# THE CYTOSKELETAL PROTEIN ADDUCIN AND ITS ROLE IN VASCULAR SMOOTH MUSCLE

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#### **CLAIRE ELIZABETH GIBBONS**

FACULTY OF MEDICAL AND HUMAN SCIENCES SCHOOL OF MEDICINE

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#### Abstract

Actin dynamics are precisely regulated by a large number of actin binding proteins which collectively alter the rates of actin filament assembly and disassembly. Spectrin, an actin cross-linking protein, forms lateral filamentous networks that are linked to the plasma membrane and are required for membrane stability and resistance to mechanical stress. Adducin binds to spectrin-actin complexes, recruiting additional spectrin molecules, thereby further stabilising the membrane. In addition, adducin can bundle and cap actin filaments, and its actions have been implicated in cytoskeletal rearrangement in a variety of cell types.

In vascular smooth muscle there is evidence that rearrangement of the actin cytoskeleton is involved in contraction and transmission of force to the extracellular matrix which leads to tissue remodelling. In addition, cytoskeletal dynamics are involved in vascular smooth muscle cell migration, proliferation and membrane dynamics. Protein kinase C (PKC), Rho-kinase, calmodulin and myosin light chain phosphatase are signalling proteins that are involved in these processes in vascular smooth muscle, and adducin is regulated by these signalling proteins in platelets and epithelial cells.

The current study provides evidence for regulation of the actin cytoskeleton by  $\alpha$ adducin in vascular smooth muscle. Both  $\alpha$ -adducin and spectrin are associated with the cytoskeleton in vascular smooth muscle cells of rat mesenteric small arteries. In response to activation by noradrenaline (NA),  $\alpha$ -adducin becomes rapidly phosphorylated on Ser 724, a site specific for PKC, and dissociates from the actin cytoskeleton and spectrin in a PKC-dependent manner. Longer exposure of vessels to NA results in dephosphorylation of  $\alpha$ -adducin on Ser 724 and its Rho-kinasedependent reassociation with the actin cytoskeleton. Concurrent with this reassociation is enhanced association between the two proteins and an increase in the proportion of spectrin associated with the actin cytoskeleton. In addition, a rise in filamentous actin is observed, which can be blocked by inhibition of PKC or Rhokinase and also by delivery of the  $\alpha$ -adducin antibody into vessels in order to inhibit the function of endogenous  $\alpha$ -adducin.

These data provide evidence for a model in which  $\alpha$ -adducin functions as an actin capping protein in resting vascular smooth muscle cells. Upon vasoconstrictor activation  $\alpha$ -adducin becomes phosphorylated by PKC, inducing its dissociation from the actin cytoskeleton allowing elongation of actin filaments and further rearrangement of the actin cytoskeleton. Following this reorganisation,  $\alpha$ -adducin reassociates with the actin cytoskeleton, possibly in response to phosphorylation by Rho-kinase, and recruits additional spectrin molecules, thus strengthening the newly formed actin filament network. These data provide further insight into the regulation of the actin cytoskeleton in vascular smooth muscle.

## Declaration

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#### Abbreviations

ADF, actin depolymerising protein

ADP, adenosine diphosphate

Arp, actin related protein

ATP, adenosine triphosphate

**BSA**, bovine serum albumin

CA1, cornu ammonis 1

Ca<sup>2+</sup>, calcium

cAMP, cyclic adenosine monophosphate

cGMP, cyclic guanosine monophosphate

CH, calponin homology

CNL, unstimulated vessels

CPI-17, C-kinase potentiated protein phosphatase-1 inhibitor

DAG, diacylglycerol

DAPI, 4'-6-Diamidino-2-phenylindole

DMEM, Dulbecco's modified eagle medium

DMSO, dimethyl sulfoxide

**DTT**, dithiothreitol

EAAT, excitatory amino acid transporter

ECL, enhanced chemiluminescence

EDTA, ethylenediaminetetraacetic acid

EGTA, ethylene glycol tetraacetic acid

ENaC, epithelial sodium channel

ERK, extracellular signal-related kinase

**ET-1**, endothelin-1

F-actin, filamentous actin

FBS, fetal bovine serum

G-actin, globular actin

GDP, guanosine diphosphate

GEF, guanine nucleotide exchange factor

GFX, GF109203X

GLUT1, glucose transporter 1

GPCR, G protein-coupled receptor

GTP, guanosine-5-triphosphate

HEK, human embryonic kidney

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hic-5, hydrogen peroxide-inducible clone 5

Hsp27, heat shock protein 27

IBMX, 3 -isobutyl-1-methylxanthine

IgG, immunoglobulin G

ILK, integrin linked kinase

IP<sub>3</sub>, inositol trisphosphate

kDa, kilodalton

KPSS, high potassium physiological salt solution

Lu/BCAM, Lutheran/basal cell-adhesion molecule

M199, medium 199

MAPK, mitogen-activated protein kinase

MARCKS, myristoylated alanine-rich C-kinase substrate

MDCK, Madin-Darby canine kidney

MHS, Milan hypertensive strain

MLC, myosin light chain

MLCK, myosin light chain kinase

MLCP, myosin light chain phosphatase

MOPS, 3-(N-morpholino) propanesulfonic acid

mRNA, messenger ribonucleic acid

MYPT1, myosin phosphatase target subunit 1

NA, noradrenaline

NMDA, N-Methyl-D-aspartate

**OCT**, optimal cutting temperature

PAK, p21-activated kinase

PAR-1, protease-activated receptor 1

PBS, phosphate buffered saline

PdBu, Phorbol 12,13-Dibutyrate

PH, pleckstrin homology

PIP<sub>2</sub>, phosphotidylinositol 4,5-bisphosphate

PI<sub>3,4</sub> P<sub>2</sub>, phosphotidylinositol 3,4-bisphosphate

PI<sub>3,4,5</sub>P<sub>3</sub>, phosphatidylinositol (3,4,5)-trisphosphate

**PIPES**, piperazine-N,N'-bis(ethanesulfonic acid)

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PMA, Phorbol 12-myristate 13-acetate

PP1c, protein phosphatase 1 catalytic subunit

**PSS**, physiological saline solution

PYK2, proline-rich tyrosine kinase

GDI, guanine nucleotide dissociation inhibitor

RMSA, rat mesenteric small arteries

**RPTE**, rat proximal tubule epithelial

RT-PCR, reverse transcription polymerase chain reaction

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser, serine

SH3, src homology 3 domain

STA<sub>2</sub>, 9,11-epithio-11,12-methanothromboxane A<sub>2</sub>

Thr, threonine

TPA, 12-O-tetradecanoylphorbol-13-acetate

Tyr, tyrosine

VASP, vasodilator-stimulated phosphoprotein

VWF, Von Willebrand factor

**Y27**, Y27632

#### **Chapter 1: Introduction**

#### 1.0 The actin cytoskeleton

The actin cytoskeleton is a strong and dynamic filamentous network, always ready to adapt to the demands of the cell under differing conditions (dos Remedios et al., 2003). In response to external signals, the cytoskeleton can be rapidly reorganised, effecting dramatic changes in cellular properties (Small et al., 1999), thus it serves central roles in a wide variety of functions including cell shape determination, motility, cell adhesion and cell-matrix interactions (Small et al., 1999).

#### **1.1 Actin treadmilling**

Actin functions fall into two categories: functions that are carried out by stable actin filaments; and functions depending on monomer-polymer transitions. Stable actin filaments are important in force generation in the sliding filaments of muscle, or during organelle transport. In addition, stable cross-linked actin meshworks support cell membranes. On the other hand, cell motility associated with lamellipodia formation (Small et al., 1999) and membrane trafficking (Lanzetti, 2007) depend on the dynamics of actin monomer-polymer interactions (Weber, 1999).

In total, six actin isoforms are present in vertebrate tissues (Kim et al., 2008a), existing as monomers (G-actin) and filaments (F-actin). Monomers are involved in polymerisation-depolymerisation-repolymerisation cycles (Barany et al., 2001), and the functions carried out by the cytoskeleton at any one time are dependent upon the ratio of G to F-actin. This dynamic turnover of actin filaments is regulated by actin associated proteins, a number of which are described below.

#### 1.2.1 Actin binding proteins

Actin binds a substantial number of proteins which collectively alter the free G-actin concentration, thus controlling the actin distribution between monomers and polymers, and the rates of actin filament assembly and disassembly. The linear elongation, shortening and architectural organisation of actin filaments are controlled in response to signalling cascades induced by extracellular stimuli. The survival and motility of cells lacking actin binding proteins that have powerful effects on actin *in vitro* imply that no single mechanism can explain actin remodelling in all cells and under all circumstances (Stossel et al., 2006). Actin binding proteins are not limited to one function, indeed many carry out a number of different functions depending on the needs of the cell at any given time.

#### 1.2.1.1 Actin filament formation

Filaments have a barbed end, onto which actin monomers are added rapidly, and a pointed end which is less kinetically active (Coluccio, 1994). Spontaneous assembly of pure actin is unfavourable due to the instability of actin dimers and trimers, but once started, filaments grow rapidly (Pollard and Borisy, 2003). Filament formation requires initiation, elongation and termination (Stossel et al., 2006) and occurs following *de novo* nucleation by the actin related protein (Arp) 2/3 complex (Lai et al., 2008) or polymerisation of actin from newly exposed filament barbed ends. Barbed ends can result from uncapping or from the action of severing proteins. Profilin serves as a polymerisation catalyst that captures actin monomers from thymosin- $\beta$ 4-actin and ushers actin onto growing barbed filament ends (Kang et al., 1999). Vasodilator-stimulated phosphoprotein (VASP) regulates dynamic actin structures such as filopodia and lamellipodia by recruiting actin monomers and

delivering them to the growing barbed filament end, thereby accelerating filament growth (Breitsprecher et al., 2008; Ferron et al., 2007).

Actin filament stabilising proteins such as tropomyosins (Porro et al., 2004), caldesmons (Hai and Gu, 2006), calponins and tropomodulin (Weber et al., 1994) promote elongation by binding the side of actin filaments, thus inhibiting actin depolymerising proteins (dos Remedios et al., 2003).

#### 1.2.1.2 Actin capping

Rapid growth at barbed ends would deplete the pool of G-actin, leading to a slower rate of polymerisation; therefore the extent of elongation must be regulated. Actin capping proteins bind to filament ends and prevent the exchange of monomers at both the pointed and barbed ends (dos Remedios et al., 2003; Pollard and Borisy, 2003). Thus, filaments grow only transiently as activation of cappers results in filament termination (Pollard and Borisy, 2003). Tropomodulins are a family of tropomyosinbinding proteins that cap the pointed end of actin filaments (Chu et al., 2003), thereby blocking elongation and depolymerisation (Weber et al., 1994). Barbed end capping proteins include adducin and CapZ (Chu et al., 2003), and it is thought that most barbed ends are capped in resting cells (Kuhlman, 2000). In muscle, control of actin filament growth is achieved by capping the barbed ends with CapZ and by capping the pointed ends with tropomodulin (Kuhlman et al., 1996). Resting erythrocytes also contain actin filaments with functional caps at both the barbed and pointed ends (Kuhlman, 2000). Indeed cytochalasin, a barbed end capper, has little effect on basal F-actin levels (DiNubile, 1999), providing evidence that most barbed ends are capped in resting cells.

#### 1.2.1.3 Branching

Nucleation and transient 70° branching of actin filaments is mediated by the Arp2/3 complex, a stable assembly of two actin-related proteins Arp2 and Arp3, which initiates new actin filaments as branches on the side of existing filaments (Pollard and Borisy, 2003).

#### 1.2.1.4 Actin cross-linking

Cross-linking proteins, such as villin and dematin, contain at least two actin binding sites, thereby forming filament bundles and three-dimensional networks (dos Remedios et al., 2003; Frank et al., 2004). Relatively small globular or rod-like proteins such as fimbrin and  $\alpha$ -actinin stabilise actin bundles, whereas larger proteins that have inherent spring-like properties, such as the filamins, instead promote high-angle filament organisation.  $\alpha$ -actinins, filamins and spectrins, a family of membrane-associated cross-linking proteins, also function as scaffolds for signalling intermediates that stimulate actin elongation; so they are well positioned to direct the orientation of elongating actin filaments. These actin cross-linking and bundling properties can be regulated by phosphorylation (Azim et al., 1995).

#### 1.2.1.5 Actin filament contraction, cargo motoring and membrane binding

Signalling pathways that induce actin elongation also increase binding of actin filaments to membrane receptors, thus bringing filament ends into close proximity with the signals that promote their elongation. These interactions serve to amplify the mass of elongating actin at the surface (Stossel et al., 2006), which is useful for locomotion. In addition, the linkage between actin filaments and membranes is important for mechanical traction against substrates and retraction of membranes for shape changes in response to contractile forces (Stossel et al., 2006).

#### 1.2.1.6 Actin filament disassembly

In order to maintain the G-actin pool, depolymerisation of existing filaments is required. Filament severing proteins shorten the average length of filaments by binding to the side of F-actin and cutting it into two pieces (dos Remedios et al., 2003), thereby producing new barbed ends (Pollard and Borisy, 2003). Proteins of the gelsolin family disrupt the interactions between actin subunits in filaments in response to calcium or phosphorylation and then tightly cap the barbed ends of the severed filaments. Proteins of the ADF (actin depolymerising factor)/cofilin family weakly sever but do not cap the barbed ends. Barbed ends generated by cofilin either serve as initiation sites for new elongation or become capped, depending upon the signals present.

#### 1.2.1.7 Actin monomer sequestration

Actin functions depending on the dynamic treadmilling of actin are sensitive to the free monomer concentration (Weber, 1999), thus a pool of G-actin is maintained in most cells in order for filament formation to occur wherever and whenever necessary in the cell (Pollard and Borisy, 2003). The volume of F-actin is limited by G-actin sequestering proteins (DiNubile, 1999) such as thymosin- $\beta$ 4 and profilin (dos Remedios et al., 2003; Mannherz et al., 2010), which bind to monomers, preventing them from spontaneous nucleation, thus sustaining the G-actin pool. Profilin catalyses the exchange of adenosine diphosphate (ADP) for adenosine triphosphate (ATP) on

actin, thereby returning subunits to the ATP-actin-profilin pool ready for another cycle of assembly (Pollard and Borisy, 2003).

The main functions carried out by actin binding proteins during the treadmilling process, as described above, are illustrated in **Figure 1.1**. Together these actions are responsible for the highly dynamic and reactive nature of the actin cytoskeleton.



Figure 1.1. The polymerisation-depolymerisation-repolymerisation cycle of actin. Initiation defines where and when actin filament elongation occurs. Nucleation and 70° branching of actin filaments is mediated by the Arp2/3 complex, which amplifies the mass of actin filaments. Relatively small globular or rod-like actin binding proteins stabilise actin bundles, whereas larger actin binding proteins that have inherent spring-like properties cross-link filaments promoting high-angle filament organisation. Actin filament **barbed-end capping** regulates the extent of actin filament elongation. Actin filament configurations are susceptible to deformation by contractile forces generated by bipolar myosin filaments. Signals contributing to actin elongation also induce **membrane attachment**, which is important for mechanical traction against substrates and retraction of membranes for shape changes. Actin filament disassembly is carried out by severing proteins or by barbed end uncapping. In addition, some proteins accelerate pointed end depolymerisation, maintaining the actin monomer pool, needed for initiation. Following this, a number of proteins are involved in monomer sequestration, which maintains a pool of monomers ready for the next cycle of *initiation* (Stossel et al., 2006)

#### 1.2.2 The cellular functions of actin treadmilling

Actin treadmilling, carried out by the proteins described above, provides cells with the ability to regionally control the polymerisation and supramolecular organisation of actin filaments, resulting in the formation of different subcompartments, each with a defined function (Small et al., 1999). For example localised bands of actin filaments can provide the structural support for cell-cell junctions (Parsons et al., 2010), and in motile cells actin filaments form the meshed framework of the protruding lamellipodia and linear filopodia (Pollard and Borisy, 2003; Small et al., 1999). Rearrangement of actin filaments can also aid in contraction (Kim et al., 2008a), ion transport (Khurana, 2000) and membrane trafficking (Lanzetti, 2007), mechanisms which are described in more detail below.

#### 1.2.2.1 Locomotion

An important property of actin is its ability to produce movement in the absence of motor proteins (dos Remedios et al., 2003). Protrusion of the leading edge of a cell toward a chemotactic stimulus or along a path of varying adhesiveness within the extracellular matrix is mediated by the actin polymerisation mechanisms described in Section 1.2.1. Cytoskeletal remodelling at the leading edge and detachment of focal contacts at the trailing edge propels the cell towards a stimulus (Gerthoffer, 2007).

#### 1.2.2.2 Contraction

Contraction of actin stress fibres is mediated by myosin, which moves antiparallel actin filaments past each other, providing the force that rearranges the actin cytoskeleton (Parsons et al., 2010). Actin polymerisation is thought to be involved in the sustained phase of smooth muscle contraction (Rembold et al., 2007), with

suggested mechanisms including maintenance of force (Kim et al., 2008a) and transmission of cross-bridge generated force to the cell membrane (Gerthoffer and Gunst, 2001). Connections between membrane adhesion junctions and actin filaments within the contractile apparatus provide a strong and rigid framework for the transmission of force generated by the interaction of myosin and actin filaments to the outside of the cell (Kim et al., 2008a). The involvement of the actin cytoskeleton in smooth muscle contraction will be discussed in more detail in Section 3.0.

#### 1.2.2.3 Ion transport

Epithelial cells can rapidly alter solute transport rates by changing the kinetics of transport proteins (Khurana, 2000). Molecular mechanisms involved in this process include regulation of the transporter itself i.e. by phosphorylation; and altering the number of transport proteins per unit area of the plasma membrane. Both these mechanisms can be regulated by the actin cytoskeleton. Ion transport function can be modulated via alterations in membrane fluidity, cell morphology and cell motility, while actin binding proteins with motor activity can regulate the passage of vesicles containing ion transport proteins through the cell, affecting delivery to the membrane (Khurana, 2000).

#### 1.2.2.4 Membrane trafficking

Signals originating at the plasma membrane control the formation and dissolution of the actin cytoskeleton at various locations throughout the cell. Such rearrangement of the actin cytoskeleton provides the forces required for a variety of cellular processes based on membrane dynamics, such as endocytosis, exocytosis, and vesicular trafficking (Lanzetti, 2007). Endocytosis involves membrane invagination, coated pit formation, coated pit sequestration, detachment of the newly formed vesicle (scission), and movement of this new endocytic compartment away from the plasma membrane into the cytosol (Qualmann et al., 2000). Actin polymerisation at endocytic sites is an early event, and F-actin dynamics are required at multiple stages including coated pit formation and vesicle scission (Lanzetti, 2007). Moreover, actin regulatory proteins have been shown to directly bind to signalling molecules known to regulate vesicle trafficking (Khurana, 2000).

During exocytosis, F-actin is rapidly assembled around secretory vesicles upon fusion with the plasma membrane, and it has been suggested that F-actin may be needed to stabilise the secretory compartment during docking with the plasma membrane (Lanzetti, 2007).

