely that PKC has a major role in the modulation of the Kv current by UTP in mesenteric artery smooth muscle cells. Hence my results in rat mesenteric arterial smooth muscle agree with those reported by Luykenaar et al (2004) that UTP modulation of Kv currents depends on a pathway other than PKC, possibly a Rho-kinase pathway and this needs to be explored further in mesenteric arteries.

4.6.2 $K_{\text{ATP}}$ current and UTP

UTP also inhibited the pinacidil activated $K_{\text{ATP}}$ current in rat mesenteric smooth muscle cells. The inhibition of the $K_{\text{ATP}}$ current was dependent on free [Ca$^{2+}$] in the pipette solution. With 100 nM free Ca$^{2+}$ the UTP reduced the current to $15 \pm 7\%$ of the control value, but with only 20 nM Ca$^{2+}$ in the pipette the reduction was only to $77 \pm 2.9\%$ of the control and was not significant.

As is the case with vascular Kv channels, $K_{\text{ATP}}$ channels are also inhibited by several vasoconstrictors in a PKC dependent manner (Hayabuchi et al., 2001a, Kubo et al., 1997, Standen and Quayle, 1998, Moon et al., 2004, Ko et al., 2008). However, as was the case with the Kv current inhibition by UTP, the inhibition of the $K_{\text{ATP}}$ current by UTP was also insensitive to PKC inhibition by the Tat-linked PKC20-28-IP, either in 100nM or in 20 nM free internal Ca$^{2+}$. It should be noted that these Tat-linked peptide inhibitors are effective at blocking the ET-1 and Ang II induced reduction of both Kv and $K_{\text{ATP}}$ currents in this preparation (Rainbow et al., 2009).

Like the Kv inhibition, $K_{\text{ATP}}$ channel modulation by UTP was also dependent on intracellular Ca$^{2+}$ which suggests that UTP is more likely to inhibit the potassium
channels under conditions where the concentration of Ca\(^{2+}\) is high. It is likely that a Ca\(^{2+}\) dependent signaling pathway is involved in UTP modulation of both Kv and K\(_{\text{ATP}}\) channels. However, inhibition of Ca\(^{2+}\) dependent PKC isoforms were generally ineffective, thus it could be proposed that Ca\(^{2+}\) dependent RhoKinase signaling might be involved.

4.6.3 NSCC current and UTP

Since Gd\(^{3+}\), a known blocker of non-selective channels (Hill et al., 2006), was effective at blocking the contraction induced by UTP the ability of UTP to activate non-selective cation current was investigated. In these experiments K\(^{+}\) was replaced by Cs\(^{+}\) which would block K\(^{+}\) currents but permeate non-selective channels (Albert et al., 2003, Hill et al., 2006). As shown in the results section, UTP did induce a current with properties similar to those reported previously for a non-selective current (Alvarez et al., 2008). The I-V relation of the current, obtained using voltage ramps, reversed close to 0 mV and displayed a fairly marked inward rectification (Fig.4.16) and steady-state recordings of the current at -60 mV were blocked by the application of Gd\(^{3+}\). The activation of this current, however, was quite variable and often transient.

The blocking effects of Gd\(^{3+}\) on Kv and K\(_{\text{ATP}}\) currents were also studied and although Gd\(^{3+}\) had no significant effect on Kv currents it did inhibit K\(_{\text{ATP}}\) current by nearly 40%.

4.6.4 Voltage gated Ca\(^{2+}\) current

These channels respond to membrane depolarization and mediate calcium influx resulting in an inward current. Voltage gated calcium channels play important roles in several intracellular processes including smooth muscle contraction. I attempted to investigate the effects of UTP on these channels but I found that the ability to observe
Ca\textsuperscript{2+} channel currents, even with 10 mM Ba\textsuperscript{2+} as the charge carrier, was too unreliable. In my preliminary experiments, I did manage to record whole cell inward currents Ba\textsuperscript{2+} currents but was unable to test the effect of UTP on these currents.
Chapter 5 Overall Discussion
5.1 Discussion

The work presented in this thesis was focused on investigating the possible mechanisms leading to UTP induced contraction in rat mesenteric arteries. UTP is not only a building block of cellular RNA but also acts as an extracellular signaling molecule and plays an important role in conditions such as migraine and hypertension (Lazarowski and Boucher, 2001). Extracellular UTP triggers a signaling cascade by activating membrane bound P2Y receptors that are members of the G protein coupled receptor family. UTP, ATP and ADP are secreted from platelets, neuronal and endothelial cells (Daniel et al., 1998). In the cardiovascular system, UTP is secreted from endothelial cells during ischemia and hypoxia (Erlinge et al., 2005).