This section demonstrates the diversity of functions in which cytoskeletal rearrangement is involved, and the ability of the cytoskeleton to adapt so quickly and fittingly to the needs of the cell is brought about by the array of actin binding proteins working in combination to make the cytoskeleton such a dynamic entity. Two such proteins, adducin and spectrin, are the focus of the current study and will be described in more detail in the following section.

#### 2.0 Adducin and spectrin

In erythrocytes, where adducin and spectrin have been most intensely studied, the two proteins interact as part of a multi-protein complex of actin binding proteins that provides linkage of the cytoskeleton to the plasma membrane (**Figure 1.2**). This complex is responsible, along with other membrane complexes, for the prominent morphologic and mechanical properties of the cell (Anong et al., 2009).



Figure 1.2. Model of the junctional complex on the human erythrocyte membrane. The junctional complex contains the membrane-spanning proteins band 3, glycophorin C, Rh complex proteins, and a glucose transporter, in addition to peripheral proteins actin, tropomyosin, tropomodulin, adducin, dematin, p55, protein 4.1, and a variety of glycolytic enzymes. This complex serves to anchor the spectrin/actin cytoskeleton to the phospholipid bilayer and along with other protein complexes, is responsible for the prominent morphologic and mechanical properties of the cell. 3, band 3; GPC, glycophorin C; Rh, Rh complex proteins; GLUT1, glucose transporter (Anong et al., 2009).

Although the membrane-associated cytoskeletal network was first identified in human erythrocytes, it has subsequently been identified on the plasma membranes of most cells (Davis et al., 2009). In erythrocytes, the mechanical properties of the cytoskeleton allow the cells to adapt rapidly to environmental changes and resist the stresses of their environment. In non-erythroid cells this feature may be important during contraction, migration, and adhesion, amongst other functions. Indeed, a number of erythrocyte proteins have been shown to play an important role in nonerythroid cells.

#### 2.1 Adducin

#### 2.1.1 Adducin structure

Adducin has three isoforms  $\alpha$ ,  $\beta$ , and  $\gamma$ , encoded by genes ADD1 ( $\alpha$ -adducin), ADD2 ( $\beta$ -adducin), and ADD3 ( $\gamma$ -adducin) (Manunta and Bianchi, 2006). The human  $\alpha$ -adducin gene, spanning approximately 85 kb consists of 16 exons (Lin et al., 1995) and is ubiquitously distributed (Citterio et al., 2003). The gene for  $\beta$ -adducin comprises 17 exons and has been localised to human chromosome 2p13-p14 (Gilligan et al., 1995). Differences in putative transcription factor binding sites in the  $\alpha$  and  $\beta$ -adducin promoters suggest that the two genes are regulated by quite different pathways (Gilligan et al., 1997). The human  $\gamma$ -adducin gene (ADD3) is composed of at least 13 introns and 14 exons and is localised on murine chromosome 19, a region that shows conserved synteny with human chromosome 10 (Citterio et al., 1999).

Each subunit contains an N-terminal 38 kilodalton (kDa) globular head domain (domain I), a neck domain (domain II) and a COOH-terminal 60-64kDa protease-sensitive tail domain (domain III) (Joshi et al., 1991). The 38kDa protease-resistant domains are highly conserved between subunits whereas the COOH-terminal protease-sensitive regions differ. At the end of the tail domain there is a 22-residue domain known as the myristoylated alanine-rich protein kinase C substrate (MARCKS)-related domain as it has high homology to the PKC substrate MARCKS (Matsuoka et al., 2000) (**Figure 1.3**).



Figure 1.3. The domain structure of an adducin subunit. Each subunit contains an *N*-terminal 38 kDa globular head domain (domain I), which is protease-resistant and highly conserved between subunits. The neck domain (domain II) contains the calmodulin binding site and is highly conserved, but protease-sensitive. Finally, the COOH-terminal 60-64kDa protease-sensitive tail domain (domain III) differs in sequence between subunits (Scaramuzzino and Morrow, 1993).

Subunits combine to form either  $\alpha$ - $\beta$  or  $\alpha$ - $\gamma$  heterodimers/tetramers (Hughes and Bennett, 1995).  $\alpha$ - $\beta$  heterodimers have been shown to function in erythrocytes where adducin is a mixture of heterodimers and heterotetramers (Hughes and Bennett, 1995), and  $\alpha$ - $\gamma$  heterodimers have been detected in renal proximal tubule epithelial cell extracts (Dong et al., 1995). In  $\alpha$ -adducin knockout mice,  $\beta$ - and  $\gamma$ -adducin are absent in red blood cells (despite normal mRNA expression), indicating that  $\alpha$ -adducin is the limiting subunit in tetramer formation. Loss of both  $\beta$ - and  $\gamma$ -adducin in the absence of the  $\alpha$ -subunit in mice provides *in vivo* evidence that both  $\alpha$ - $\beta$  and  $\alpha$ - $\gamma$  heterodimers and heterotetramers form in the mouse red blood cell membrane, but that stable  $\beta$ - $\gamma$  complexes do not (Robledo et al., 2008). In  $\beta$ -adducin knockout mice, a substantial (up to 80%) decrease in  $\alpha$ -adducin expression is observed, with an upregulation of  $\gamma$ -adducin (Marro et al., 2000; Muro et al., 2000; Porro et al., 2004), suggesting that  $\alpha$ - $\gamma$  dimers take the place of  $\alpha$ - $\beta$  dimers. In  $\beta$ - $\gamma$  adducin knock-out

mice, the amount of  $\alpha$ -adducin is reduced to barely detectable levels. However, the stability of  $\alpha$ -adducin in the absence of a heterologous binding partner varies considerably between tissues. The amount of  $\alpha$ -adducin is modestly reduced (~15%) in the kidney, while in the spleen and brain it is reduced by ~50%, suggesting that the structural properties of adducin differ significantly between erythroid and various nonerythroid cell types (Sahr et al., 2009).

The globular core of each adducin subunit has the ability to form dimers and tetramers (Joshi et al., 1991); however it is unable to bind calmodulin and does not interact with actin. Adducin binds to actin via its COOH-terminal tail (Hughes and Bennett, 1995) and it has been suggested that MARCKS-related domains mediate direct contact with actin ends. In addition to the MARCKS domain, adducin's interactions with spectrin and actin require an oligomerisation site localised in its neck domain (Li et al., 1998). Capping activity requires the intact adducin molecule and is not provided by the N-terminal globular head domains alone or by the COOH-terminal extended tail domains (Kuhlman et al., 1996). A model has been suggested in which four head domains contact one another to form a globular core, which caps the end of actin filaments (Ferrandi et al., 1999), with extended interacting  $\alpha$ - and  $\beta$ -adducin tails (Hughes and Bennett, 1995) that participate in the lateral contacts between several actin filaments, recruiting additional spectrin units to the actin filaments (Ferrandi et al., 1999).



Figure 1.4 Interactions between adducin, spectrin and actin. Adducin forms complexes between spectrin and actin, promoting the association of spectrin with actin filaments. The four head domains of adducin form a globular core, which caps the end of actin filaments. The extended tail domains form contacts between several actin filaments and recruit additional spectrin molecules (Pariser et al., 2005b).

#### 2.1.2 Adducin function

Adducin blocks elongation and depolymerisation at the barbed ends of actin filaments, thus functioning as a barbed end capping protein (Kuhlman et al., 1996). It also bundles actin filaments into lateral arrays, the adducin forming cross-bridges between the filaments (Mische et al., 1987). In addition, adducin binds tightly to spectrin-actin complexes but with much less affinity to spectrin or actin alone (Gardner and Bennett, 1987). Once bound to spectrin-actin complexes adducin promotes assembly of additional spectrin molecules onto actin filaments, exhibiting a preference for the fast growing ends of actin filaments for its spectrin recruiting function (Li et al., 1998).

Its effects on the cytoskeleton have been implicated in a number of cellular processes in nucleated cells including oogenesis (Pinto-Correia et al., 1991), transepithelial fluid secretion (Beyenbach et al., 2009), cell proliferation (Bowen et al., 2003), T lymphocyte signalling (Lu et al., 2004) and mediating key cell movements in the developing chick embryo (Akai and Storey, 2002).

#### 2.1.2.1 Adducin knock-out mice

As yet, there is no knockout lacking all three adducin subunits, however there are several knockout mice lacking one or two adducin subunits, which give some indication of adducin's function.

 $\beta$ -adducin knockout mice exhibit deficiencies in erythrocyte function, with red blood cells resembling those from patients with hereditary spherocytosis (Porro et al., 2004). In addition, the mice exhibit behavioural, motor coordination and learning deficits implicating adducin in mechanisms underlying synaptic plasticity, motor coordination performance and learning behaviours (Porro et al., 2010). Double knock-out mice lacking  $\beta$ -adducin and the headpiece of dematin show a decrease in actin filament number in erythrocytes, revealing an essential role of dematin and adducin in the maintenance of erythrocyte shape and membrane stability (Chen et al., 2007b). Furthermore, retention of the spectrin-actin complex under low ionic conditions is significantly reduced in these mice, further supporting a role for adducin in stabilising/regulating spectrin-actin interactions (Chen et al., 2007b).

Cells from  $\alpha$ -adducin mice also display features characteristic of red blood cells in hereditary spherocytosis (Robledo et al., 2008). In addition, the mice display growth retardation at birth and throughout adulthood. Approximately 50% of knockouts develop lethal communicating hydrocephalus with striking dilation of the lateral,
third, and fourth ventricles. These data indicate that adducin plays a role in red blood cell membrane stability and in cerebrospinal fluid homeostasis.

However, adducin knock-out mice also exhibit upregulation of other cytoskeletal proteins including CapZ and tropomodulin suggesting compensatory mechanisms are in place, possibly preventing the manifestation of more severe phenotypes (Porro et al., 2004)

#### 2.1.2.2 Adducin and hypertension

Adducin has been implicated in the pathogenesis of hypertension. The Milan hypertensive strain (MHS) of rat has several single nucleotide polymorphisms in the three adducin genes giving rise to a hypertensive phenotype (Salardi et al., 1988). The proposed mechanism for regulation of blood pressure by adducin involves differentially modulated renal  $Na^+/K^+/CI^-$  cotransport (Ferrari et al., 1992), thereby altering sodium reabsorption (Efendiev et al., 2004). Adducin is thought to be involved in the endocytosis of membrane proteins involved in renal ion transport including the Na-K pump (Torielli et al., 2008) and aquaporins (Procino et al., 2011).

As this section illustrates, adducin is implicated in a wide variety of cellular functions. Knockout mice reveal a significant role for the  $\alpha$  and  $\beta$  subunits in stabilising/regulating spectrin-actin interactions and stabilising the membrane, while there is a body of evidence to suggest that it may be important in regulating the renal Na-K pump in order to control blood pressure. However it is clear that, although it is involved in actin capping, actin bundling, actin cross-linking and spectrin recruitment; its functions can be adapted depending on the cell type or indeed the needs of the cell at any given time and in any situation. In this way it appears to be as dynamic as the cytoskeleton it helps to regulate.

#### 2.1.3 Regulation of adducin and its functions by intracellular signalling pathways

Adducin was first purified from human erythrocytes based on calmodulin binding activity (Matsuoka et al., 1996) and it is now known to be a substrate for PKC, protein kinase A (PKA) and Rho-kinase, amongst others.

## 2.1.3.1 Phosphorylation by protein kinases C and A

Ser 726 and Ser 713 in the C-terminal MARCKS-related domains of  $\alpha$ - and  $\beta$ -adducin respectively, are the major PKC phosphorylation sites (Matsuoka et al., 1996). In addition to phosphorylation on Ser 726, PKA phosphorylates  $\alpha$ -adducin on Ser 408, Ser 436, and Ser 481 in the neck domain, reducing the affinity of adducin for F-actin. The PKA-unique sites of  $\alpha$ -adducin are not present in  $\beta$ - and  $\gamma$ -adducin, thus represent a specialised feature of the  $\alpha$  subunit (Matsuoka et al., 1996).

Phosphorylation of adducin by PKC is often correlated with changes in subcellular distribution. In resting cells, adducin is either tightly associated with the cytoskeleton (Barkalow et al., 2003; Dong et al., 1995) or localised at the plasma membrane, in some cases at sites of cell-cell contact (Dong et al., 1995; Kaiser et al., 1989). However, phosphorylation by PKC often induces dissociation from the cytoskeleton or plasma membrane. *In vitro* PKC phosphorylation decreases adducin binding to phosphatidylserine, which indicates that phosphorylation may interfere with membrane binding *in vivo* (Fowler et al., 1998a). Phosphatidylinositol 3,4-bisphosphate (PI<sub>3,4</sub>  $P_2$ ), phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), and

phosphatidylinositol (3,4,5)-trisphosphate (PI<sub>3,4,5</sub>P<sub>3</sub>) also induce dissociation of a portion of the adducin bound to the cytoskeleton in platelets (Barkalow et al., 2003). In proximal tubules of Eker rats with renal carcinomas, increased adducin phosphorylation is coupled with loss of its association with the membrane (Fowler et al., 1998b). Changes in subcellular distribution and phosphorylation levels of adducin are also seen in murine models of amyotrophic lateral sclerosis (Shan et al., 2005), suggesting a role for adducin in neuron death (Hu et al., 2003). Phorbol esters induce adducin phosphorylation in rat proximal tubule epithelial (RPTE) cells and the phosphorylated forms are preferentially recovered in the soluble fraction (Dong et al., 1995), in contrast to unphosphorylated forms, which are found in the Triton X-100insoluble cytoskeletal fraction. Similarly in platelets, adducin localises to the Tritonsoluble cytosolic fraction once phosphorylated (Gilligan et al., 2002). Deletion of the small G-proteins Rac1 and Rac2 leads to a profound disruption of actin assembly in erythrocytes, coupled with increased phosphorylation of adducin on Ser 724. The phosphorylated form is more readily extractable with Triton X-100, indicating weaker association with the cytoskeleton (Kalfa et al., 2006).

PKC-induced dissociation from the cytoskeleton has implications for adducin's actin binding functions. Phosphorylation by PKC leads to diminished adducin activity in terms of its actin capping and spectrin recruiting capabilities (Matsuoka et al., 1998). Indeed, platelet activation induces adducin phosphorylation which releases it from Factin and spectrin, facilitating the centralisation of the membrane skeleton during platelet spreading. Release of adducin from F-actin exposes actin barbed ends that contribute to the actin assembly reactions in lamellipodia and filopodia (Barkalow et al., 2003).

#### 2.1.3.2 Phosphorylation by Rho-kinase

In addition to phosphorylation in the tail domain, adducin is phosphorylated by Rhokinase in the neck domain on Thr 445 and Thr 480 (Fukata et al., 1999). In contrast to regulation by PKC, phosphorylation of adducin by Rho-kinase enhances the interaction of adducin with actin filaments *in vitro* (Kimura et al., 1998). Indeed, phosphorylation of adducin on Thr 445 in platelets leads to its redistribution to the cytoskeletal fraction (Tamaru et al., 2005). Differential cytoskeletal targeting has been shown in platelets, in which adducin phosphorylated on Thr 445 was selectively isolated in the cytoskeletal fraction, whereas adducin phosphorylated on Ser 726 was mainly present in the Triton-soluble fraction (Tamaru et al., 2005).

Although PKC and Rho-kinase phosphorylation have, until now, only been studied in isolation, a model has been proposed in which PKC and Rho-kinase may work together to regulate adducin's actin binding functions as illustrated in **Figure 1.5**; upon the activation of Rho-kinase by Rho during the action of extracellular stimuli,  $\alpha$ -adducin is phosphorylated on Thr 445/Thr 480. Phosphorylated  $\alpha$ -adducin binds to F-actin, and then facilitates the recruitment of spectrin to F-actin. On the other hand, PKC phosphorylates  $\alpha$ -adducin on Ser 726, and then inhibits the activity of adducin in recruiting spectrin to F-actin.



Figure 1.5. Model for the regulation of adducin by Rho-kinase and PKC. The spectrin-actin network is linked to the plasma membrane via associations with membrane-spanning proteins. Upon Rho-kinase activation by extracellular stimuli, adducin becomes phosphorylated on Thr 445 and Thr 480, inducing its binding to F-actin. Adducin then facilitates the recruitment of additional spectrin molecules to the F-actin network. Conversely when PKC is activated by extracellular stimuli, adducin is phosphorylated on Ser 726. Phosphorylation on Ser 726 induces removal of adducin from F-actin, thereby inhibiting its spectrin recruiting activities, possibly leading to the disassembly of the spectrin-F-actin meshwork (Fukata et al., 1999).

# 2.1.3.3 Phosphorylation by Fyn

Fyn is a Src-family tyrosine kinase involved in neuronal development, transmission, and plasticity in the mammalian central nervous system (Pariser et al., 2005a). Fyn phosphorylates  $\beta$ -adducin at Tyr 489 inducing its translocation to the cell periphery, and co localisation with actin filaments and Fyn itself (Shima et al., 2001).

## 2.1.3.4 Regulation by calmodulin

In addition to the phosphorylation site for PKC, the MARCKS domain of adducin contains the calmodulin binding site (Matsuoka et al., 1998). Use of recombinant  $\beta$ -adducin peptides generated from partial cDNA clones identified strong calmodulin-binding activity within the protease-sensitive domain of adducin in residues 425–461. This region of the molecule is highly conserved between mouse, rat, and human and shares structural features with calmodulin-binding sequences in other proteins

(Scaramuzzino and Morrow, 1993). Calmodulin binding is inhibited by phosphorylation of  $\beta$ -adducin by PKA and PKC. A novel difference between adducin and other capping proteins is that it is down-regulated by calmodulin in the presence of calcium (Ca<sup>2+</sup>) (Kuhlman et al., 1996). Indeed, *in vitro* the actin bundling, actin capping and spectrin recruiting properties of adducin are diminished in the presence of Ca<sup>2+</sup> and calmodulin (Scaramuzzino and Morrow, 1993). Calmodulin binding activity may be more active in  $\beta$ -adducin than  $\alpha$ -adducin (Matsuoka et al., 1996).

#### 2.1.3.5 Interactions with myosin light chain phosphatase

Adducin has been shown to interact with the ankyrin repeat domain of the myosin binding subunit of myosin light chain phosphatase (MLCP) *in vitro* and *in vivo*. Indeed the two proteins co-localise at cell-cell junctions of Madin-Darby canine kidney (MDCK) cells. MLCP also dephosphorylates  $\alpha$ -adducin after phosphorylation by Rho-kinase (Kimura et al., 1998).

# 2.1.3.6 Adducin cleavage

It has been suggested that increased phosphorylation of  $\alpha$ -adducin and its subsequent dissociation from the cytoskeleton leads to caspase-3-mediated cleavage to form a 74kDa protein. This product is seen in apoptotic RPTE cells following cisplatin treatment (van de Water et al., 2000) and supports a model in which dissociation of  $\alpha$ -adducin from the cytoskeleton is rendered irreversible by caspase-3-mediated cleavage as shown in **Figure 1.6**.

# Spectrin/Actin/Adducin interactions



Figure 1.6. Model for the dissociation of adducin from actin, and degradation by caspase-3. Adducin forms complexes between spectrin and actin promoting the association of spectrin with actin filaments. Agonist-induced phosphorylation of adducin on Ser 713 and Ser 726 reduces the affinity of adducin for actin filaments, inducing filament dissociation and increasing cleavage and degradation of adducin by caspase 3 (Pariser et al., 2005b).

Adducin phosphorylation also leads to calpain-mediated cleavage of adducin during platelet activation with either thrombin, calcium ionophore (A23187), or phorbol 12-myristate 13-acetate. The PKC-phosphorylated form is proteolysed at a significantly

faster rate than the unphosphorylated form (Gilligan et al., 2002). As these experiments were carried out in the absence of binding proteins such as spectrin and actin, the data suggest that PKC causes a conformational change of adducin rendering it susceptible to proteolysis.

The regulatory processes described above have been associated with a number of cellular functions in a variety of cell types, demonstrating the wide ranging effects of adducin. The functions in which these mechanisms are involved are shown in **Table 1.1**.

Cell/tissue type	Phosphorylation mechanism	Function	Reference
Human intestinal epithelial (HT-29) cells	Phosphorylation by PKC	Cytoskeletal disorganisation in response to the cytotoxic enterotoxin Act	(Galindo et al., 2005)
THP-1 cells	Phosphorylation by PKC	Disruption of the cytoskeleton during mycobacterial infection	(Hestvik et al., 2003)
Neurons	Phosphorylation by PKC	Cocaine-induced F- actin formation in medium spiny neurons expressing the dopamine receptor	(Lavaur et al., 2009)
Bronchiolar and alveolar type II (ATII) epithelial cells in murine lung sections	Phosphorylation by PKC	Cell proliferation following inhalation of asbestos	(Lounsbury et al., 2002)
MDCK cells	Phosphorylation by PKC	Cell motility and spreading	(Chen et al., 2007a)
Metastatic mammary tumour cells	Phosphorylation by PKC	Migration, adhesion and extravasion involved in tumor metastasis	(Kiley et al., 1999)
Erythrocytes	Phosphorylation by PKC	Decreased erythrocyte deformability in sickle cell disease	(George et al., 2010)
Vascular smooth muscle	Phosphorylation by PKC	Vascular smooth muscle contraction	(Je et al., 2004)
Guinea pig outer hair cells	Phosphorylation by Rho-kinase and PKC	Shape change involved in outer hair cell electromotility	(Zhang et al., 2003)
MDCK cells	Phosphorylation by Rho-kinase	Membrane ruffling	(Fukata et al., 1999)
Rat brain	Phosphorylation by Fyn	Hypothalamic regulation of food intake and energy homeostasis	(Gotoh et al., 2008)
HeLa cells	Phosphorylation by PKC	Pleiotrophin-induced cytoskeletal destabilisation and loss of cell-cell adhesion	(Pariser et al., 2005b)

 Table 1.1. The functions of adducin phosphorylation

#### 2.1.4 Adducin expression

Northern blot analysis and reverse transcriptase (RT)-PCR have demonstrated ubiquitous expression of  $\alpha$  and  $\gamma$  adducin (Matsuoka et al., 2000), whereas  $\beta$ -adducin is found mostly in erythrocytes, bone marrow and brain (Gilligan et al., 1999). Interestingly, no tissue expresses one adducin gene without a second, suggesting that adducin does not function as a monomer (Gilligan et al., 1999). The wide expression of adducin suggests that it may function in a number of different cell types.

# 2.2 Spectrin

Spectrin is the defining element of the almost ubiquitous membrane-associated cytoskeletal protein network in animal cells (Das et al., 2006).

# 2.2.1 Spectrin structure

Spectrin has two isoforms,  $\alpha$  and  $\beta$ ; flexible rods, approximately 0.2 microns in length. The spectrin repeat domains of  $\alpha$  and  $\beta$  spectrin associate end-to-end to form heterodimers. Heterodimers associate laterally in an antiparallel fashion to form tetramers (Baines, 2009) (**Figure 1.7**). In  $\alpha$ -spectrins, a Src homology 3 (SH3) domain is inserted between repeats 9 and 10, while a calmodulin-like domain containing four EF hands binds to calcium (Baines, 2009). Variation in the structure of the C-terminal regions of spectrin correlates with differential intracellular targeting (Hayes et al., 2000). At the N terminus of  $\beta$ -spectrin there is a pair of calponin homology (CH) domains which together form an actin binding domain. The actin binding domain also binds protein 4.1, and the formation of a three-way complex between protein 4.1, spectrin and actin effectively strengthens the interaction of spectrin with actin (Baines, 2010). A pleckstrin homology (PH) domain binds to inositol lipids and inositol triphosphate (Das et al., 2006). The  $\alpha$  and  $\beta$  subunits combine via two repeats close to the N-terminus of  $\alpha$ -spectrin and the C-terminus of  $\beta$ -spectrin (Baines, 2009). The  $\beta$ -spectrin N-terminal domain plus the first two  $\alpha$ -helical domains are required for participation in spectrin/adducin/actin complexes.



Figure 1.7. Domain structure of spectrin. Two a spectrins (top left) and five  $\beta$  spectrins (top right) are shown. Spectrin molecules are composed of modular units called spectrin repeats (yellow), along with ankyrin binding domains (purple), Src homology domains (blue), EF hand domains (red) and calmodulin binding domains (green) which promote interactions with binding targets important for spectrin function. The pleckstrin homology domain (black) promotes association with the plasma membrane, and the actin binding domain (grey) tethers the spectrin-based membrane skeleton to the underlying actin cytoskeleton. The spectrin tetramer (bottom left) is the fundamental unit of the spectrin-based membrane skeleton. Spectrin repeat domains of  $\alpha$  and  $\beta$  spectrin associate end to end to form heterodimers; heterodimers then associate laterally to form tetramers (Bennett and Healy, 2009).

The most abundant protein motifs of both  $\alpha$ - and  $\beta$ -spectrins are spectrin repeats, repeating units of approximately 106 amino acids, which form three-helix coiled-coils and are present in 21 copies in  $\alpha$ -spectrins and either 16–30 copies in  $\beta$ -spectrins. The principal function of the spectrin repeat is to confer elasticity to spectrin, allowing it to act as a molecular spring or shock absorber (Davis et al., 2009; De Matteis and Morrow, 2000). The repeats can also serve structural purposes, tethering integral and

cytosolic proteins with high spatial precision, and forming a `switchboard' for interactions with multiple signalling proteins. The binding properties of the repeats perform important roles in controlling spacing of proteins (De Matteis and Morrow, 2000), leading to the assembly of complex, multiprotein structures involved both in cytoskeletal architecture and in forming large signal transduction complexes (Djinovic-Carugo et al., 2002).

#### 2.2.2 Spectrin function

Nonerythroid spectrins are proposed to have roles in cell adhesion, establishment of cell polarity, and attachment of other cytoskeletal structures to the plasma membrane. Spectrins commonly assemble into planar cytoskeletal sheets composed of spectrin molecules cross-linked by short actin filaments, forming a hexagonal network that underlays membrane surfaces (Bennett and Healy, 2008) (**Figure 1.8**).



Figure 1.8. The spectrin cytoskeleton of human erythrocytes. Spectrin tetramers (illustrated in Figure 1.8) associate end-to-end to generate extended macromolecules, forming a two-dimensional submembrane network directly underneath the plasma membrane. Spectrin molecules form junctional complexes with ankyrin and short actin filaments to generate a hexagonal pattern (Dubreuil, 2006).

This arrangement forms the basis for the two main functions attributed to the spectrin cytoskeleton: the maintenance of membrane structural integrity and the formation of

discrete membrane domains. Indeed, one consistently observed effect in spectrin gene knockout experiments is that loss of spectrin leads to a failure of interacting membrane proteins to accumulate at the appropriate site (Das et al., 2006).

#### 2.2.2.1 Organisation of membrane domains

In addition to precisely regulating which molecules cross the plasma membrane, it is also important to regulate where substances cross the membrane, thus cells have evolved mechanisms to confine and stabilise transport proteins within specific subdomains of the plasma membrane (Dubreuil, 2006). Spectrin is coupled to the inner surface of the erythrocyte membrane primarily through association with ankyrin (Bennett and Healy, 2009). Whereas the spectrin cytoskeleton of erythrocytes is uniformly distributed beneath the plasma membrane, spectrin and ankyrin are compartmentalised within specific subdomains of the plasma membrane in polarised cells such as neurons (Lazarides and Nelson, 1983), kidney cells (Drenckhahn et al., 1985; Nelson and Veshnock, 1986), and muscle (Craig and Pardo, 1983). A number of membrane proteins bind to ankyrin through their cytoplasmic domains, enabling them to interact with the spectrin scaffold, while others bind directly to spectrin (Figure 1.9). Nonerythroid spectrins are therefore proposed to have roles in determining the composition and function of specialised regions of the plasma membrane. An interaction with the spectrin cytoskeleton may block internalisation of the membrane protein by the endocytic pathway or lateral diffusion within the plasma membrane (Dubreuil, 2006).



**Figure 1.9. Formation of stable membrane domains.** A number of membrane proteins (depicted here by different shapes) bind to ankyrin (green) through their cytoplasmic domains, enabling them to interact with the spectrin scaffold (gold). Such interaction with the spectrin cytoskeleton may block internalisation by the endocytic pathway, or diffusion within the plasma membrane, thereby promoting stable accumulation within a specific plasma membrane domain (Dubreuil, 2006).

Nonerythroid spectrins have been shown to interact with a host of different membrane proteins (Bennett and Baines, 2001) including N-Methyl-D-aspartate (NMDA) receptors (Wechsler and Teichberg, 1998), cyclic guanosine monophosphate (cGMP)-gated cation channels (Molday et al., 1990), the epithelial sodium channel ENaC (Rotin et al., 1994), the glutamate excitatory amino acid transporter EAAT4 (Ikeda et al., 2006), CD45 (Pradhan and Morrow, 2002), and the Na-K pump (Nelson and Veshnock, 1987). Moreover, spectrin, along with ankyrin, directly participates in delivery of E-cadherin to the lateral membrane (Kizhatil et al., 2007). In this context, the spectrin-based network has been implicated as a membrane protein-sorting machine (Beck and Nelson, 1996).

#### 2.2.2.2 Membrane structural integrity

The interaction of the spectrin-based protein network with the cytoplasmic surface of the membrane controls the elasticity of the bilayer membrane and erythrocyte shape. Spectrin is also required for resistance to mechanical stresses, as evidenced by haemolytic anaemia hereditary spherocytosis which results from deficiency of spectrin or ankyrin or their failure to assemble on the plasma membrane of erythrocytes. In this case, cells are osmotically fragile and lose membrane spontaneously. Moreover, elimination of spectrin expression in the nematode worm *Caenorhabditis elegans* results in paralysis. In addition the structure of the muscles is greatly disrupted: the sarcomeres become disorganised, the sarcoplasmic reticulum is lost, and the muscles pull away from the body wall. The nervous system is also disrupted by elimination of spectrin expression: axons break as they grow, presumably because they cannot withstand the movement of the animal, and new processes grow randomly from the nerve cell bodies to replace them. In addition, adducin and spectrin are required for stabilisation of lateral membranes in human bronchial epithelial cells, as demonstrated by depletion of adducin which results in increased detergent solubility of spectrin accompanied by reduced height and disruption of the lateral membrane (Abdi and Bennett, 2008). In erythrocytes, a novel band 3-adducin-spectrin bridge that connects the spectrin/actin/protein 4.1 junctional complex to the membrane bilayer is believed to be important for membrane stability and it has been suggested that this bridge may be subject to regulation by multiple signalling pathways (Anong et al., 2009). A linkage involving dematin, GLUT1 and adducin has been described as a further mechanism for linkage of the junctional complex to the plasma membrane (Khan et al., 2008).

## 2.2.2.3 Regulation of actin dynamics

More recently, spectrin has been implicated in the regulation of actin dynamics. Interaction with Lutheran/basal cell-adhesion molecule (Lu/BCAM), found in erythroid and endothelial cells, was shown to be necessary for stress fibre formation in MDCK cells during spreading on laminin 511/521, with suggestion that spectrin acts as a signal relay between laminin 511/521 and actin (Collec et al., 2011). Moreover, in cornu ammonis 1 (CA1) pyramidal neurons in hippocampal slice cultures, expression of the actin binding domain of spectrin induced actin bundling which significantly increased the size of the spine head, indicating a possible effect in modulating both the morphological and functional dynamics of dendritic spines (Nestor et al., 2011). It has also been suggested that spectrin may stabilise treadmilling filaments during neurite outgrowth so that growth at the end of filaments pushes out the leading edge of the cell (Baines, 2010).

## 2.2.3 Regulation of spectrin by phosphorylation

There have been contrasting reports regarding the effect of phosphorylation on spectrin function. Wei *et al.* reported that the phosphorylation of spectrin is 'silent' and exhibits no measurable effect on its ability to self-associate or to interact with other cytoskeletal proteins (Wei and Tao, 1993). However, Manno *et al.* reported that membrane mechanical stability is exquisitely regulated by phosphorylation of  $\beta$ -spectrin by membrane-bound casein kinase I; increased phosphorylation of  $\beta$ -spectrin decreases membrane mechanical stability while decreased phosphorylation increases membrane mechanical stability (Manno et al., 1995).

In vitro studies indicate that calcium-dependent protease I (calpain I) cleaves spectrin in the middle of the  $\alpha$ -subunit and in the COOH-terminal third of the  $\beta$ -subunit (Harris and Morrow, 1990). Cleavage at the  $\beta$  site requires calmodulin, which binds with high affinity to a single site in the  $\alpha$  subunit (Harris and Morrow, 1990). Three functional states appear to exist: (i) intact spectrin, which constitutively forms tetramers and binds F-actin; (ii)  $\alpha$ -cleaved spectrin, which loses its ability to selfassociate and bind F-actin in the presence of calmodulin; and (iii)  $\alpha$ ,  $\beta$ -cleaved spectrin, a form that is incompetent to establish tetramers or bind actin (Harris and Morrow, 1990).

The functions of spectrin and adducin are well established and there are numerous examples of how these proteins, along with other cytoskeletal proteins, play a fundamental role in the distinctive functions of cells (Baines, 2010). As a consequence, defects in these proteins caused by mutation or by modifications are associated with numerous disease states including hereditary anaemia, heart disease and hypertension.

Despite the obvious importance of adducin and spectrin in regulating actin cytoskeletal dynamics and consequently the functions carried out by the actin cytoskeleton, there has been little research into the role of adducin and spectrin in resistance arteries, tissues that are known to rely on cytoskeletal dynamics for a number of fundamental functions. This thesis will focus on adducin and spectrin in the regulation of the cytoskeleton in vascular smooth muscle, the structure and function of which are described in more detail in Section 3.0

#### 3.0 Vascular smooth muscle

Small artery walls consist of an outer tunica adventitia containing connective tissue, fibroblasts, mast cells and nerve elements; a central tunica media containing the smooth muscle cells, bounded on the luminal side by the internal elastic lamina; and an inner tunica intima, made up of endothelial cells (Mulvany, 1990) (**Figure 1.10**).



Figure 1.10. Electron micrograph of a transverse section of guinea-pig mesenteric arteriole. S, smooth muscle cells; E, endothelial cells; EF: elastic fibres, i.e. the internal elastic lamina; N, nerve bundles; F, fibroblast; L, lumen. The scale bar represents  $5\mu m$  (Yamamoto et al., 2001).

#### 3.1 Smooth muscle cell structure

Vascular smooth muscle cells are elongated, spindle shaped cells that surround the endothelial monolayer (Lincoln et al., 2001; van den Akker et al., 2010). Vascular smooth muscle is the least specialised type of muscle and is phenotypically and morphologically more similar to nonmuscle cells than to striated or cardiac muscle in that smooth muscle does not have distinct myofibrils (Cipolla et al., 2002). The

smooth muscle internal scaffolding consists of three classes of filamentous assembly: microtubules (300 Å diameter), intermediate filaments (70–110 Å diameter) and actin microfilaments (70 Å diameter) (Sakai et al., 2009). The actin microfilaments and intermediate filaments form two distinct systems: the cytoskeleton, which comprises predominantly nonmuscle actin (and intermediate filaments), and the actin filaments of the smooth muscle contractile apparatus that interact with smooth muscle myosin (Flavahan et al., 2005). Actin is the most abundant protein in smooth muscle (Kim et al., 2008a), and four of the six actin isoforms are present: two smooth muscle type isoforms ( $\alpha$  and  $\gamma$ ) and two cytoplasmic isoforms ( $\beta$  and  $\gamma$ ) (Kim et al., 2008a). Vascular smooth muscle contains a substantial pool of G-actin, unlike striated muscle in which actin monomers contribute only ~10% of the total actin content (Koltsova et al., 2008).

Macromolecular adhesion junctions, dense bodies, form on the intracellular side of the plasma membrane at the junctions between actin filaments and the extracellular matrix (Gunst and Zhang, 2008). At these junctions actin filaments are connected to integrins via actin cross-linking proteins that bind to the cytoplasmic tails of integrins (Gunst and Zhang, 2008). Integrins connect the extracellular matrix with the cytoskeleton and other intracellular signalling molecules that accumulate at sites of focal adhesions (Martinez-Lemus et al., 2009). Dense bodies form the mechanical base of the cell (van den Akker et al., 2010), possibly playing a role in transmission of force between cells and the extracellular matrix (Kim et al., 2008a; Mulvany, 1990). Through integrin receptors, the extracellular events can be communicated into the cell and vice versa (Kim et al., 2008a) (**Figure 1.11**).



Figure 1.11. Molecular organisation of integrin/cytoskeletal adhesion junctions in smooth muscle. Actin filaments (yellow) are linked to integrin proteins (blue/green) via' linker' proteins such as α-actinin and talin, both of which can cross-link actin filaments and bind to integrin heterodimers. Scaffolding proteins, such as ILK and paxillin, regulate assembly of protein complexes at adhesion junctions in response to contractile stimulation. Proteins that assemble into macromolecular complexes at adhesion junctions, such as Arp 2/3, N-WASp and cdc42, regulate actin polymerisation. HSP, heat shock protein; ILK, integrin-linked kinase; FAK, focal adhesion kinase; N-WASp, neuronal Wiskott-Aldrich syndrome protein (Gunst and Zhang, 2008).

# 3.2 The role of the actin cytoskeleton in vascular smooth muscle

As in other cell types, the actin cytoskeleton is a highly dynamic structure in vascular smooth muscle, playing a role in various functions including contraction, migration, membrane trafficking, maintained tone and vascular plasticity (van den Akker et al., 2010). A number of these processes are described further in this section.

#### 3.2.1 Smooth muscle cell contraction

Smooth muscle cells receive neural stimulation from the autonomic nervous system, in addition to hormonal and autocrine/paracrine actions and changes in load or length (Hilgers and Webb, 2005). Neural, autocrine and paracrine signals are received by membrane spanning receptors including G-protein coupled receptors (GPCRs) and receptor tyrosine kinases.