In the vasculature UTP is released to the luminal side of the blood vessel and has little or limited interaction with the intact smooth muscle cells that are wrapped in the layers of the walls of the vessel. However, as a result of inflammation or disruption of the endothelial cells, UTP has access to the smooth muscle cells and thereby causes vasoconstriction (Boarder and Hourani, 1998). Because of the role of UTP in subarachnoid hemorrhage and migraine (Lazarowski and Boucher, 2001) many research groups have focused their attention on understanding the mechanisms whereby UTP induces contraction of cerebral arteries (Jaggar and Nelson, 2000, Lacza et al., 2001, Marrelli et al., 1999, Ralevic and Burnstock, 1991, Schilling et al., 1995, Sugihara et al., 2011, Syyong et al., 2009, Zhao et al., 2008). However, less work has been done investigating the contraction produced by UTP in other vascular beds. The research I have presented in this thesis was focused on understanding the range of possible
mechanisms leading to the UTP induced contraction of rat mesenteric arterial smooth muscle.

5.1.1 UTP induced contraction depends largely on extracellular Ca\(^{2+}\) influx

UTP is a ligand for P2Y receptors and in smooth muscle these are likely to be P2Y\(_2\) and possibly P2Y\(_4\) and P2Y\(_6\) receptors (Bultmann et al., 1999, Buvinic et al., 2002, Chootip et al., 2005, Gitterman and Evans, 2000, Harper et al., 1998, Hill and Sturek, 2002, Kumari et al., 2003, Morris et al., 2010, Erlinge et al., 2005). However, a recent report suggests the possibility of a dual signaling pathway leading to vasoconstriction by UTP in rat aorta, where UTP activates P2X\(_1\)-like receptors in addition to P2Y receptors (Sugihara et al., 2011). If the P2Y receptors are located on the endothelial cells, nucleotides causes a relaxation of the blood vessels, otherwise, if they are located on smooth muscles then contraction occurs (Miyagi et al., 1996).

In my experiments I found UTP to be a potent vasoconstrictor that induced a sustained and concentration-dependent contraction of mesenteric arteries. The vasoconstriction induced by 100 µM UTP was reproducible even after several applications.

UTP induced contraction was abolished in the absence of extracellular Ca\(^{2+}\), however, contraction was restored upon returning Ca\(^{2+}\) to the bath solution. As expected, the contraction evoked by 60 mM K\(^+\), which occurs because of membrane depolarization, was also completely abolished in the absence of Ca\(^{2+}\). UTP induced contraction that is largely due to Ca\(^{2+}\) influx has been reported previously in mesenteric arteries (Juul et al., 1992).
5.1.2 Voltage gated Ca\(^{2+}\) channels are partly involved in UTP induced contraction

Agonist induced contraction often depends on depolarization of the membrane potential and Ca\(^{2+}\) influx from the extracellular space either through voltage gated Ca\(^{2+}\) channels or non-selective cation channels (Horowitz et al., 1996, Khalil and van Breemen, 1988, Le et al., 1993). Some vasoconstrictors, including noradrenaline, have the ability to increase the open probability of voltage gated Ca\(^{2+}\) channels (Horowitz et al., 1996). Noradrenaline activates L-type current only in rabbit mesenteric arterial smooth muscle cells and was unable to modulate the T-type Ca\(^{2+}\) current (Helliwell and Large, 1997).

To examine possible Ca\(^{2+}\) influx pathways contributing to UTP induced contraction I focused my research on investigating the roles of L-type Ca\(^{2+}\) channels and non-selective cation channels in this response. Electrophysiological studies on smooth muscle cells have revealed two major components to the Ca\(^{2+}\) current, namely L- and T-type Ca\(^{2+}\) channels (Horowitz et al., 1996). Currents flowing through these channels can be distinguished by their activation and inactivation profiles. The T-type current activated differentially than L-type currents and activated as well as inactivated at more negative potentials (Horowitz et al., 1996) (Benham et al., 1987). Both L- and T-type channels are blocked by cadmium, however, the L-type Ca\(^{2+}\) channel is much more sensitive to dihydropyridines, bezothiazepines, and phenylalkylamines (Bean et al., 1986, Loirand et al., 1986, Benham et al., 1987). In my experiments cadmium effectively blocked the UTP induced contraction by 81%; whereas application of the diltiazem (benzothiazepine) reduced the contraction by 41% only. However, Diltiazem significantly blocked the 60 mM K\(^{+}\) induced contraction within same experimental conditions. Despite the differential results it appears that UTP induced contraction partly depends voltage gated Ca\(^{2+}\) channels. Similar results have been reported for rat
cerebral arteries where UTP induced contraction is only partly dependent on depolarization and activation of voltage gated Ca\(^{2+}\) channels (Luykenaar et al., 2004).

### 5.1.3 UTP mediates contraction via PLC activation

The phospholipase C signaling pathway is often the initial trigger of a vasoconstrictor induced contraction in vascular smooth muscle (Clarke et al., 2008b) (Delarue et al., 2005, Montmayeur et al., 2011). UTP activates P2Y receptors and possibly the P2Y\(_2\) receptor in mesenteric arteries which is a Gq linked receptor that activates PLC (Iwamuro et al., 1999) with the subsequent formation of IP\(_3\) and DAG from PIP\(_2\). UTP has been shown to increase the concentration of intracellular Ca\(^{2+}\) and IP\(_3\) levels in mesenteric artery smooth muscle cells (Nelson et al., 2008, Morris et al., 2010).

Phospholipase C (PLC) is a family of 11 isoenzymes and in vascular smooth muscle \(\beta_1\), \(\beta_2\), \(\delta_1\), and \(\gamma_1\) have been indentified (Hyun et al., 2002). To ascertain whether UTP activates PLC in mesenteric arteries I examined the effect that pharmacologically inhibiting PLC had on the UTP induced contraction. The blocker I used was U73122 which has been used frequently to dissect out the role of PLC in agonist stimulated signaling (Peppiatt-Wildman et al., 2007). Both UTP and 60 mM K\(^+\) induced responses were significantly blocked after pre-incubating mesenteric arteries with 10 \(\mu\)M U73122. The block of the UTP response is in agreement with the hypothesis that UTP activates PLC via activation of a Gq linked receptor; however, the inhibition of the 60 mM K\(^+\) induced contraction by U73122 was unexpected and suggests the possibility that U73122 has effects additional to blocking PLC.

Agonist stimulated Ca\(^{2+}\) release from internal stores following IP\(_3\) formation is often characterized by Ca\(^{2+}\) waves and Ca\(^{2+}\) oscillations (Berridge, 2008). Jaggar et al
investigated UTP induced contraction and its relationship with Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves and reported that UTP increases the frequency of calcium waves in rat basilar arteries whereas it inhibits Ca\(^{2+}\) spark frequency in the same tissue (Jaggar and Nelson, 2000).

5.1.4 **Is PKC involved in generating the UTP induced contraction?**

Protein kinase C, a family of related isoenzymes, plays an important role in agonist induced contraction by various mechanisms including increasing the sensitivity of the myofilaments to intracellular Ca\(^{2+}\) (Khalil, 2010, Khalil and van Breemen, 1988, Salamanca and Khalil, 2005) and inhibiting K\(^+\) channels leading to depolarization (Khalil, 2010, Khalil and van Breemen, 1988). PKC catalyzes the phosphorylation of the regulatory protein CPI-17 to increase the sensitivity of the contractile filaments by suppressing the activity of MLC phosphatase and increases vascular smooth muscle contraction (Kanashiro and Khalil, 1998, Khalil, 2010, Khalil and van Breemen, 1988). Activation of PKC\(\alpha\) is reported to phosphorylate a regulatory protein, calponin, that is a thin filament protein associated with actin and is involved in the regulation of smooth muscle contraction (Kaneko et al., 2000, Nagumo et al., 1994, Parker et al., 1994, Winder et al., 1998, Winder and Walsh, 1993, Winder et al., 1993). PKC mediated phosphorylation of the calponin could also enhance vascular smooth muscle contraction (Bi et al., 2005, Patil et al., 2004, Sohn et al., 2001, Somara and Bitar, 2008, Tani and Matsumoto, 2004, Woodsome et al., 2001).