## 3.2.1.1 Regulation of myosin light chain phosphorylation

Activation of cell surface GPCRs leads to the hydrolysis of PIP<sub>2</sub> forming inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> mediates the release of Ca<sup>2+</sup> from intracellular stores. Following an increase in intracellular Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ), Ca<sup>2+</sup> interacts with the four EF-hands of calmodulin forming a Ca<sup>2+</sup>-calmodulin complex which is capable of activating myosin light chain kinase (MLCK) (Barany et al., 1990). The active MLCK phosphorylates myosin light chain (MLC) on Ser 19, initiating actin-myosin interaction and allowing myosin ATPase to be activated by actin (Akata, 2007; Kim et al., 2008a) thereby promoting vascular smooth muscle contraction (Salamanca and Khalil, 2005) (**Figure 1.12**).

Figure *1.12*. Calciumdependent vascular smooth muscle contraction. Activation of membranespanning receptors leads to PIP<sub>2</sub> hydrolysis, forming IP<sub>3</sub>.  $IP_3$  mediates the release of calcium from intracellular stores. Intracellular calcium interacts with CaM, activating MLCK, which in turn phosphorylates MLC on Ser 19. Actin-myosin interaction allows myosin-ATPase to be activated by actin, thereby promoting contraction. Conversely, dephosphorylation of myosin induces relaxation. PLC, phospholipase *C*: *PIP*<sub>2</sub>; phosphatidylinositol bisphosphate; **IP**<sub>3</sub>, inositol trisphosphate; SR, sarcoplasmic reticulum; CaM, calmodulin; MLCK, myosin light chain kinase.



MLC dephosphorylation is catalysed by MLCP. In addition to the well-studied pathways that activate MLCK during receptor activation, the  $Ca^{2+}$  sensitivity of MLC phosphorylation is increased via signalling pathways that inhibit MLCP (Kitazawa et al., 1991; Kitazawa et al., 1989). This  $Ca^{2+}$  sensitisation is carried out by a number of signalling proteins including PKC and Rho-kinase.

## > Rho-kinase

Rho A, an upstream regulator of Rho-kinase, is involved in agonist-induced contractile effects in mesenteric arterial smooth muscle (Hirata et al., 1992). Rho

cycles between an inactive guanosine diphosphate (GDP) form, present in the cytosol, and an active guanosine-5-triphosphate (GTP) form, associated with the membrane (Somlyo and Somlyo, 2003) (**Figure 1.13**).



Figure 1.13. The Rho GTPase cycle. Rho GTPases cycle between an active (GTPbound) and an inactive (GDP-bound) conformation. In the active state, they interact with one of over 60 target effector proteins. The cycle is regulated guanine nucleotide exchange factors (GEFs), which catalyse nucleotide exchange and mediate activation; GTPase-activating proteins (GAPs), which stimulate GTP hydrolysis leading to inactivation; and guanine nucleotide exchange inhibitors (GDIs), which extract the inactive GTPase from membranes (Etienne-Manneville and Hall, 2002).

In resting cells, Rho GDP dissociation inhibitor (Rho GDI) binds to Rho-GDP and transfers it from the membrane to the cytosol (Fukata et al., 2001). In stimulated cells, Rho-GDP is converted to Rho-GTP through the action of guanine nucleotide exchange factors (GEFs). Rho-GTP is then targeted to the cell membrane via its C-terminal tail (Fukata et al., 2001). Rho-GTP activates Rho-kinase, which binds to the myosin phosphatase target subunit (MYPT1) of MLCP to inhibit its activity (Kimura et al., 1996). Moreover, a pyridine derivative, Y27632, which inhibits Rho-kinase, selectively inhibits smooth muscle contraction by inhibiting Ca<sup>2+</sup> sensitisation (Uehata et al., 1997).

## Protein kinase C

The PKC family, which includes  $Ca^{2+}$ -dependent (conventional) PKCs ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), and  $Ca^{2+}$ -independent (novel) PKCs ( $\delta$ ,  $\varepsilon$ ,  $\eta$ ,  $\theta$ ) (Salamanca and Khalil, 2005), plays an important role in myogenic contraction of rat small arteries (Karibe et al., 1997). The PKC activator DAG and other activators of PKC such as phorbol 12,13-Dibutyrate (PdBu) and 12-O-tetradecanoylphorbol-13-acetate (TPA) induce  $Ca^{2+}$  sensitisation in isolated intact and  $\alpha$ -toxin permeabilised arteries (Gailly et al., 1997; Nishimura et al., 1990). This occurs via phosphorylation of MLCP which inhibits the interaction of the two MLCP subunits, MYPT1 and protein phosphatase 1 catalytic subunit (PP1c) (Toth et al., 2000), thereby inhibiting dephosphorylation of MLC (Hirano et al., 2004). Activation of PKC indirectly by noradrenaline (NA) also increases the  $Ca^{2+}$  sensitivity of vascular smooth muscle myofilaments (Nishimura et al., 1990). Furthermore GF109203X, an inhibitor of conventional and novel PKCs, completely abolishes  $Ca^{2+}$  sensitisation by PdBu (Gailly et al., 1997).

# > Other proteins

Other proteins involved in  $Ca^{2+}$  sensitisation include C-kinase-potentiated protein phosphatase-1 inhibitor (CPI-17), which inhibits MLCP activity following its phosphorylation (Hirano, 2007) and arachidonic acid which increases the level of force and MLC phosphorylation at constant  $Ca^{2+}$  in permeabilised smooth muscle, and slows relaxation and MLC dephosphorylation (Gong et al., 1992). ZIP kinase, integrin-linked kinase and p21-activated protein kinase (PAK) also phosphorylate smooth muscle MLC in a  $Ca^{2+}$ /calmodulin independent manner, significantly increasing the actin-activated ATPase activity of myosin (Hirano, 2007; Vetterkind and Morgan, 2009).

#### 3.2.1.2 The role of the actin cytoskeleton in contraction

Smooth muscle slowly develops force and maintains maximal tension for extended intervals at a much lower energy cost than the energy expended in similar contractions in skeletal muscle (Fultz et al., 2000). These properties have been uncoupled from the well-studied pathways leading to MLC phosphorylation and actin-activated myosin ATPase activity; leading to considerable interest in additional factors that may contribute to the contractile properties of smooth muscle, particularly cytoskeletal rearrangement.

In intact, unstimulated, contractile smooth muscle, actin is present predominantly as F-actin, with approximately 80% of total actin existing as filaments (Kim et al., 2008a). Upon  $\alpha$ -adrenergic receptor stimulation or increased intravascular pressure, the percentage of F-actin can increase, and the proportion of G-actin decrease, demonstrating dynamic receptor-induced reorganisation of the actin cytoskeleton (Cipolla et al., 2002). Agents that inhibit actin polymerisation, such as latrunculin A (Cipolla et al., 2002) and cytochalasin D, reduce contraction in mesenteric small arteries without significantly affecting MLC phosphorylation or  $[Ca^{2+}]_i$  (Shaw et al., 2003). Cytochalasins B and D also inhibit carbachol-induced contraction in bovine tracheal smooth muscle (Tseng et al., 1997). Furthermore, inhibition of actin polymerisation by *Clostridium botulinum* C2 toxin (which ADP ribosylates G-actin) impairs the contraction of smooth muscle isolated from guinea pig ileum (Mauss et al., 1989). As F-actin is not a substrate for C2 toxin, these findings provide additional evidence for G to F-actin transition in smooth muscle contraction.

Conflicting results on agonist-induced cytoskeletal rearrangement in smooth muscle have been reported. In cultured airway smooth muscle cells actin polymerisation increases after stimulation with carbachol (Togashi et al., 1998). Moreover,  $\alpha$ adrenergic agonists induce phosphorylation of a number of adhesion plaque proteins expected to lead to cytoskeletal rearrangement (Kim et al., 2008a). Paxillin undergoes tyrosine phosphorylation in response to the contractile stimulation of tracheal smooth muscle, and tracheal smooth muscle strips treated with paxillin antisense oligonucleotides display markedly reduced tension development. Moreover, paxillindepleted smooth muscle tissues display vastly reduced actin dynamics, including inhibition of the reduction in G-actin in response to acetylcholine stimulation (Tang et al., 2002). In rat mesenteric small arteries (RMSA), both proline-rich tyrosine kinase (PYK2) and paxillin associate with the actin cytoskeleton following NA stimulation (Ohanian et al., 2005). The paxillin homologue hydrogen peroxide-inducible clone-5 (Hic-5) is also expressed in RMSA and is phosphorylated by NA, inducing its redistribution to the cytosol in a Src-dependent manner. NA-induced activation and association of Hic-5 with heat shock protein 27 (Hsp27) are required for the reorganisation of the actin cytoskeleton and force development in small arteries (Srinivasan et al., 2008). Indeed, a number of these changes occur concomitantly with increased F-actin formation and contraction of vessels.

In contrast to the findings described above, Flavahan *et al.* reported that phenylephrine had no effect on actin rearrangement in mouse tail arterioles, despite observing pressure-induced F-actin formation (Flavahan et al., 2005). However, the rearrangement of the cytoskeleton in vascular smooth muscle is both isoform- and stimulus-dependent (Kim et al., 2008b). The  $\gamma$ -actin isoform, the least abundant isoform in smooth muscle, is the most dynamic actin, showing significant isoformdependent remodelling during phenylephrine-induced contractions, in contrast to  $\alpha$ smooth muscle actin (Kim et al., 2008b). Moreover,  $\alpha$ - and  $\beta$ -actin behave differently in response to stimulation with PdBu in A7r5 smooth muscle cells. Upon stimulation  $\beta$ -actin is retained in stress cables which shorten during the interval of cell constriction and hold the cell in the contracted configuration. By comparison,  $\alpha$ -actin stress cables disassemble and reform in association with  $\alpha$ -actinin into column-like structures which ring the cell periphery and appear to be associated with remaining  $\alpha$ actin filament structure (Fultz et al., 2000). Thus, the contrasting reports of agonistinduced cytoskeletal rearrangement may involve different actin isoforms; rearrangement of particular actin isoforms may be involved in agonist-induced contraction, whereas others may be required for non-contractile cellular functions.

#### 3.2.2 Smooth muscle cell migration

Smooth muscle cells are capable of changing their attachments, both between themselves and the surrounding extracellular matrix, thereby enabling active adjustment of their position within the vascular wall (Martinez-Lemus et al., 2009). Repositioning of smooth muscle cells is suggested to redistribute wall stress to noncontractile vessel elements, thereby minimising smooth muscle cell energy expenditure. Such smooth muscle cell migration occurs in response to stimulation of cell surface receptors and is needed during vascular development, in response to vascular injury, and during atherogenesis (Gerthoffer, 2007). Actin polymerisation, triggered by early signalling events, is fundamental to vascular smooth muscle migration. Many promigratory and antimigratory molecules exist in smooth muscle including small biogenic amines, peptide growth factors, cytokines, and extracellular matrix components. Physical forces including blood flow, sheer stress, and matrix stiffness can also influence vascular smooth muscle migration. The GPCR agonists, angiotensin II (Bell and Madri, 1990) and NA (Bell and Madri, 1989) have been shown to influence vascular smooth muscle migration. Indeed, a number of signalling proteins that are involved in smooth muscle contraction are also important for smooth muscle cell migration including rho (Liu et al., 2002), PAK (Gerthoffer, 2007) and integrin-linked kinase (ILK) (Esfandiarei et al., 2010).

# 3.2.3 Membrane trafficking in vascular smooth muscle

In smooth muscle, transmembrane receptors are predominantly localised in specialised regions of the plasma membrane, which also contain signalling and adaptor proteins to facilitate signalling pathways from these domains (Mukherjee 2006). Receptor tyrosine kinases promote numerous downstream signalling pathways leading to vascular permeability, proliferation, migration and differentiation. Following ligand binding, receptors are internalised via clathrin-dependent or caveolar pathways, either to downregulate the signalling pathway or to signal from endosomes; actin polymerisation is required for this process (Mukherjee 2006), as described in Section 1.2.2.4.

## 3.2.4 Small artery remodelling

Vascular remodelling reflects any change in vascular structure, including changes in lumen diameter, wall thickness and wall composition (van den Akker et al., 2010). At the cellular and molecular level this includes cytoskeletal organisation, cell-to-cell connections, and extracellular matrix composition (Martinez-Lemus et al., 2009). Remodelling may occur as a result of persistent functional responses (Bakker et al., 2004); in the early phases of the activation process, cells are engaged in quick responses that cannot rely on the synthesis of new proteins, but that are probably dependent on molecules that are present and post-translationally modified (Boccardi et al., 2007).

The extracellular matrix-integrin-cytoskeleton linkage plays an essential role in the mechanosensory apparatus, which enables vascular smooth muscle cells to detect and respond to changes in intraluminal pressure, allowing eutrophic inward remodelling of resistance arteries in hypertension (Heerkens et al., 2007). Chronic vasoconstriction in vitro results in the inward remodelling of small arteries and the wall-to-lumen ratio is increased in hypertension. Following prolonged constriction, vascular smooth muscle cells auto-regulate their length, changing their position within the arterial wall as vascular diameter remains reduced (Heerkens et al., 2007; Martinez-Lemus et al., 2009). Indeed, during prolonged exposure to NA (4 hours), acute cell shortening is followed by cellular elongation (Martinez-Lemus et al., 2009). Migration is fixation of extracellular matrix components terminated by by surface transglutaminases (Heerkens et al., 2007). It has been proposed that the sequence of events from vasoconstriction, to intracellular reorganisation of the cytoskeleton, to cellular repositioning and, eventually, a change in the passive vessel diameter, should not be considered as separate events but rather as a continuum (Martinez-Lemus et al., 2009). These events rely on overlapping pathways and depend on the same structural elements, which form the cytoskeleton-integrin-matrix axis.

## 3.4 Summary

Clearly cytoskeletal dynamics are instrumental in a number of smooth muscle cellular functions. Despite this, relatively little is known about the structure of the cytoskeleton in smooth muscle, and the molecular mechanisms by which it is regulated. Actin filament formation has emerged as an essential cellular event that substantially regulates force development in vascular smooth muscle in response to chemical and mechanical stimulation (Tang and Anfinogenova, 2008), in addition to demonstrating involvement in migration, proliferation and membrane dynamics. The focus of the current study will be the actin binding proteins adducin and spectrin and the role they may play in the regulation of the all-important cytoskeleton.

## 4.0 Aims and Objectives

# 4.1 Aims

The overall aims of this project were to characterise adducin in rat mesenteric small arteries and to define its involvement in regulation of the actin cytoskeleton.

# 4.2 Objectives

*Hypothesis*: adducin helps to regulate the actin cytoskeleton in vascular smooth muscle, possibility aiding in the cytoskeletal rearrangement known to be important in vascular smooth muscle contraction.

The initial objective was to describe the presence and distribution of adducin isoforms in resting vessels, and to investigate their interactions with spectrin. Following this, regulation of adducin's phosphorylation state and distribution in response to vasoconstrictor activation was to be investigated. In addition, the functional role of adducin in regulating the actin cytoskeleton was to be studied.

# **Chapter 2: Materials and Methods**

# **1.0 Introduction**

The methods outlined in this chapter were used to characterise the cytoskeletal proteins adducin and spectrin in vascular smooth muscle.

# 2.0 Materials and Methods

# 2.1. Rat mesenteric small arteries

# 2.1.1. Animals and tissue dissection

Adult female Sprague Dawley rats (approx 200–300g weight, age range 6–10 weeks) were housed in the animal facility of the University of Manchester. The animals were killed by stunning and cervical dislocation. The required tissue samples were taken and immediately placed in ice cold physiological salt solution (PSS) and kept on ice. The tissue was then pinned out in plastic dishes coated with Sylgard, and immersed in PSS as shown in **Figure 2.1**.



Figure 2.1. Rat mesentery pinned out in a plastic dish coated with Sylgard.

Mesenteric small arteries (internal diameter  $<300\mu$ m) were dissected free from surrounding fat and connective tissue. For each experiment, the entire mesentery of one rat was dissected and the vessels were divided into the required number of treatment groups.

# 2.1.2 Vessel stimulation

Each group of vessels was incubated in an eppendorf tube containing 500µl medium 199 (M199) (Invitrogen, Paisley, UK) at 37°C for 30 min prior to stimulation. To stimulate vessels, noradrenaline (NA) or endothelin-1 (ET-1) were added to the media to give final concentrations of 15µM or 100nM, respectively. The contents of the eppendorf were mixed gently, and left to incubate at 37 °C for the required length of time.

## 2.1.3 Homogenisation of samples and determination of protein concentration

Unless otherwise stated, following stimulation vessels were transferred into a glass homogeniser containing 100–200µl of ice cold homogenisation buffer, and ground using a glass pestle until fully dissolved (approximately 30sec-1 min). Lysates were then transferred to eppendorf tubes and the homogeniser was rinsed with  $40\mu$ l of homogenisation buffer to collect any homogenate residue. This  $40\mu$ l of homogenisation buffer was then added to the lysate. The method of Bradford (Bradford, 1976) was used to measure protein concentration. 2.5µl of homogenised protein sample was diluted in 797.5µl of dH<sub>2</sub>O and mixed with 200µl of Bio-Rad protein assay dye reagent to give a final volume of 1ml. A spectrophotometer was used to read the absorbance of each sample at 595nm. Protein concentration was determined by comparison to a standard curve.

## 2.1.4 Subcellular fractionation

Each group of arteries (approximately 5-15 arteries per group, depending on the number of groups in each experiment) was homogenised in 120µl ice cold MOPS homogenisation buffer, as described in Section 2.1.3. The homogeniser was then rinsed with 40µl MOPS buffer, which was added to the homogenised sample. 12µl total homogenate from each group was kept and its protein concentration measured using the method of Bradford (Section 2.1.3). The remaining sample was centrifuged in a Beckman ultracentrifuge at 800xg at 4°C for 15 min to remove nuclei and debris. The supernatant was transferred to a fresh eppendorf tube on ice. The pellet was resuspended in 50µl of 2% sodium dodecyl sulphate (SDS) and 12.5µl of 5x Laemmli buffer to form a nuclei and debris sample, and stored at -20°C. The supernatant was then centrifuged at 150,000xg at 4°C for 75 min. Following centrifugation, the supernatant was transferred to a fresh eppendorf and its volume measured. One quarter volume of 5x Laemmli buffer was added to the supernatant to form *fraction 1*: cytosol. The pellet was resuspended in MOPS buffer with 1% Triton X-100 (volume of MOPS = volume of supernatant removed in previous step) and incubated on ice for 30 min followed by centrifugation at 150,000xg for 75 min at 4°C. The supernatant was transferred to a fresh eppendorf and its volume measured; one quarter volume of 5x Laemmli buffer was added to form fraction 2: Triton X-100-soluble membrane fraction. The pellet was resuspended in 50µl of 2% SDS and 12.5µl of 5x Laemmli buffer to form fraction 3: Triton X-100-insoluble membrane fraction (Ohanian et al., 1997; Srinivasan et al., 2008; Storrie and Madden, 1990; Walker et al., 2001). 20µl each of fractions 1, 2 and 3 were run on 9% SDS-polyacrylamide gels, as described below in Section 2.1.7. Data were expressed as a proportion of the total protein (sum of signal detected in fractions 1, 2 and 3).

#### 2.1.5 Immunoprecipitation

Following stimulation as described in Section 2.1.2, each group of arteries (approximately 5–15 arteries per group, depending on the number of groups in each experiment) was homogenised in 200µl 0.1% Triton X-100 in MOPS buffer, as described in Section 2.1.3. The homogeniser was then rinsed with 40µl MOPS buffer, which was added to the homogenised sample. Samples were incubated on a rotary mixer for 30 min at 4°C before centrifugation at 500xg for 10 min at 4°C to remove unhomogenised material. The pellet was discarded and protein concentration of the supernatant was measured, using the Bradford assay described in Section 2.1.3. Protein concentrations were adjusted to 1mg/ml. Equal volumes of each sample (approximately 200µl) were incubated with 60µl of albumin agarose at 4°C with agitation for 30 min, followed by centrifugation at 12,000xg for 5 min at 4°C. Samples were pre-cleared with 25µl of immunoglobulin G (IgG) magnetic beads (New England Biolabs, Massachusetts, USA), with incubation for 30 min at 4°C with agitation. A magnet was used to remove the magnetic beads, and supernatants were incubated with 5µg of 1° antibody overnight at 4°C with agitation. The following day 25µl of IgG magnetic beads were added to each sample and incubated for 30 min at 4°C with agitation. A magnet was used to separate beads from the supernatant. Supernatant was removed and kept on ice while beads were washed three times with 200µl 0.1% Triton X-100 in phosphate buffered saline (PBS), using the magnet to separate beads from the wash buffer each time. Proteins were eluted from the beads using 10µl of 2% SDS per sample; 2.5µl of 5x Laemmli buffer was then added to each sample. 20µl each of the immunoprecipitate and supernatant were run on 9% SDS-polyacrylamide gels, as described below in Section 2.1.7. Data were expressed as the ratio of spectrin immunoprecipitate to adducin immunoprecipitate, as measured by the amount of adducin and spectrin detected by western blotting, measured by densitometry. Antibodies and concentrations used for immunoprecipitation are listed in **Table 2.1**.

Antibody	Туре	Concentration
α-adducin	Polyclonal	1: 1000
Spectrin	Polyclonal	1: 1000

Table 2.1 Antibody concentrations used for immunoprecipitation.

#### 2.1.6 G-actin/F-actin in vivo assay

The G-actin/F-actin *in vivo* assay kit (Cytoskeleton, Denver, USA) was used to create G-actin and F-actin fractions. All steps were carried out at 37°C in order to minimise the breakdown of actin filaments. Following treatment with 15 $\mu$ M NA as described in Section 2.1.2, each group of arteries was transferred to an eppendorf tube containing 100 $\mu$ l of F-actin stabilisation buffer (warmed to 37°C), and homogenised using dissection scissors until fully dissolved (approximately 1–2 min). Samples were centrifuged at 800xg for 15 min to remove nuclei and debris. The pellet was discarded and the protein concentration of the supernatant was measured using the Bradford assay, as described in Section 2.1.3. Protein levels were adjusted so that the protein concentration was equal in all samples, before centrifugation at 50,000rpm for 1 hour. Supernatants were removed and placed immediately on ice to form the G-actin fraction. Pellets containing F-actin were resuspended in 100 $\mu$ l of 1 $\mu$ M cytochalasin D and incubated for 1 hour on ice to form the F-actin fraction. One quarter volume of 5x Laemmli buffer was added to both G-actin and F-actin fractions. 40 $\mu$ l of both fractions were run on 9% SDS-polyacrylamide gels and probed with a G-actin
antibody. Data were expressed as the ratio of F-actin to G-actin as measured by the signal detected by the G-actin antibody in each fraction, measured by densitometry.

## 2.1.7 Immunoblotting

Lysates, pre-mixed with 5x Laemmli buffer, were thawed where necessary and heated at 95°C for 5 min. Between 10 and 40µl of each sample (depending on the assay) was run on 9% SDS-polyacrylamide gels, along with 5µl of a 20-200kDa molecular weight marker loaded at each end of the gel. Gels were run at 110V for approximately 1h, or until the dye front reached the bottom of the gel. Following this proteins were transferred electrophoretically from the gel onto nitrocellulose membrane, in blotting buffer for 90 min at 45V on a magnetic stirrer. Nitrocellulose membranes were then incubated in 10ml 1% (w/v) bovine serum albumin or 5% (w/v) non-fat milk for 30-60 min at room temperature with gentle agitation, followed by incubation in 10ml of the 1° antibody at 4°C overnight or at room temperature for 1 hour with gentle agitation. Membranes were washed in 0.1% Tween-20 in Tris Buffered Saline (TBS) for 45 min (3 x 15 min washes) before being incubated in horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature with gentle agitation. Membranes were washed again for 10 min in Tween-20-TBS and then developed with the enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Rockford, USA), as described in the manufacturer's instructions. Where appropriate, membranes were stripped by incubation for 45 min at 90°C in 40 ml strip buffer with 280µl of  $\beta$ -mercaptoethanol, washed in 20ml 0.1% Tween-20 in TBS for 15-30 min, and reprobed for total protein. Alternatively, the lower half of the membrane was probed with actin antibody to ensure equal loading.

Immunoreactivity was quantified by densitometry. Primary and secondary antibodies and concentrations used for western blotting are listed in **Table 2.2**.

Antibody	Туре	Concentration	Secondary antibody	Secondary antibody concentration
Laboratory- developed α-adducin	Polyclonal	1:1000	Rabbit	1:10000
Abcam α-adducin	Polyclonal	1:1000	Rabbit	1:10000
β-adducin	Polyclonal	1:100-1:1000	Rabbit	1:10000
γ-adducin	Polyclonal	1:500	Rabbit	1:10000
4.1	Polyclonal	1:1000	Rabbit	1:10000
Spectrin	Polyclonal	1:1000	Rabbit	1:10000
662 P-add	Polyclonal	1:1000	Rabbit	1:10000
445 P-Add	Polyclonal	1:1000	Rabbit	1:10000
G-actin	Polyclonal	1:500	Rabbit	1:10000
Actin	Polyclonal	1:2500	Rabbit	1:10000

Table 2.2 Antibodies and concentrations used for western blotting.

# 2.1.8 Chariot transfection method

Chariot protein delivery reagent (Active Motif, California, USA) was used to deliver antibodies into RMSAs to assess protein function. Dissected vessels were divided into two groups, and each group was incubated in 500µl of Leibovitz's medium (Invitrogen, Paisley) for 30 min at 37°C. Meanwhile, 5µg of antibody was mixed gently with 6µl Chariot reagent in 100µl PBS. In addition, 5µg of rabbit serum (control) was mixed gently with 6µl Chariot reagent in 100µl PBS in a separate tube. Both samples were incubated at room temperature for 30 min. Following their 30 min incubation at 37°C, arteries were transferred to fresh eppendorf tubes, and lids were punctured with a needle to allow air circulation. The first set of vessels was overlaid with 200µl of the Chariot-antibody mixture, while the second set was overlaid with 200µl of the Chariot-serum mixture as a control. 400µl of Leibovitz's medium was added to each tube and arteries were incubated for 1 hour at 37°C. A further 750µl of Leibovitz's medium was then added to each tube, followed by additional 2 hour incubation at 37°C. Arteries were either transferred to 500µl of M199 and incubated at 37°C before being stimulated with NA as described in Section 2.1.2 and processed for F:G actin fractions as described in Section 2.1.6; or cannulated and mounted in a pressure myograph as described below.

# 2.1.9 Myograph experiments

Contractile responses of RMSAs were measured using pressure myography (Living Systems). Segments of small artery were cannulated and mounted in a Living Systems pressure myograph. After equilibration at 20mmHg and 37°C in PSS (pH 7.4, gassed with 5% CO<sub>2</sub> in air), intraluminal pressure was raised to 70mmHg, and the vessel left to stabilise for 15 min before addition of 50mM KPSS (high potassium PSS, molar substitution with NaCl) or cumulative concentrations of NA (0.6, 3, and 15 $\mu$ M) at 2 min intervals. Following agonist treatment the arteries were incubated in Ca<sup>2+</sup> free PSS containing 2mM EGTA and passive lumen diameter recorded. To normalise responses for differences in starting vessel diameter the data were expressed as % of passive lumen diameter. A dual-chamber organ bath was used, allowing study of paired arterial segments in each experiment: a control artery that had received rabbit serum and a test artery that had received anti- $\alpha$ -adducin or anti-spectrin. The

concentrations of agonist used were chosen to cause minimal, sub-maximal, and maximal contraction. The total protocol took 90 min, thus minimising the time for degradation of antibodies.

### 2.1.10 Protein kinase inhibition

# 2.1.10.1. Bisindolylmaleimide I (GF109203X)

To inhibit PKC activity, the inhibitor GF109203X was used at 1 $\mu$ M, a concentration at which it abolished aldosterone-mediated contraction in RMSA (Michea et al., 2005). GF109203X was titrated to a minimum concentration of 1 $\mu$ M in M199 (0.01% final dimethyl sulfoxide [DMSO] concentration). Arteries were incubated for 30 min prior to stimulation, and then treated and homogenised as described in Sections 2.1.2 and 2.1.3.

#### 2.1.10.2. Go6976

In freshly isolated rat mesenteric smooth muscle cells, addition of 1µM Go6976 reduced the inhibition of Kv currents by ET-1, suggesting the involvement of conventional PKCs in vasoconstrictor-induced vascular smooth muscle signalling pathways (Rainbow et al., 2009). Accordingly, Go6976 was used at 1µM to inhibit conventional PKC isotypes ( $\alpha$ ,  $\beta$  and  $\gamma$ ). For a final concentration of 1µM, Go6976 was diluted in M199 (0.1% final DMSO concentration). Arteries were incubated for 30 min prior to stimulation, and then treated and homogenised as described in Sections 2.1.2 and 2.1.3.

### 2.1.10.3. Y27632

The Rho-kinase inhibitor Y27632 competes with ATP for binding to the catalytic site on Rho-kinase, thereby inactivating the phosphotransferase activity of the protein. The inhibitory constant of Y27632 is  $0.2-0.3\mu$ M for Rho-kinase and  $10\mu$ M for PKC. Thus its significantly greater (100-fold) activity against Rho-kinase than against PKCs is considered to be specific for evaluating specific Rho-kinase functions (Somlyo and Somlyo, 2003). In intact resistance arteries  $10\mu$ M Y27632 was used to decrease angiotensin II-induced contraction and significantly diminish extracellular signalrelated kinase (ERK) 1/2 activity (Matrougui et al., 2001). In the current study, Y27632 was titrated to a minimum concentration of  $10\mu$ M to inhibit Rho-kinase. For a final concentration of  $10\mu$ M the stock was diluted in M199 (0.1% final DMSO concentration). Arteries were incubated for 10 min prior to stimulation, and then treated and homogenised as described in Sections 2.1.2 and 2.1.3.

#### 2.1.10.4. H89

To inhibit PKA, H89 was used at a concentration of  $1\mu$ M, a concentration at which it inhibited phenylephrine-induced maintained increases in intracellular calcium (Utz et al., 1999). For a final concentration of  $1\mu$ M the stock was diluted in M199 (0.01% final DMSO concentration). Arteries were incubated for 10 min prior to stimulation, and then treated and homogenised as described in Sections 2.1.2 and 2.1.3.

### 2.1.10.5 DMSO control

In order to rule out any effect of DMSO, an experiment was carried out in which RMSA samples were treated with DMSO alone, at the highest concentration used for each particular inhibitor

### 2.1.11 Conditions for removal of extracellular calcium

To determine the effect of calcium influx on intracellular signalling, RMSA were incubated in  $Ca^{2+}$ -free HEPES buffer containing 1mM EGTA for 10 min prior to stimulation with NA or ET as described in Sections 2.1.2 and 2.1.3.

### 2.1.12 Conditions for activation of protein kinase A

### 2.1.12.1 IBMX

IBMX was used at a concentration of  $50\mu$ M to inhibit cyclic adenosine monophosphate (cAMP) degradation and therefore increase PKA activation. In rat vascular smooth muscle cells, this concentration has been shown to elevate basal levels of cAMP (Kubalak and Webb, 1993). For a final concentration of  $50\mu$ M the stock was diluted in M199 (0.01% final DMSO concentration). Arteries were incubated for 60 min, and then treated and homogenised as described in Sections 2.1.2 and 2.1.3.

## 2.1.12.2 Dibutyryl – cAMP

In order to determine the effect of PKA on  $\alpha$ -adducin phosphorylation, a cell permeable cAMP (dibutyryl-cAMP) was used at a concentration of 50 $\mu$ M (soluble in H<sub>2</sub>O). Arteries were incubated for 10 min, before being treated and homogenised as described in Sections 2.1.2 and 2.1.3.

## 2.1.13 Immunostaining

RMSA were dissected free from fat and connective tissue as described in Section 2.1.1 before being fixed in 10% paraformaldehyde in PBS. A tin foil container, cylindrical in shape, was placed in a vessel containing liquid nitrogen and half filled

with an optimal cutting temperature (OCT) compound (Raymond Lamb Eastbourne, UK). Dissected, fixed arteries were embedded in the OCT compound and stored at -  $80^{\circ}$ C. Frozen samples were cut into 5µm-thick cryosections using a cryostat, mounted on poly-D-lysine coated slides (Thermo Fisher Scientific, Rockford, USA) and stored at - $20^{\circ}$ C.

### 2.1.13.1 Cryosection staining

Slides containing RMSA sections were placed on a heat block and heated at  $60^{\circ}$ C for 10 min, followed by gentle washing in PBS + 50mM NH<sub>4</sub>Cl. Sections were then dehydrated and rehydrated by briefly placing slides in concentrations of ethanol from 30%–100% in the following order:

$$30\% \rightarrow 50\% \rightarrow 75\% \rightarrow 90\% \rightarrow 100\% \rightarrow 90\% \rightarrow 75\% \rightarrow 50\% \rightarrow 30\%$$

Sections were rinsed in 1% bovine serum albumin (BSA) solution before being incubated with primary antibody, diluted in 0.1% BSA, 0.3% Triton X-100 solution, overnight at 4°C. The following day, sections were washed in 0.1% BSA, 0.05% saponin, 0.2% gelatin solution (3 x 10 min) followed by incubation in either Alexa Fluor 488-conjugated (Molecular Probes, Invitrogen, Paisley, UK) or Texas-red-conjugated secondary antibody (Jackson labs, West Grove, PA, USA) for 1 hour in the dark. All subsequent incubations were done in the dark. Sections were washed in 0.1% BSA, 0.05% saponin, 0.2% gelatin solution (3 x 10 min), and then in PBS for 10 min. Fluorescent mounting medium (DAKO, California, USA) was added before sections were covered with glass coverslips and sealed. Slides were viewed using a Leica DM5000 B microscope, and photographed using a Leica DFC350FX Camera or

viewed on a Delta Vision RT (Applied Precision) restoration microscope using a 60x/1.42 Plan Apo objective and the Sedat filter set (Chroma 86000). Images were collected using a Coolsnap HQ (Photometrics) camera with a Z optical spacing of 0.2µm. Raw images were then deconvolved using the Softworx software. All images shown are representative of several independent experiments.

# 2.1.13.2 Conditions for use of antibodies

Using cultured smooth muscle cells, antibodies were titrated to a minimum concentration for all immunofluorescence experiments. Cells were stained with a range of primary antibody concentrations from 1: 200 to 1: 1000 and a concentration giving a clean, clear signal was chosen for each antibody. Antibodies and stains and the concentrations used for immunofluorescence are listed in **Table 2.3**.

			Secondary	Secondary
Antibody	Туре	Concentration	antibody	antibody
				concentration
Laboratory-	Polyclonal	1:200	Rabbit	1:400
developed α-adducin				
γ-adducin	Polyclonal	1:100	Rabbit	1:400
Phospho-adducin	Polyclonal	1:100	Rabbit	1:400
(Ser 724)				
Spectrin	Polyclonal	1:200	Rabbit	1:400
		_	_	
4.1	Polyclonal	1:100	Rabbit	1:400
Von Willebrand	Polyclonal	1:100	Rabbit	1:400
Factor (VWF)				
Phalloidin	Stain	1:1000	-	-
G-actin	Polyclonal	1:50	Rabbit	1:400
SMC actin	Monoclonal	1:400	Mouse	1:400

 Table 2.3 Antibodies and stains used for immunofluorescence experiments.

#### 2.2 Smooth muscle cells

#### 2.2.1. Isolating cells

Aortic smooth muscle cells were isolated from adult female Sprague Dawley rats. Aortas were removed and placed immediately in ice cold PSS and kept on ice. Surrounding fat was dissected and aortas were washed 4 times in 10ml serum free Dulbecco's modified eagle media (DMEM) (Invitrogen, Paisley, UK) supplemented with 0.375% Na bicarbonate (Invitrogen, Paisley, UK), 2mM glutamine (Invitrogen, Paisley, UK), 1x antibiotic-antimycotic (Invitrogen, Paisley, UK), 0.5µg/ml fungizome amphotericin B (Invitrogen, Paisley, UK). Aortas were then incubated at 37°C for 15 min in 10mg/ml collagenase (Lorne Laboratories, Reading, UK) in serum free DMEM (Solution 1), followed by further dissection to remove any remaining fat and adhering tissue. Lumens were rinsed by slicing aorta open and rinsing with serum-free DMEM. This was followed by a further 15 min incubation at 37°C in 2mg/ml collagenase (Lorne Laboratories, Reading, UK) in serum free DMEM (Solution 2). The adventitial sleeve was then removed using two pairs of forceps and the aorta was transferred to fresh Solution 2 and chopped finely. Incubation at 37°C was carried out for 2 hours with occasional agitation. All subsequent steps were carried out in a tissue culture hood. The sample was centrifuged at 700xg for 5 min and the pellet was resuspended in DMEM supplemented with 30% heat-inactivated fetal bovine serum (FBS) (Hyclone, Cramlington, UK), 0.375% Na bicarbonate, 2mM glutamine. 1x antibiotic-antimycotic, 0.5µg/ml fungizome amphotericin B. Supernatant was centrifuged again at 700xg for 5 min to pellet any remaining cells and the pellet was resuspended as described above. The two suspensions were pooled and transferred to sterile 35mm cell culture dishes. Media volume was increased to 5ml and cells were placed in an incubator at  $37^{\circ}$ C, 5% CO<sub>2</sub> for 3–4 days before use.

### 2.2.2 Smooth muscle cell culture

Vascular smooth muscle cells were cultured in 35mm dishes containing 5ml of DMEM supplemented with 10% heat-inactivated FBS, 0.375% Na bicarbonate, 2mM glutamine, 1x antibiotic-antimycotic, and 0.5µg/ml fungizome amphotericin B. Every 2 days, 2.5ml of media was removed and replaced with 2.5ml of fresh media. A complete media change was carried out every 4–5 days until cells were ready for use.