Because of the potential role of PKC in UTP stimulated contraction, I studied UTP induced contraction in rat mesenteric arteries using a range of PKC inhibitors. Unfortunately, the results obtained with different PKC inhibitors were very mixed.
making it difficult to reach any conclusions about the involvement of PKC in UTP induced contraction. A commonly used PKC inhibitor, chelerythrine, was used initially and this reduced the UTP induced contraction significantly. Since chelerythrine, reduced the 60 mM K⁺ induced contraction also, a more specific blocker of PKC was used. Gö6983 is another broad spectrum PKC inhibitor that inhibits the isoforms PKCα, PKCβ, PKCγ, PKCδ, PKCζ and PKCμ with IC₅₀ values of 7, 7, 6, 10, 60 and 20000 nM respectively (Gschwendt et al., 1996). Gö6983 (1 μM) significantly blocked the UTP induced contraction by more than 50% in 3 mesenteric arterial segments without any effect on 60 mM K⁺ induced contraction, suggesting a role for at least one of the isoforms of PKC blocked by Gö6983.

To investigate the role of PKC in more detail I used a range of peptide inhibitors, many of which target specific PKC isoforms (Rainbow et al., 2009). PKC20-28 is a pseudosubstrate sequence that acts as a PKC inhibitor. Pretreatment of arterial segments with a cell permeable form of this peptide PKC inhibitor, myristoylated-PKC20-28-IP only inhibited the UTP induced contraction by 28.8%. PKC20-28-IP has been used previously in our lab to block PKC sensitive modulation of K⁺ currents in mesenteric smooth muscle cells (Hayabuchi et al., 2001a, Hayabuchi et al., 2001b, Rainbow et al., 2006).

To further investigate the role of PKC in UTP induced contraction I used an alternative Tat peptide-mediated method of making the PKC inhibitor peptides cell permeable (Brooks et al., 2005). In this method the PKC inhibitor peptides were conjugated with the HIV Tat-derived peptide (47-57) using disulphide conjugation at the N terminal of the cysteine residues (Rainbow et al., 2009). Only very low concentrations of this
carrier Tat peptide is required to translocate into the cell and in the reducing environment of the cell the conjugated peptide is released thereby accumulating the unconjugated PKC inhibitors in the cell (Rainbow et al., 2009, Brooks et al., 2005). Linking the PKC inhibitor peptides with the Tat peptide sequence was done by Dr. R.I. Norman of the Department of Cardiovascular Sciences at the University.

The effects of the myristoylated PKC20-28-IP (40 μM) as well as its Tat linked chimera Tat-PKC20-28-IP (100 nM) reduced the UTP induced contraction by a similar amount, 21% and 17 % respectively. Under the same conditions the Tat-PKC20-28-IP reduced the ET-1 induced contraction by 40%, suggesting that PKC has a smaller role in the UTP induced contraction.

Among various PKC isoforms PKC α, β, δ, and ε are found in mesenteric arteries (Mueed et al., 2005). In cultured mesenteric smooth muscle cells GFP-linked PKCα, PKCδ and PKCε have all been shown to be translocated to the membrane following application of either Ang II, ET-1 or UTP (Nelson et al., 2008). PKCα and PKCε have been shown to mediate contractions, and the inhibition of Kv current, induced by ET-1 and Ang-II respectively in rat mesenteric arteries (Rainbow et al., 2009). These results indicate that each of these vasoconstrictors can target a distinct PKC isoform to mediate contraction of mesenteric arteries. To evaluate the role of different PKC isoforms in mediating UTP induced contraction, various Tat-PKC inhibitor peptides against PKCα, β1, β11, δ, and ε were used. No significant reduction of UTP induced contraction was seen after pre-incubation of mesenteric arteries with either of these Tat PKC inhibitor peptides. Tat-PKCα and Tat-PKCε, under conditions similar to those used in my experiments, significantly reduced the contraction induced by ET-1 and Ang-II
respectively (Rainbow et al., 2009). In the experiments presented in this thesis I found that Tat-PKCα-IP blocked the ET-1 induced contraction by almost 70%, but its effect on the UTP induced contraction was not significant.