#### 2.2.3 Conditions for stimulation of smooth muscle cells

Cells were grown to 60–70% confluence in 35mm dishes and serum starved in serumfree media for 24 hours prior to use. To treat with NA, 7.5 $\mu$ l of 10mM NA was added to the 5ml of media in the culture dish for a final concentration of 15 $\mu$ M NA. Control dishes were treated with the same concentration of H<sub>2</sub>O. Cells were incubated at 37°C for the required amount of time, following which the media was removed and cells were rinsed in PBS.

#### 2.2.4. Immunofluorescence

Cells were grown on glass coverslips in 35mm dishes and treated as described above before 1–2 ml of 4% paraformaldehyde was added to dishes for 30 min to fix the cells. Cells were treated with 1–2 drops of 10% horse serum for 30 min at room temperature. 100–200 $\mu$ l of primary antibody in horse block (PBS + 0.1% Triton X-100 + 0.1% horse serum) was then placed on top of each coverslip and cells were incubated for 1 hour at room temperature followed by gentle washing in PBS. This was followed by incubation for 45 min at room temperature with 100–200 $\mu$ l of secondary antibody in horse block plus Hoescht stain (1: 10000) and phalloidin (1: 1000) where appropriate. Cells were washed gently again in PBS and mounted on glass slides with fluorescent mounting fluid (DakoCytomation, Ely, Cambridgeshire, UK).

### 2.3 Presentation of data and statistical analysis

Data are presented as mean  $\pm$  S.E. and statistical significance was determined by oneway analysis of variance (Tukey post hoc test or Dunnett correction for multiple comparisons) or paired *t* test using GraphPad Prism software.

# **2.4 Materials**

NA, albumin-agarose, Go6976 and Kodak biomax XAR film were purchased from Sigma Aldrich (Dorset, UK). Nitropure Nitrocellulose membrane (0.45 micron) was purchased from Genetic Research Instrumentation (Essex, UK) Endothelin, Phorbol 12,13-Dibutyrate (PdBu), Bisindolylmaleimide I (GF109203X), H-89 and Y-27632 were purchased from Calbiochem (EMD Biosciences, Inc. San Diego, CA). IBMX and dcAMP were a kind gift from Andrew Trafford.

# 2.4.1 Antibodies and stains

The phospho-adducin (Thr 445),  $\gamma$ -adducin and  $\beta$ -adducin antibodies were purchased from Santa Cruz Biotechnology (California, USA). The  $\alpha$ -adducin antibody was from Abcam (Cambridge, UK). The phospho-adducin (Ser 726) antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA) and the G-actin antibody was from Cytoskeleton (Denver, USA). Phalloidin and the smooth muscle cell actin antibody were from Sigma Aldrich (Dorset, UK). Horseradish peroxidise-conjugated secondary antibodies were from Jackson Laboratories (West Grove, PA, USA). The laboratory-developed  $\alpha$ -adducin antibody was a kind gift from John Hartwig, the spectrin and 4.1 antibodies were from Anthony Baines.

# 2.4.2 Buffers

- Physiological salt solution (PSS) contained (g/L): NaCl (6.954), KCl (0.35), NaHCO<sub>3</sub> (2.1), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.288), KH<sub>2</sub>PO<sub>4</sub> (0.161), K<sub>2</sub>EDTA (0.0011), Glucose (0.991), CaCl<sub>2</sub>.2H<sub>2</sub>O (0.368).
- MOPS Homogenisation Buffer (pH 7.2) contained: 0.15M NaCl, 20mM MOPS pH 7.2, 0.25M Sucrose, 1mM dithiothreitol (DTT), 1mM EGTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 200µM NaPyrophosphate and 1 Complete Mini protease inhibitor tablet (Roche Diagnostics).
- RIPA Buffer (pH 7.2) contained: 0.15M NaCl, 50mM Tris pH 7.2, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 5% glycerol, 1mM DTT, 50µM Na<sub>3</sub>VO<sub>4</sub>, 200µM NaPyrophosphate and 1 Complete Mini protease inhibitor tablet.
- **10X Running Buffer** contained (g/L): Tris (30), Glycine (145), sodium dodecyl sulphate (10).
- **10X TBS** (pH 8) contained (g/L): Tris (18.2) and NaCl (87).
- Membrane Stain contained 0.1% Napthol Blue Black, 10% Methanol and 2% Acetic acid.

- 10X PBS (pH 7.4) contained (g/L): NaCl (80), KCl (2), Na<sub>2</sub>HPO<sub>4</sub> (14.4), KH<sub>2</sub>PO<sub>4</sub> (2.4).
- Laemmli buffer contained: 0.32M Tris (pH 6.8), 5% (w/v) sodium dodecyl sulfate, 25% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 1% (w/v) bromophenol blue)
- **1% BSA solution** contained: 1% BSA, 0.05% saponin, 0.2% gelatin diluted in PBS
- F-actin stabilisation buffer contained: 50 mM PIPES pH 6.9, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 5% (v/v) Glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercapto-ethanol, 0.001% Antifoam C
- Blotting buffer contained: 25mM Tris, 200mM Glycine, 15% (v/v) methanol)

Chapter 3: Noradrenaline treatment elicits subcellular redistribution of cytoskeletal proteins in rat mesenteric small arteries

# **1.0 Introduction**

Dynamic actin filament turnover allows vessels to adapt rapidly in response to environmental changes, thus playing a vital role in various vascular smooth muscle functions. The actin-associated proteins adducin, spectrin and 4.1 are known to contribute to such cytoskeletal dynamics and have been well characterised in erythrocytes and other non-muscle cells. However their role in cytoskeletal regulation in vascular smooth muscle has not yet been determined. This chapter investigates the presence and localisation of a number of cytoskeletal proteins, including adducin and spectrin, in RMSA. The effect of vasoconstrictor activation on the subcellular localisation and interactions of  $\alpha$ -adducin and spectrin are also determined.

#### 2.0 Methods

The protocols for the experimental techniques used to gain the results described in this chapter are detailed in section 2.1 of Chapter 2.

### **3.0 Results**

### 3.1 Identification of cytoskeletal proteins in rat mesenteric small arteries

# 3.1.1 Investigation of the presence of adducin isoforms in rat mesenteric small arteries

Adducin has three isoforms,  $\alpha$ ,  $\beta$ , and  $\gamma$  (Manunta et al., 1996), and functions as  $\alpha$ - $\beta/\alpha$ - $\gamma$  dimers or tetramers (Hughes and Bennett, 1995). In the current study, isoformspecific antibodies were used to investigate the presence of adducin isoforms in RMSA. The presence of the  $\alpha$ -adducin isoform was demonstrated by immunochemical analysis with two  $\alpha$ -adducin antibodies: a laboratory-developed polyclonal  $\alpha$ -adducin antibody (described in Chapter 2), **Figure 3.1Ai**, and a commercial polyclonal  $\alpha$ -adducin antibody (**Figure 3.1Aii**). Both antibodies detected a band of approximately 120kDa. In addition, using a polyclonal  $\gamma$ -adducin antibody, a band of approximately 94kDa was identified (**Figure 3.1B**). Immunoblotting was also carried out using a polyclonal  $\beta$ -adducin antibody; however antigen reactivity was very poor.

# 3.1.2 Investigation of a-adducin localisation in rat mesenteric small arteries

In order to investigate the localisation of  $\alpha$ -adducin in RMSA, vessel cryosections were stained with a laboratory-developed  $\alpha$ -adducin antibody (**Figure 3.2A**). The staining (green), illustrates the presence of  $\alpha$ -adducin throughout vascular smooth muscle cells. Very little  $\alpha$ -adducin was present in endothelial cells as shown by comparison with von willebrand factor (VWF) (green) and smooth muscle cell actin (red) staining in **Figure 3.2B**. However, matched IgG controls will be required in future experiments to rule out non-specific staining and confirm the specificity of the adducin antibody.



Figure 3.1  $\alpha$ - and  $\gamma$ -adducin are present in rat mesenteric small artery extracts. Untreated vessels were homogenised in RIPA buffer, and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. Membranes were probed with isoform-specific adducin antibodies. Ai. A laboratory-developed  $\alpha$ -adducin antibody detected a band of approximately 120kDa. Aii. A commercial polyclonal  $\alpha$ -adducin antibody detected a band of approximately 120kDa. B. A polyclonal  $\gamma$ -adducin antibody detected a band of approximately 94kDa. Blots shown are representative of three experiments.

 $\alpha$ -adducin (green)

 $\alpha$ -adducin (green)

A.



Von Willebrand Factor (green)

Figure 3.2 a-adducin is localised in smooth muscle cells of rat mesenteric small arteries. Frozen RMSAs were cut into 5µm-thick cryosections and mounted on poly-D-lysine coated slides. A. Sections were stained with the laboratory-developed  $\alpha$ adducin antibody (green).  $\alpha$ -adducin was seen in vascular smooth muscle cells throughout the section, however very little was present in endothelial cells. B. Sections were stained with smooth muscle cell actin (red) and Von Willebrand Factor (green) to define smooth muscle cells and endothelial cells within the cryosection. Pictures shown are representative of three experiments.

### 3.1.3 Investigation of spectrin localisation in rat mesenteric small arteries

Using a polyclonal spectrin antibody, a band of approximately 200kDa was identified in RMSA extracts using immunochemical analysis (**Figure 3.3A**). The same antibody was used to stain RMSA cryosections and spectrin could be seen in smooth muscle cells with a similar distribution to that of  $\alpha$ -adducin (**Figure 3.3B**).

#### 3.1.4 Investigation of protein 4.1 localisation in rat mesenteric small arteries

Using a polyclonal protein 4.1 antibody, a band of approximately 80kDa was identified in RMSA extracts using immunochemical analysis (**Figure 3.4A**). The same antibody was used to stain RMSA cryosections, however in this case the majority of the protein 4.1 was located in endothelial cells with very little located in smooth muscle cells (**Figure 3.4B**).

Again, matched IgG controls will be required in future experiments to rule out nonspecific staining and confirm the specificity of the spectrin and protein 4.1 antibodies.





Figure 3.3 Spectrin is present in rat mesenteric small arteries. A. Untreated vessels were homogenised in RIPA buffer, and  $20\mu l$  of lysate was loaded onto a 9% SDS polyacrylamide gel. Membranes were probed with a polyclonal spectrin antibody. A band of approximately 200kDa was detected. **B.** RMSA cryosections (5µm thick) were stained with the polyclonal spectrin antibody (green). Spectrin can be seen in smooth muscle cells with a small amount present in endothelial cells. Pictures shown are representative of three experiments.





Figure 3.4 Protein 4.1 is present in rat mesenteric small arteries. A. Untreated vessels were homogenised in RIPA buffer, and  $20\mu l$  of lysate was loaded onto a 9% SDS polyacrylamide gel. Membranes were probed with a polyclonal 4.1 antibody. A band of approximately 80kDa was detected. **B.** RMSA cryosections ( $5\mu m$  thick) were stained with the polyclonal protein 4.1 antibody (green) and smooth muscle cell actin (red). Protein 4.1 can be seen mainly in the endothelial cells with minimal staining in the smooth muscle cells. Pictures shown are representative of three experiments.

# **3.2** Investigation of the subcellular distribution of cytoskeletal proteins in vascular smooth muscle

# 3.2.1 Investigation of the subcellular distribution of $\alpha$ -adducin in rat mesenteric small arteries

To determine whether  $\alpha$ -adducin is associated with the actin cytoskeleton in vascular smooth muscle, its subcellular distribution in small artery extracts was investigated. RMSA lysates were separated into subcellular components as described in Chapter 2. A representative western blot displaying  $\alpha$ -adducin in cytosolic, Triton X-100-soluble, and Triton X-100-insoluble fractions is shown in **Figure 3.5A**, and densitometric data are shown in **Figure 3.5B**. In unstimulated tissue, 57.0% of adducin was present in the Triton X-100-insoluble (cytoskeletal) fraction, significantly more than the 11.3% present in the Triton X-100-soluble fraction and the 31.7% in the cytosolic fraction (p<0.001).



Figure 3.5 a-adducin is associated with the actin cytoskeleton in rat mesenteric arteries. Untreated RMSA homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions.  $30\mu$ l of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the laboratory-developed  $\alpha$ -adducin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows percentage of total antigen present in each fraction. A mean of 57.0% of antigen was present in the cytoskeletal fraction with 31.7% in the cytosolic fraction and 11.3% in the Triton X-100-soluble fraction. P-values are difference from cytosol: \*\* p < 0.01; \*\*\* p < 0.001; n=36, where n includes all vessels dissected from one animal.

### 3.2.2 Investigation of the distribution of $\gamma$ -adducin in rat mesenteric small arteries

Following fractionation of untreated RMSA as described above, immunoblotting was carried out with the polyclonal  $\gamma$ -adducin antibody. A representative immunoblot showing the subcellular distribution of  $\gamma$ -adducin is shown in **Figure 3.6A**, and densitometric data are shown in **Figure 3.6B**. In unstimulated tissue, 76.9% of antigen was present in the cytosolic fraction, significantly more than the 7.4% associated with the Triton X-100-insoluble cytoskeleton and the 15.7% in the Triton X-100-soluble membrane fraction (p<0.001).

# 3.2.3 Investigation of the subcellular distribution of spectrin in rat mesenteric small arteries

To determine the subcellular localisation of spectrin in smooth muscle cells, differential centrifugation was carried out in RMSA and subcellular fractions were used for immunoblotting with the polyclonal spectrin antibody. A representative immunoblot is shown in **Figure 3.7A**, and densitometric data are shown in **Figure 3.7B**. In unstimulated tissue, the distribution of spectrin was similar to that of  $\alpha$ -adducin; the majority of spectrin was located in the Triton X-100-insoluble cytoskeleton (62.4%). This proportion was significantly higher than the levels in the cytosolic and Triton X-100-soluble fractions (19.8% and 17.8%, respectively), (P<0.001).



Figure 3.6. The majority of  $\gamma$ -adducin is present in the cytosolic fraction in rat mesenteric arteries. Untreated RMSA homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100soluble and cytoskeletal fractions.  $30\mu$ l of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the  $\gamma$ adducin antibody at a concentration of 1: 500. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. Data are presented as mean  $\pm$ S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Graph shows percentage of total antigen present in each fraction. A mean of 76.9% of antigen was present in the cytosolic fraction, significantly more than the 7.4% in the Triton X-100soluble fraction and the 15.7% in the cytoskeletal fraction. P-values are difference from cytosol: \*\*\* p<0.001; n=11, where n includes all vessels dissected from one animal.



Figure 3.7. The majority of spectrin is present in the cytoskeletal fraction in rat mesenteric arteries. Untreated RMSA homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions.  $30\mu$ l of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the spectrin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Graph shows percentage of total antigen present in each fraction. A mean of 19.8% of antigen was present in the cytosolic fraction and 17.8% was present in the Triton X-100-soluble fraction. P-values are difference from cytoskeleton: p<0.001; n=26, where n includes all vessels dissected from one animal.

**3.3** Temporal effects of noradrenaline on the subcellular distribution of cytoskeletal proteins in rat mesenteric small arteries

# 3.3.1 Investigation of the effect of 1 min noradrenaline treatment on the subcellular distribution of $\alpha$ -adducin and spectrin

3.3.1.1 The effect of 1 min noradrenaline treatment on the subcellular distribution of  $\alpha$ -adducin

A number of studies have described NA-induced changes in the subcellular distribution of signalling molecules in RMSA (Clarke et al., 2007; Ohanian et al., 2005; Srinivasan et al., 2008). To investigate whether NA treatment alters the subcellular distribution of  $\alpha$ -adducin in RMSA, vessels were treated with NA (15µM) for 1 min before carrying out subcellular fractionation. Following NA treatment, there was an increase in the proportion of  $\alpha$ -adducin present in the cytosolic fraction (1 a.u. in resting vessels vs 2.94 a.u. in NA-treated vessels, P<0.001, **Figure 3.8A**). However, there was no significant change in the proportion of  $\alpha$ -adducin associated with the Triton X-100-soluble membrane (1 a.u. in resting vessels *vs* 1.13 a.u. in NA-treated vessels, **Figure 3.8B**). Nevertheless, the proportion of  $\alpha$ -adducin in the cytoskeletal fraction was slightly but significantly lower in NA-treated vessels than in non-stimulated vessels (1 a.u. in non-stimulated resting vessels vs 0.70 a.u. in NA-treated vessels, p<0.001, **Figure 3.8C**), suggesting NA-induced dissociation of  $\alpha$ -adducin from the cytoskeleton.

# 3.3.1.2 The effect of protein kinase inhibition on the noradrenaline-induced dissociation of $\alpha$ -adducin from the cytoskeleton

In other cell types  $\alpha$ -adducin is regulated by PKC and Rho-kinase, proteins that are known to be important in smooth muscle function. Accordingly we investigated the

effect of protein kinase inhibition on NA-induced dissociation of α-adducin from the cytoskeleton. Vessels were pre-treated with either the PKC inhibitor GF109203X (1µM) or the Rho-kinase inhibitor Y27632 (10µM) before incubation with NA for 1 min (**Figure 3.8**). In the presence of GF109203X, NA no longer induced a significant increase in cytosolic α-adducin (1 a.u. in unstimulated vessels vs 1.58 a.u. in NA-treated vessels pre-treated with GF109203X, ns, **Figure 3.8C**), or a significant reduction in cytoskeletal α-adducin (1 a.u. in unstimulated vessels vs 0.92 a.u. in NA-treated vessels pre-treated with GF109203X, ns). However, in the presence of Y27632, NA continued to induce a significant increase in cytosolic α-adducin (1 a.u. in unstimulated vessels pre-treated with Y27632, NA continued to induce a significant increase in cytosolic α-adducin (1 a.u. in unstimulated vessels vs 2.64 a.u. in NA-treated vessels pre-treated with Y27632, P<0.01, **Figure 3.8A**) and a small but significant reduction in cytoskeletal α-adducin (1 a.u. in unstimulated vessels pre-incubated with Y27632, P<0.05, **Figure 3.8C**).



Figure 3.8 Noradrenaline induces protein kinase C-dependent changes in the subcellular distribution of  $\alpha$ -adducin. RMSAs were treated with 15µM NA for 1 min in the presence or absence of 1µM GF109203X or 10µM Y27632 and homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions.  $30\mu l$  of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the spectrin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. To control for variability in the proportion of spectrin present in the three fractions across different experiments, data were then corrected to CNL samples, where CNL was 1.00 (arbitrary units). Data are presented as mean ± S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. NA induced an increase in the proportion of  $\alpha$ -adducin in the cytosol, an effect which was inhibited by GF109203X, but not by Y27632. B. NA had very little effect on the proportion of  $\alpha$ -adducin in the Triton X-10- soluble fraction. C. NA reduced the proportion of  $\alpha$ -adducin present in the cytoskeletal fraction, an effect which was inhibited by GF109203X, but not by Y27632. P-values are difference from untreated vessels (CNL): \*\*\* p<0.0001, \*\* p<0.001, \* *p*<0.05.; *n*=4.

3.3.1.3 The effect of 1 min noradrenaline treatment on the subcellular distribution of spectrin

As  $\alpha$ -adducin and spectrin had a similar subcellular distribution in resting vessels, and NA treatment for 1 min induced a change in the subcellular distribution of  $\alpha$ -adducin, the effect of NA treatment on the subcellular distribution of spectrin was then investigated. In contrast to its effect on  $\alpha$ -adducin, NA treatment for 1 min had very little effect on spectrin distribution in the cytosol (1 a.u. in resting vessels vs 1.21 a.u. in NA-treated vessels, ns, **Figure 3.9A**), the Triton X-100-soluble membrane (1 a.u. in resting vessels vs 1.04 a.u, in NA-treated vessels, ns, **Figure 3.9B**) or the Triton X-100-insoluble cytoskeleton (1.00 a.u. in resting vessels vs 1.00 a.u. in NA-treated vessels, ns, **Figure 3.9C**).



Figure 3.9 Noradrenaline treatment for 1 min has very little effect on the subcellular distribution of spectrin. RMSAs were treated with NA and homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions.  $30\mu l$  of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the spectrin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. To control for variability in the proportion of spectrin present in the three fractions across different experiments, data were then corrected to CNL samples, where CNL was 1.00 (arbitrary units). Data are presented as mean  $\pm$  S.E. and statistical significance was determined by Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. Graph shows the change in the amount of spectrin present in the cytosolic fraction following NA stimulation.NA had very little effect on the proportion of cytosolic **B**. Graph shows the change in the amount of spectrin present in the Triton X-100-soluble fraction following NA stimulation.NA had very little effect on the proportion of Triton X-100-soluble spectrin. C. Graph shows the change in the amount of spectrin present in the cytoskeletal fraction following NA stimulation. NA had no effect on the proportion of cytoskeletal spectrin; n=10, where n includes all vessels dissected from one animal.

# 3.3.2 Investigation of the effect of 5 min noradrenaline treatment on the subcellular distribution of $\alpha$ -adducin and spectrin

3.3.2.1 The effect of 5 min noradrenaline treatment on the subcellular distribution of  $\alpha$ -adducin

To determine whether the changes in  $\alpha$ -adducin distribution were sustained, vessels were treated with NA for either 1 min or 5 min before subcellular fractionation was carried out. Changes in the cytoskeletal and cytosolic distribution after NA treatment for 1 min were as described in Section 3.4.1; NA induced a significant increase in cytosolic  $\alpha$ -adducin (p<0.01) and a significant reduction in cytoskeletal  $\alpha$ -adducin (p<0.01) (Figure 3.10A and C, respectively). However after NA treatment for 5 min, the proportion of a-adducin present in the cytosolic fraction was no longer significantly higher than in unstimulated vessels (1 a.u. in unstimulated vessels vs 1.64 a.u. in NA 5 min-treated vessels, ns), Figure 3.10A. Moreover, after NA treatment for 5 min the proportion of  $\alpha$ -adducin in the cytoskeletal fraction was no longer significantly lower than in unstimulated vessels (1 a.u. in unstimulated vessels vs 0.98 a.u. in NA 5 min-treated vessels, ns). The resulting subcellular distribution of  $\alpha$ -adducin was similar to that in non-stimulated vessels, suggesting that NA treatment for 5 min induces reassociation of a-adducin with the cytoskeleton subsequent to its initial dissociation. No significant change was seen in the proportion of  $\alpha$ -adducin in the Triton-X-100 soluble fraction (Figure 3.10B).

# 3.3.2.2 The effect of Rho-kinase inhibition on noradrenaline-induced reassociation of $\alpha$ -adducin with the cytoskeleton

The NA-induced dissociation of  $\alpha$ -adducin from the cytoskeleton was PKCdependent. This made it difficult to determine the PKC dependence of its subsequent reassociation with the cytoskeleton, as adding GF109203X as a pre-treatment inhibited the initial NA-induced distribution changes. However, NA-induced dissociation of a-adducin from the cytoskeleton was independent of Rho-kinase; therefore we were able to investigate the effect of Rho-kinase inhibition on the subsequent reassociation of  $\alpha$ -adducin with the cytoskeleton. Accordingly, vessels were pre-incubated with Y27632 before being treated with NA for 5 min (Figure **3.10**). In the presence of Y27632, NA treatment for 5 min induced a significant increase in cytosolic  $\alpha$ -adducin (1 a.u. in unstimulated vessels vs 4.05 a.u in NA 5 min-treated vessels pre-treated with Y27632, p<0.001, Figure 3.10A) and a significant reduction in cytoskeletal a-adducin (1 a.u. in unstimulated vessels vs 0.65 a.u. in vessels treated with NA (5 min) in the presence of Y27632, p<0.001, Figure **3.10C**). Put simply, the subcellular distribution of  $\alpha$ -adducin after treatment with NA for 5 min in the presence of Y27632 was similar to that after treatment with NA for 1 min, suggesting that NA (5 min)-induced reassociation of  $\alpha$ -adducin with the cytoskeleton is dependent upon Rho-kinase. Although a notable reduction was seen in the proportion of  $\alpha$ -adducin in the Triton X-100-soluble fraction in the presence of both inhibitors, these changes were not significant (Figure 3.10B).

# 3.3.2.3 The effect of 5 min noradrenaline treatment on the subcellular distribution of spectrin

Although NA treatment for 1 min had very little effect on the subcellular distribution of spectrin it was important to determine any effects of NA treatment for 5 min on the subcellular distribution of spectrin. Vessels were treated for 5 min with NA and subjected to fractionation as before (**Figure 3.11**). After treatment with NA for 5min, a significant reduction in the proportion of spectrin in the cytosol was seen (1 a.u. in

untreated vessels vs 0.40 a.u. in NA-treated vessels, p<0.05, Figure 3.11A), coupled with an increase in the proportion of spectrin in the cytoskeletal fraction (1 a.u. in untreated vessels vs 1.43 a.u. in NA-treated vessels, p<0.05, Figure 3.11C). A marked increase in the proportion of spectrin in the Triton X-100-soluble fraction was also observed in response to NA treatment for 5 min (1 a.u. in untreated vessels vs 1.52 a.u. in NA-treated vessels, Figure 3.11B); however this increase was not significant.

# 3.3.2.4 The effect of protein kinase inhibition on the noradrenaline-induced increase in cytoskeletal spectrin

There is no evidence in the literature to suggest that spectrin is regulated by either PKC or Rho-kinase, however adducin is known to promote the association of spectrin with actin (Gardner and Bennett, 1987) and as the previous sections show, the localisation of α-adducin is clearly regulated by both PKC and Rho-kinase in RMSA. Therefore the effect of pre-incubation with either GF109203X or Y27632 on spectrin distribution was investigated. In the presence of GF109203X or Y27632, NA (5 min) failed to induce a reduction in cytosolic spectrin (1 a.u. in untreated vessels, 1.69 a.u. in NA-treated vessels pre-treated with GF109203X, 2.25 a.u. in NA-treated vessels pre-treated with Y27632, ns, Figure 3.11A) or an increase in cytoskeletal spectrin (1 a.u. in untreated vessels, 0.72 a.u. in NA-treated vessels pre-treated with GF109203X, 0.70 a.u. in NA-treated vessels pre-treated with Y27632, ns, Figure 3.11C), indicating that the NA-induced changes in spectrin distribution rely on both PKC and Rho-kinase. Marked increases were seen in the proportion of spectrin in the Triton X-100-soluble fraction in the presence of both inhibitors (1 a.u. in untreated vessels, 3.88 a.u. in NA-treated vessels pre-treated with GF109203X, 4.52 a.u. in NA-treated vessels pre-treated with Y27632, ns, Figure 3.11B); however these effects were not significant.



Figure 3.10 Noradrenaline treatment for 5 min induces Rho-kinase-dependent changes in the subcellular distribution of  $\alpha$ -adducin. RMSAs were treated with 15µM NA in the presence/absence of 10µM Y27632, and homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions. 30µl of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the laboratory-developed  $\alpha$ -adducin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. To control for variability in the proportion of spectrin present in the three fractions across different experiments, data were then corrected to CNL samples, where CNL was 1.00 (arbitrary units). Data are presented as mean  $\pm$  S.E. and statistical significance was determined by Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. Graph shows the change in the amount of  $\alpha$ -adducin present in the cytosolic fraction. Following an increase in cytosolic  $\alpha$ -adducin after 1 min NA treatment, the level was reduced in response to 5 min treatment, an effect which was inhibited by Y27632. B. Graph shows the change in the amount of  $\alpha$ -adducin present in the cytosolic Triton X-100-soluble fraction.NA had no significant effect on the proportion of  $\alpha$ -adducin in the Triton X-100-soluble fraction. C. Graph shows the change in the amount of  $\alpha$ -adducin present in the cytoskeletal fraction. NA treatment for 1 min reduced the proportion of  $\alpha$ -adducin present in the cytoskeletal fraction, which was then increased following min NA treatment, an effect which was inhibited by Y27632. P-values are difference from untreated vessels (CNL): \*\*\* p < 0.0001, \*\* p < 0.001; n=4, where n includes all vessels dissected from one animal.



Figure 3.11 Noradrenaline treatment for 5 min induces protein kinase C- and Rho-kinasedependent changes in the subcellular distribution of spectrin. RMSAs were treated with  $15 \mu M$  NA in the presence/absence of  $1 \mu M$  GF109203X or  $10 \mu M$  Y27632, and homogenates were subjected to differential centrifugation to separate subcellular components to form cytosolic, Triton X-100-soluble and cytoskeletal fractions. 30µl of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the spectrin antibody at a concentration of 1: 1000. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. To control for variability in the proportion of spectrin present in the three fractions across different experiments, data were then corrected to CNL samples, where CNL was 1.00 (arbitrary units). Data are presented as mean  $\pm$  S.E. and statistical significance was determined by Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. NA treatment for 5 min reduced cytosolic spectrin, an effect which was blocked by both GF109203X and Y27632. B. NA increased the proportion of spectrin in the Triton X-100soluble fraction, and a further increase was seen in the presence of GF109203X or Y27632, however the effects were not significant. C. NA treatment for 5 min increased cytoskeletal spectrin, an effect which was blocked by both GF109203X and Y27632. P-values are difference from untreated vessels (CNL): \* p < 0.05, n=3, where n includes all vessels dissected from one animal.

### **3.4 Investigation of the interaction between α-adducin and spectrin**

The data presented in the previous figures show that both  $\alpha$ -adducin and spectrin are present in RMSAs and that their subcellular distribution is similar in non-stimulated vessels. *In vitro*, adducin forms a complex with the fast growing ends of actin filaments in order to recruit spectrin (Li et al., 1998). Accordingly, the interaction between the two proteins in RMSA was investigated.

# 3.4.1 Investigation of the interaction between $\alpha$ -adducin and spectrin in nonstimulated rat mesenteric small arteries

RMSA lysates were incubated with the  $\alpha$ -adducin antibody, the spectrin antibody or rabbit IgG, and probed for  $\alpha$ -adducin and spectrin pulldown. In non-stimulated vessels, a small amount of spectrin was present in adducin immunoprecipitates. However, no  $\alpha$ -adducin was present in spectrin immunoprecipitates. No spectrin was detected in lysates which had been incubated with rabbit IgG as a control (**Figure 3.12**).

# 3.4.2 Investigation of the effect of noradrenaline treatment for 1 min on the interaction between $\alpha$ -adducin and spectrin

The current study has demonstrated that treatment with NA for 1 min alters the subcellular distribution of  $\alpha$ -adducin but has little effect on spectrin distribution. To determine whether the change in  $\alpha$ -adducin distribution was coupled with a change in association between  $\alpha$ -adducin and spectrin, lysates were stimulated with NA for 1 min before incubation with the  $\alpha$ -adducin antibody. A representative immunoblot is shown in **Figure 3.13A**. Densitometric data are shown in **Figure 3.13B**. The level of spectrin present in the  $\alpha$ -adducin immunoprecipitates decreased following stimulation
with NA for 1 min (P<0.001). In the presence of GF109203X, NA failed to induce a reduction in the amount of spectrin present in  $\alpha$ -adducin immunoprecipitates.

# 3.4.3 Investigation of the effect of noradrenaline treatment for 5 min on the association between $\alpha$ -adducin and spectrin

Section 3.3 demonstrated NA (5 min)-induced increases in cytoskeletal  $\alpha$ -adducin and spectrin. Therefore the association between the two proteins after 5 min stimulation was also investigated. Treatment with NA for 5 min induced a large increase in the amount of spectrin present in  $\alpha$ -adducin immunoprecipitates (P<0.05), suggesting an increase in association between the two proteins (**Figure 3.14**). Further investigation into the Rho-kinase-dependency of this increase in association produced inconclusive results (data not shown).



Figure 3.12 Adducin and spectrin interact in non-stimulated rat mesenteric small arteries. Untreated RMSAs were homogenised and immunoprecipitation was carried out with rabbit IgG,  $\alpha$ -adducin antibody or spectrin antibody. 10µl each of immunoprecipitate (IP) and supernatant (SN) samples were loaded onto 9% SDS polyacrylamide gels and immunoblotting was carried out with both  $\alpha$ -adducin and spectrin antibodies. Neither  $\alpha$ -adducin nor spectrin were present in samples which had been incubated with rabbit IgG. A small amount of spectrin was pulled down following incubation with the  $\alpha$ -adducin antibody. No  $\alpha$ -adducin was detected in samples which had been incubated with spectrin antibody. Blot shown is representative of three experiments. IP, immunoprecipitate; SN, supernatant.



Figure 3.13 Noradrenaline treatment for 1 min reduces the interaction between aadducin and spectrin. RMSAs were treated with  $15\mu$ M NA for 1 min in the presence and absence of  $1\mu$ M GF109203X. Vessels were then homogenised and immunoprecipitation was carried out with  $\alpha$ -adducin antibody.  $10\mu$ l each of immunoprecipitate (IP) and supernatant (SN) samples were loaded onto 9% SDS polyacrylamide gels and immunoblotting was carried out with both  $\alpha$ -adducin and spectrin antibodies. Data are presented as mean  $\pm$  S.E. Means were calculated as the ratio of spectrin antigen: adducin antigen and all data were corrected to CNL (where CNL is 1.0 arbitrary unit) for individual experiments. Statistical significance was determined by paired t-test. A. A representative immunoblot is shown. B. Graph shows the amount of spectrin present in untreated, NA-treated and NA-and GF109203X-treated vessels. NA reduced the amount of spectrin present in  $\alpha$ -adducin immunoprecipitates, an effect which was inhibited by GF109203X. P-values are difference from untreated vessels (CNL): P<0.001; n=5, where n includes all vessels dissected from one animal. IP, immunoprecipitate; SN, supernatant.



Figure 3.14 Treatment with noradrenaline for 5 min increases the association between a-adducin and spectrin. RMSAs were treated with  $15\mu$ M NA for 5 min before being homogenised. Immunoprecipitation was carried out with  $\alpha$ -adducin antibody.  $10\mu$ l of immunoprecipitate (IP) sample was loaded onto 9% SDS polyacrylamide gels and immunoblotting was carried out with both  $\alpha$ -adducin and spectrin antibodies. Data are presented as mean  $\pm$  S.E. Means were calculated as the ratio of spectrin antigen: adducin antigen and all data were corrected to CNL (where CNL is 1.0 arbitrary unit) for individual experiments. Statistical significance was determined by paired t-test. A. A representative immunoblot is shown. B. Chart shows the amount of spectrin present in untreated (CNL) and NA-treated vessels. NA significantly increased the amount of spectrin present in  $\alpha$ -adducin immunoprecipitates, P-values are difference from CNL: P<0.05; n=4, where n includes all vessels dissected from one animal. IP, immunoprecipitate.

#### 4.0 Discussion

Vasoconstrictors induce actin filament formation in vascular smooth muscle tissue and cultured smooth muscle cells, and recently there has been evidence that this cytoskeletal rearrangement may be instrumental in sustaining vascular smooth muscle contraction (Barany et al., 2001). Moreover, cytoskeletal rearrangement is vital for other cellular processes in smooth muscle such as small artery remodelling (van den Akker et al., 2010), migration (Gerthoffer, 2007), and proliferation (Ho and Bendeck, 2009), yet the presence and function of the actin binding proteins adducin and spectrin in smooth muscle have not been studied.

The current study demonstrates the presence of adducin in vascular smooth muscle tissue along with spectrin and protein 4.1. The  $\alpha$ -isoform of adducin is ubiquitously expressed and it has been reported that the  $\gamma$ -isoform is also expressed in a number of cell types (Matsuoka et al., 2000). Expression of the  $\beta$ -isoform however is less common and has been studied mostly in erythrocytes and brain (Gilligan et al., 1999). In the current study, two polyclonal antibodies were used to detect the presence of  $\alpha$ -adducin in RMSA and both detected a band of approximately 120kDa. The  $\alpha$ -adducin isoform has been detected in other cell types such as fibroblasts, T lymphocytes, erythrocytes and platelets at approximately 120kDa (Gilligan et al., 2002; Joshi and Bennett, 1990; Lu et al., 2004; Waseem and Palfrey, 1990). In addition, a  $\gamma$ -adducin antibody detected a band of approximately 94kDa on western blots. This correlates to the size of  $\gamma$ -adducin in a number of other tissues including rat kidney (Dong et al., 1995), platelets (Gilligan et al., 2002) and brain (Yang et al., 2002). As the selectivity of the laboratory-developed  $\alpha$ -adducin antibody had been confirmed in a previous study (Barkalow et al., 2003), and the commercial  $\alpha$ -adducin and  $\gamma$ -adducin antibodies

gave convincing bands of the correct size, it was concluded that the  $\alpha$ - and  $\gamma$ -isoforms were present in RMSA. However, in order to further strengthen the evidence for the presence of  $\alpha$ - and  $\gamma$ -adducin, peptide block experiments should be carried out in which the nitrocellulose membrane is probed with the antigen used to produce the antibody prior to western blotting with the adducin antibody. Absence of the adducin band under these conditions would provide more conclusive evidence for the presence of the protein as it would eliminate the possibility that non-specific binding may be contributing to the adducin signal observed.