It is difficult to interpret my results regarding the involvement of PKC in mediating the UTP induced contraction of mesenteric arteries. The results as they stand indicate that PKC has only a limited role in producing the UTP induced contraction, although obtaining a clear control that can overcome possible variations in the concentration of PKC inhibitors inside the cells is lacking. It should, however, be noted that Luykenaar et al. (2004) report that the UTP induced contraction of rat cerebral arteries is independent of PKC and they suggest that Rho kinase plays a major role in this tissue (Luykenaar et al., 2004).

5.1.5 UTP activates non selective cation channel

Various studies have suggested that UTP activates non selective cation channels (Alvarez et al., 2008, Baek et al., 2008, Reading et al., 2005). Electrophysiological studies reveal that UTP induces a sustained, non selective cation current in rat cardiomyocytes by the activation of TRPC3/7 via a PLC mediated pathway (Alvarez et al., 2008).

My result showing that 100 μM Gd\(^{3+}\), a known blocker of non-selective cation channels (Hill et al., 2006), blocks the UTP induced contraction is consistent with an activation of a non-selective depolarizing current by UTP. This rather high concentration also blocked the 60 mM K\(^+\) induced contraction, whereas 10 μM Gd\(^{3+}\) effectively blocked the UTP induced contraction with little or no effect on the 60 mM K\(^+\) induced contraction (Figure 3-17). In the light of these observations, and of the incomplete block
of the response by the L-type Ca\(^{2+}\) channel blocker diltiazem, it appears that UTP induced contraction may be mediated, at least partly, by Ca\(^{2+}\) influx via non selective cation channels. The activation of a non-selective cation current by UTP was confirmed using whole-cell current measurements. During some of these measurements voltage-ramps were applied and the resulting instantaneous I-V curves obtained had a similar rectification profile and reversal potential (near 0 mV) to those recorded in vascular smooth muscle cells previously (Albert et al., 2003).

5.1.6 UTP modulates Kv current
Vasoconstrictors such as 4-AP, ET-1 and Ang-II induced contraction is partly due to the inhibition of voltage gated potassium channels which may in turn cause membrane depolarization, an increase in intracellular Ca\(^{2+}\) concentration and force generation. Several voltage gated potassium channel blockers have been identified so far that prove themselves as effective therapeutic agents. The voltage gated potassium channel blockers could be used in a variety of pathological conditions like hypertension, cardiac arrhythmia, stroke. (Beech, 1997, Dohi et al., 1994, Goonetilleke and Quayle, 2010, Mackie et al., 2008, Surti and Jan, 2005).

Luykenaar and colleagues report that UTP induced contraction and depolarization in rat cerebral arteries are partly mediated by the suppression of a Kv current (Surti and Jan, 2005, Luykenaar and Welsh, 2007, Luykenaar et al., 2004). To study the possible involvement of Kv channel inhibition by UTP I measured whole-cell Kv currents. The Kv currents recorded with 100 nM free Ca\(^{2+}\) in the pipette revealed that UTP suppressed the Kv current at potentials more positive than -10 mV. The activation curve for the Kv current also revealed the ability of UTP to suppress the Kv current at more positive
potentials, while surprisingly at potentials negative to -10 mV the Kv current was increased. Because of the range of Kv subunits expressed in smooth muscle (Cox, 2005) there is the possibility that UTP may modulate the different channel types differentially.

Studies on the mechanism of vasoconstriction often reveal that vasoconstrictors such as 5-HT, ET-1, and Ang-II modulate K⁺ channels by the PLC and PKC signaling pathways and that inhibition of K⁺ channels, including Kv channels, leads to depolarization and contraction (Ko et al., 2010). Including 5 μM of the PLC inhibitor U73122 in the pipette attenuated the reduction of Kv currents by UTP provided there was sufficient Ca²⁺ (100 nM) in the pipette. There was also a slight reduction in the average currents observed with U73122 in the pipette compared to control currents (figure 4.7C), suggesting that U73122 has effects in addition to blocking PLC. Potential additional effects of U73122 were also hinted at by its ability to abolish the contraction induced by 60 mM K⁺. Although U73122 has been used widely to study the signaling mediated by phospholipase C there have been reports by some research groups on its nonspecific blocking effects (Walker et al., 1998).

Ion channels and pumps are known to be regulated by the various isoforms of PKC in smooth muscle cells (Salamanca and Khalil, 2005). Vasoconstrictors in several preparations such as airway smooth muscle, cerebral and mesenteric arteries exert their effect partly through inhibiting Kv channel activity in a PKC dependent manner (Hayabuchi et al., 2001b, Cheng et al., 2007, Cole et al., 1996, Ko et al., 2008, Mackie et al., 2008, Park et al., 2005a, Poole and Furness, 2007, Rainbow et al., 2006, Rainbow et al., 2009, Standen and Quayle, 1998). In contrast to many of these reports, pretreating mesenteric smooth muscle cells with 100 nM Tat PKC20-28-IP for at least 10 minutes
did not significantly prevent UTP suppression of the Kv current. Similarly pretreating the cells with either Tat PKCe-IP or Tat PKCa-IP did not prevent the effects of UTP on Kv current. Similarly Luykenaar (2004) reported that in rat cerebral arteries UTP contraction, as well as its effect on Kv current suppression, was mediated by a PKC independent signaling mechanism which was proposed to be a RhoKinase mediated signaling pathway (Luykenaar et al., 2004, Luykenaar and Welsh, 2007).

5.1.7 UTP modulates $K_{ATP}$ current

ATP sensitive potassium channels are widely expressed in smooth muscle including mesenteric arterial smooth muscle cells and play an important role in the regulation of membrane potential (Standen et al., 1989, Nelson et al., 1990, Nelson and Quayle, 1995, Quayle et al., 1997, Quayle and Standen, 1994). Ang-II and some other vasoconstrictors possess the ability to suppress the $K_{ATP}$ channel activity causing depolarization of smooth muscle. Standen et al (1989) were the first to report these channels in smooth muscle and recorded single channel $K_{ATP}$ currents from rabbit mesenteric arterial smooth muscle cells (Standen et al., 1989).

$K_{ATP}$ channels are activated by a fall in the intracellular ATP and a rise in ADP but they are also the target of several vasoactive compounds, being inhibited by vasoconstrictors such as Ang II and ET-1 in a PKC dependent manner (Kubo et al., 1997, Hayabuchi et al., 2001a) and activated by vasodilators such as calcitonin gene related peptide via activation of PKA (Wellman et al., 1998).

In this investigation I examined whether UTP was able to inhibit the $K_{ATP}$ current in mesenteric smooth muscle cells and whether this involved PKC. The experimental conditions used to isolate the $K_{ATP}$ current were similar to those used previously where
modest internal ATP (1 mM) and ADP (0.1 mM) concentrations enabled background $K_{\text{ATP}}$ channel activity whilst maintaining enough ATP for any phosphorylation reactions to occur (Hayabuchi et al., 2001a). In addition, a holding potential of -60 mV and a low intracellular free Ca$^{2+}$ (100 nM) ensured that contamination of the $K_{\text{ATP}}$ current recorded by BK and Kv channels was minimal. To examine the inhibition of the $K_{\text{ATP}}$ current it was necessary to increase its amplitude over the background activity by using pinacidil, which is a well known activator of these channels. This pinacidil activated $K_{\text{ATP}}$ current was quite stable over time but on application of UTP it was significantly reduced. The effect of UTP on $K_{\text{ATP}}$ current suppression was quite robust when 100 nM free Ca$^{2+}$ was used in the pipette solution. However, the effect of UTP on $K_{\text{ATP}}$ current suppression was less in low (20 nM) intracellular free Ca$^{2+}$.

Similar to Kv channels of vascular smooth muscle, many vasoconstrictors suppress $K_{\text{ATP}}$ channel activity in a PKC dependent manner (Kubo et al., 1997, Standen and Quayle, 1998, Moon et al., 2004, Ko et al., 2008). In contrast to this the inhibition of the $K_{\text{ATP}}$ current by UTP appeared insensitive to PKC inhibition by the Tat-linked PKC$_{20-28}$-IP, either in 100 nM or in 20 nM free internal Ca$^{2+}$. However, inclusion of PKC$_{20-28}$-IP in the pipette has been shown to block $K_{\text{ATP}}$ modulation by Ang II (Hayabuchi et al., 2001a) and both Tat-PKC$_{20}$-IP and Tat-PKC$_{\epsilon}$-IP at 100nM blocked the Kv inhibition by ET-1 and Ang II respectively in this tissue (Rainbow et al., 2009). These results, together with those obtained for the Kv current suggest that inhibition of Kv and $K_{\text{ATP}}$ currents by UTP are not mediated through PKC.
5.2 Conclusion and future perspectives

UTP induced a sustained and dose dependent contraction in rat mesenteric arteries which was reproducible on repeated applications and abolished upon removal of extracellular Ca\(^{2+}\). The UTP induced sustained contraction was partly dependent on voltage gated Ca\(^{2+}\) channels as shown by the partial block of contraction with diltiazem; however, non selective cation channels seem involved as UTP induced contraction was greatly reduced by Gd\(^{3+}\), a known blocker of non selective cation channels. Additionally, whole cell current recordings revealed that UTP induced a non selective cation current which, under the conditions I used, had a reversal potential close to 0 mV; hence it appears that non selective cation channels are largely involved in UTP induced contraction.

Kv channels regulate membrane potential and are activated due to depolarization and therefore have a negative feedback role limit the extent of depolarization and hence contraction (Cole et al., 1996, Prior et al., 1998, Shimoda et al., 1998, Cox, 2005). UTP suppressed the Kv current at more depolarized potentials with 100 nM free Ca\(^{2+}\) in the pipette. However, little or no suppression of the Kv current by UTP was observed at low intracellular free Ca\(^{2+}\) (20 nM). My results are consistent with those reported by Luykenaar et al (2004), who reported similar Kv current suppression by UTP in rat cerebral arteries (Luykenaar et al., 2004). In addition to the inhibitory effect of UTP at positive potentials, it was seen that UTP elevates the current slightly at membrane potentials negative to –10 mV. The activation curve also revealed that UTP has potentiating effects at negative potentials which could reflect the possibility that UTP
modulates certain Kv channel subunits differently (Cox, 2005). It would be interesting to further dissect the effect of UTP on other Kv channel subunits.

The results obtained from measuring UTP induced contraction as well as nodulation of ionic currents suggest that PLC activation is important in mediating these effects. This is also supported by previous reports that UTP activates P2Y receptors (possibly P2Y$_2$ and P2Y$_4$) which are G-protein coupled receptor (Abbracchio et al., 2003) which activates PLC and mobilizes the intracellular Ca$^{2+}$ from the intracellular stores by the activation of IP$_3$.

An inhibitory effect of UTP on K$_{ATP}$ channel activity was also observed and was more pronounced than its effects on the Kv current. UTP significantly blocked the pinacidil activated K$_{ATP}$ current in the presence of 100 nM free Ca$^{2+}$ which suggests that suppression of K$_{ATP}$ channel activity may be one of the reasons for the sustained contraction induced by UTP. However, this sustained contraction as well as the modulation of Kv and K$_{ATP}$ channel activity by UTP seems independent of PKC activation. This is also in agreement with the results of Luykenaar et al (2004) who report that UTP induced depolarization, as well as Kv current suppression, was independent of PKC activation. Luykenaar and colleagues also proposed that Rhokinase partly mediates the UTP induced constriction of rat cerebral arteries by modulating Kv current (Luykenaar et al., 2004, Luykenaar and Welsh, 2007). It would be interesting to investigate whether Rhokinase has a role in the UTP induced contraction of rat mesenteric arteries. Other potassium channels such as BK and Kir also regulate the membrane potential and thus contraction and it would be interesting to study the effect of UTP on these ion channels in rat mesenteric arteries.
Chapter 6 

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