The  $\beta$ -adducin antibody used in this study is a polyclonal antibody raised against amino acids 581–700 of human  $\beta$ -adducin which should detect  $\beta$ -adducin of mouse, rat and human origin. Several attempts were made to produce a clear and consistent signal with this antibody; however the only band produced was one at 120kDa, the same molecular weight as the band correlating to  $\alpha$ -adducin. In brain,  $\beta$ -adducin has a molecular weight of 110kDa (Costessi et al., 2006), while in erythrocytes and spleen its molecular weight is 97kDa (Joshi et al., 1991). The size of the band detected by the  $\beta$ -adducin antibody in the current study and the fact that the signal was produced inconsistently, led to the conclusion that the  $\beta$ -adducin antibody could be crossreacting with the  $\alpha$ -isoform, however further investigation is needed to confirm the absence of  $\beta$ -adducin in vascular smooth muscle. Nonetheless, our results along with the results of Citterio et al, who only detected the  $\alpha$ - and  $\gamma$ -adducin genes ADD1 and ADD3 in human vascular smooth muscle cells and found that the  $\beta$ -adducin genes ADD2  $\beta$ 1 and ADD2  $\beta$ 2 were not present (Citterio et al., 2003), suggest that only two adducin isoforms,  $\alpha$ - and  $\gamma$ , are present in RMSA. The presence of both  $\alpha$  and  $\gamma$  isoforms in the current study may suggest that adducin functions as an  $\alpha$ - $\gamma$  heteromer in RMSA. However, the results demonstrate a large difference in the subcellular distribution of  $\alpha$ - and  $\gamma$ -adducin, with the majority of  $\alpha$ adducin present in the cytoskeletal fraction and most of the  $\gamma$ -adducin detected in the cytosolic fraction. The need for heterologous binding partners for  $\alpha$ -adducin was brought into question by the  $\beta$ -  $\gamma$  adducin knockout study of Sahr *et al.* (Sahr et al., 2009). In nonerythroid tissues analysed such as the kidney,  $\alpha$ -adducin was considerably more stable in the absence of  $\beta$ - or  $\gamma$ -adducin. The apparent stability of  $\alpha$ -adducin in the absence of  $\beta$ - or  $\gamma$ -adducin may be due to stabilisation of the  $\alpha$ isoform through interactions with other membrane components or  $\alpha$ -adducin homooligomer formation. Interestingly, recombinant adducin constructs can form homodimers, although their presence has not been detected in vivo (Bennett and Baines, 2001). The large difference in the subcellular distributions of  $\alpha$  and  $\gamma$  adducin presented here would support the idea that  $\alpha$ -adducin may be acting as a homodimer in smooth muscle tissue. In agreement with the data presented here,  $\alpha$ - and  $\gamma$ -adducin localise to the apical and basal membranes of renal proximal tubules respectively, suggesting that the two isoforms may have distinct structural and functional properties (Fowler et al., 1998b).

It should be noted here that although the subcellular fractionation protocol used is a well established method, in the experiments presented here no lane controls were used to probe membranes for cytosolic, Triton X-100-soluble and cytoskeletal proteins. For future experiments, this should be carried out to ensure that cytosolic, Triton X-100-soluble and cytoskeletal fractions have been accurately separated.

As spectrin was present in RMSA in the current study, it was interesting to detect a band of the correct size in RMSA lysates using a protein 4.1 antibody. However, further investigation revealed that the majority of protein 4.1 in RMSA was expressed in the endothelial cells; therefore further investigation was not carried out.

The  $\alpha$ -adducin isoform was chosen for further investigation in the current study, as its importance has been demonstrated in a number of studies. Tripodi *et al.* performed a number of mutation studies in which adducin mutated only on the  $\beta$  subunit behaved similarly to normal adducin, whereas adducin mutated only on the  $\alpha$  subunit exhibited an intermediate behaviour (Tripodi et al., 1996). In  $\alpha$ -adducin knockout mice,  $\beta$ - and  $\gamma$ -adducin are also absent in red blood cells (despite normal mRNA expression), indicating that  $\alpha$ -adducin is the limiting subunit in tetramer formation (Robledo et al., 2008). Spectrin was also chosen for further investigation, due to its documented interactions with adducin in other cell types. However it will be of interest to further investigate the function of the other cytoskeletal proteins detected in smooth muscle in this chapter, particularly  $\gamma$ -adducin. Furthermore, it will be important to determine the presence of proteins that have not been investigated here, but are known to be involved in cytoskeletal rearrangement in other cell types e.g. ankyrin, tropomyosin and tropomodulin.

Adducin has been shown to associate with the cytoskeleton or the plasma membrane in a number of cell lines (Barkalow et al., 2003; Kaiser et al., 1989; Kimura et al., 1998; Nehls et al., 1991). Here, it is clear from fractionation studies that the majority of both  $\alpha$ -adducin and spectrin is associated with the Triton X-100 insoluble cytoskeleton in unstimulated RMSA, in agreement with its distribution in other cell types. Moreover, immunoprecipitation experiments suggested an association between  $\alpha$ -adducin and spectrin in non-stimulated vessels. It is important to mention that these proteins may be part of a multi-protein complex, which can be pulled down by the  $\alpha$ -adducin antibody; indeed this is likely given the number of proteins thought to interact with actin to regulate cytoskeletal dynamics (dos Remedios et al., 2003). Therefore the interaction between  $\alpha$ -adducin and spectrin may be an indirect one.

Interestingly, **RMSA** lysates incubated with spectrin antibody during immunoprecipitation experiments did not contain detectable levels of  $\alpha$ -adducin. This may be due to competition for the binding site on spectrin between the spectrin antibody and  $\alpha$ -adducin i.e. both  $\alpha$ -adducin and the spectrin antibody bind spectrin at the same site. It should also be noted that two different  $\alpha$ -adducin antibodies were used for the immunoprecipitation experiments and both exhibited detectable levels of spectrin in their immunoprecipitates. Additionally, no spectrin was detected in the rabbit IgG control immunoprecipitates, strengthening the evidence supporting an interaction between the two proteins. The technical difficulty in demonstrating interactions between cytoskeletal proteins should be mentioned here. Spectrin and adducin, by their very nature, are both insoluble in vivo and steps to make these proteins soluble tend to disrupt the interactions being studied (Dubreuil, 2006). Indeed, the immunoprecipitation results shown here were captured following many rounds of methodology optimisation, including differing concentrations of primary antibody, secondary antibody and IgG magnetic beads. Yet, still at times the experiments gave inconsistent results as described later; therefore the results should be interpreted with caution. Despite this, the large proportion of  $\alpha$ -adducin and spectrin associated with the cytoskeleton, as shown by subcellular fractionation, along

with the possibility that the two proteins associate, as shown by immunoprecipitation, provide evidence for a resting vessel in which  $\alpha$ -adducin and spectrin localise to the actin cytoskeleton, and suggest that the two proteins may interact.

Having detected  $\alpha$ -adducin and spectrin in RMSA and determined their distribution and interactions in resting vessels, it was of interest to investigate their regulation by physiological agonists. NA activates a number of signalling pathways in vascular smooth muscle and both PKC and Rho-kinase translocate to the membrane after such adrenergic activation (Taggart et al., 1999). As adducin is regulated by PKC and Rhokinase in other cell types, it was of interest to investigate any NA-induced changes in α-adducin distribution in RMSA. NA treatment for 1 min induced an increase in the proportion of  $\alpha$ -adducin present in the cytosolic fraction and a reduction in cytoskeletal  $\alpha$ -adducin, suggesting dissociation of  $\alpha$ -adducin from the cytoskeleton. In support of this idea, a reduction in association between  $\alpha$ -adducin and spectrin was also seen in response to 1 min NA stimulation, a treatment which had very little effect on spectrin distribution. This correlates with the effects of NA on other cytoskeletal proteins such as paxillin and PYK2 which relocate to the actin cytoskeleton upon NA activation (Ohanian et al., 2005). In addition, Hic-5 translocates to the cytosol upon NA activation in parallel with the redistribution of Hsp27 from the cytosol to the actin cytoskeleton (Srinivasan et al., 2008). The effects of NA described in the studies of Ohanian (Ohanian et al., 2005) and Srinivisan (Srinivasan et al., 2008) are important for rearrangement of the cytoskeleton in RMSA, indicating that NA-induced changes in distribution of signalling proteins lead to important cytoskeletal functions. The data described in this chapter illustrate NA-induced changes in distribution of  $\alpha$ -adducin;

the functional importance of this redistribution will be further investigated in Chapter 5.

The initial NA-induced decrease in  $\alpha$ -adducin associated with the cytoskeleton, seen in the current study, supports a model in which adducin acts as an actin capping protein, which is removed from actin filaments upon stimulation with NA, exposing the barbed ends of the filaments and allowing their elongation. Indeed, other actin capping proteins are regulated by such signalling pathways. CapZ, which is present in all eukaryotic cells, is regulated by second messengers PIP<sub>2</sub>, which induces the removal of CapZ from filament ends resulting in localised areas of increased free barbed ends (Schafer et al., 1996). Although it is not known whether CapZ is expressed in RMSA, it is expressed in saphenous vein smooth muscle (McGregor et al., 2004), raising the possibility that the presence of a second barbed end actin capping protein, namely adducin, is redundant. However in adducin knockout mice, CapZ is upregulated (Porro et al., 2004), suggesting it may be part of a compensatory mechanism to reduce disruption due to the absence of adducin. Upregulation of CapZ in these mice suggests that the two proteins carry out similar functions and raises the possibility that both proteins perform barbed end capping functions in smooth muscle, possibly responding to different signalling pathways depending on the cellular function they are involved in. Receptor-mediated signalling pathways may evoke changes in actin dynamics with different efficacies, utilising a number of barbed-end capping proteins. For example in protease-activated receptor 1 (PAR-1)-activated platelets, the majority of free barbed ends result from gelsolin severing and uncapping via the Arp2/3 complex and adducin contributes only ~10% of the barbed ends produced. In contrast, in phorbol 12-myristate 13-acetate (PMA)-activated platelets adducin contributes over 60% of the new filament barbed ends, reflecting the different signalling activities induced by differential receptor activation (Barkalow et al., 2003).

NA-induced dissociation of adducin from the cytoskeleton could be inhibited by pretreatment with GF109203X, but not Y27632. Moreover, the reduction in association between the two proteins could be inhibited by pre-treatment with GF109203X, further implicating PKC in the dissociation of adducin from the cytoskeleton. Indeed, regulation of adducin distribution by PKC has been demonstrated in a number of cell types including platelets and RPTE cells. It is known that adducin is phosphorylated by PKC in other cell types, therefore phosphorylation may be involved in the redistribution of adducin observed in the current study; however this will be investigated further in Chapter 4.

The NA-induced dissociation of adducin from the cytoskeleton reported in this chapter occurs very rapidly, and yet the effects of NA on RMSA are much longer lasting. Phosphorylation of other signalling proteins such as paxillin, PYK2 and Hsp27 is maintained for up to 20 min in response to NA in RMSA (Ohanian et al., 2005; Srinivasan et al., 2008), carbachol-induced redistribution of PKC and Rho-kinase is sustained for up to 10 min in intact differentiated smooth muscle cells (Taggart et al., 1999). Here, NA treatment for 5 min induced an increase in the proportion of  $\alpha$ -adducin associated with the cytoskeleton in relation to that after NA treatment for 1 min. Indeed, the subcellular distribution after 5 min NA treatment was very similar to that in resting vessels. These data indicate that following PKC-dependent dissociation from the cytoskeleton, adducin reassociates with the

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cytoskeleton. A simultaneous increase in the proportion of spectrin present in the cytoskeleton was shown, in addition to an increase in the amount of spectrin detected in  $\alpha$ -adducin immunoprecipitates, suggesting increased association between the two proteins.

It has been shown *in vitro* and in cell culture models that phosphorylation of adducin by Rho-kinase enhances its interaction with the actin cytoskeleton (Kimura et al., 1998; Tamaru et al., 2005). This notion supports the data in the current chapter which indicate that translocation of  $\alpha$ -adducin to the cytoskeleton is dependent upon Rhokinase. Whether or not this involves direct adducin phosphorylation by Rho-kinase will be investigated further in Chapter 4.

Data discussed so far suggest a model in which NA induces PKC-dependent dissociation of adducin from the cytoskeleton followed by Rho-kinase-dependent reassociation with the cytoskeleton. Interestingly, both PKC and Rho-kinase were required for the NA-induced increase in cytoskeletal spectrin. As no evidence could be found in the literature to suggest that spectrin is regulated by either PKC or Rho-kinase, the data described here may indicate that both stages of  $\alpha$ -adducin translocation are needed in order for spectrin to increase its association with the cytoskeleton. In support of this model, Gardner and Bennett (Gardner and Bennett, 1987) showed that adducin binds tightly *in vitro* to spectrin-actin complexes but with much less affinity either to spectrin or actin alone, and suggested that adducin participates in an ordered assembly pathway involving the formation of spectrin-actin complexes, followed by the binding of adducin to these complexes.

In conclusion, this chapter has provided evidence for a model in which  $\alpha$ -adducin acts as an actin capping protein in resting vessels. NA treatment elicits PKC-dependent dissociation of  $\alpha$ -adducin from the cytoskeleton, possibly to allow cytoskeletal rearrangement. Following sustained NA treatment,  $\alpha$ -adducin reassociates with the actin cytoskeleton in a Rho-kinase-dependent manner. In addition, a concurrent increase in cytoskeletal spectrin is observed. The following chapters will investigate the involvement of phosphorylation in these processes and the effect they have on smooth muscle function. Chapter 4: The phosphorylation status of  $\alpha$ -adducin is regulated by vasoconstrictor activation in rat mesenteric arteries

#### **1.0 Introduction**

The previous chapter described PKC- and Rho-kinase-dependent changes in the subcellular localisation of  $\alpha$ -adducin in response to NA. Both NA and ET-1 activate PKC and Rho-kinase in smooth muscle (Taggart et al., 1999; Wynne et al., 2009) and it has been shown in a number of cell types that  $\alpha$ -adducin is phosphorylated by PKC on Ser 713 and Ser 726 located in the C-terminal MARCKS domain (Matsuoka et al., 1998; Waseem and Palfrey, 1990). In addition, adducin can be phosphorylated by Rho-kinase on Thr 445 and Thr 480 in its neck and tail domain (Kimura et al., 1998). Two of these phosphorylation sites, Ser 726 (Ser 724 in rat) and Thr 445, are known to be important in determining the cellular localisation and functions of adducin in other cell types. Accordingly, the effect of NA and ET-1 treatment on  $\alpha$ -adducin phosphorylation on Ser 724 and Thr 445 was investigated.

#### 2.0 Methods

The protocols for the experimental techniques used to gain the results described in this chapter are detailed in section 2.1 of Chapter 2.

#### 3.0 Results

#### 3.1 Optimisation of the phospho-adducin (Ser 724) antibody

In order to optimise the signal with the phospho-adducin (Ser 724) antibody, RMSA were stimulated for 2 min with Phorbol 12,13-Dibutyrate (PdBu, 1 $\mu$ M), which activates PKC and therefore should lead to increased  $\alpha$ -adducin phosphorylation on Ser 724. Immunoblot analysis showed that the level of  $\alpha$ -adducin phosphorylation on Ser 724 increased after activation for 1 min with 1 $\mu$ M PdBu (**Figure 4.1**). This increase in phosphorylation could be blocked by pre-incubation with the PKC inhibitor GF109203X (1 $\mu$ M). Consequently, these conditions were used for all further immunoblotting with the phospho-adducin (Ser 724) antibody.





**Figure 4.1 Optimisation of the phospho-adducin (Ser 724) antibody.** RMSAs were incubated with 1µM PdBu for 1 min in the presence or absence of the PKC inhibitor GF109203X (1µM). Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ -adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724):  $\alpha$ -adducin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. PdBu significantly increased  $\alpha$ -adducin phosphorylation on Ser 724. The presence of GF109203X inhibited the PdBu-induced increase in  $\alpha$ -adducin phosphorylation. P values are difference from untreated vessels (CNL): P<0.05; n=4, where n includes all vessels dissected from one animal. PdBu, Phorbol 12,13-Dibutyrate.

# 3.2 Temporal aspects of vasoconstrictor-induced $\alpha$ -adducin phosphorylation on Ser 724

Having established that  $\alpha$ -adducin could be phosphorylated in RMSA through direct activation of PKC, it was then important to determine whether more physiological agonists could induce phosphorylation on Ser 724. In RMSA, PKC inhibition reduces NA- and ET-1-induced MLC phosphorylation and contraction suggesting that these vasoconstrictors mediate their contractile effects, at least in part, via PKC (Buus et al., 1998; Sakurada et al., 2003; Yoshida et al., 1994). To determine whether NA- or ET-1-induced  $\alpha$ -adducin phosphorylation could play any role in the contractile response, the phosphorylation time course was investigated.

## 3.2.1 Investigation of the time course of $\alpha$ -adducin phosphorylation on Ser 724 in response to noradrenaline

Arteries were incubated for time points from 30 sec to 60 min with NA ( $15\mu$ M) and homogenised samples were analysed using the phospho-adducin (Ser 724) antibody. As there are limitations on the number of samples that can be prepared from one animal, two time courses were carried out. Initially, the time points were 1 min, 5 min, 10 min and 60 min. The time course of  $\alpha$ -adducin phosphorylation induced by the vasoconstrictors varied between animals, therefore individual time courses are shown in **Figure 4.2**. Results from four individual animals (**Figures 4.2 A–D**) show an initial peak in  $\alpha$ -adducin phosphorylation between 30 sec and 5 min, with the phosphorylation level returning to baseline or near baseline by 60 min. As the earlier time points appeared to be important, a further time course was carried out with time points 30 sec, 1 min, 2.5 min and 5 min. Again, variation in the phosphorylation time course was seen between animals therefore individual experiments are shown in **Figure 4.3** (**A–D**). In all four experiments, an initial peak in phosphorylation was seen within 1 min, followed by a fall in the level of phosphorylation. In two animals the phosphorylation level continued to fall and returned to or close to baseline (C and D) after the initial peak, however in two animals (A and B) the phosphorylation increased again after the fall, failing to return to baseline within 5 min. To determine an average phosphorylation time course all results were combined. The average data (**Figure 4.4**) reveals an initial peak in phosphorylation followed by a steady decline back down to baseline phosphorylation levels. Phosphorylation at the two initial time points (30 sec and 1 min) was significantly higher than the level in untreated vessels (p<0.001 and p<0.05 respectively). In addition the 5 min, 10 min and 60 min time points were significantly lower than phosphorylation levels at 30 sec (p<0.05).



Figure 4.2 Time course of a-adducin phosphorylation in response to noradrenaline. RMSAs were incubated with  $15\mu$ M NA for 1 min, 5 min, 10 min or 60 min. Vessels were homogenised in RIPA buffer and  $20\mu$ l of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The a-adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): actin. Four separate experiments are shown (A–D). NA induced an increase in a-adducin phosphorylation on Ser 724 in all four experiments. In two experiments (A and B) the level of phosphorylation dropped after the initial increase and a second peak was recorded. In two experiments (C and D) the phosphorylation level dropped after the initial peak and remained around baseline level.



Figure 4.3 Early time course of a-adducin phosphorylation in response to noradrenaline. RMSAs were incubated with  $15\mu$ M NA for 30 sec, 1 min, 2.5 min or 5 min. Vessels were homogenised in RIPA buffer and  $20\mu$ l of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ -adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): actin. Four separate experiments are shown (A–D). NA induced an increase in phosphorylation in all four cases which was followed by a drop in phosphorylation. In two experiments, the phosphorylation level increased again (A and B) and in two the phosphorylation level returned to or close to baseline (C and D).



Figure 4.4 Average a-adducin phosphorylation time course in response to noradrenaline. Results from figures 4.2 and 4.3 were combined to produce a bar chart demonstrating the average NA-induced  $\alpha$ -adducin phosphorylation time course. Data were corrected to CNL for each individual experiment and are presented as mean  $\pm$  S.E. Statistical significance was demonstrated by one-way analysis of variance (Tukey post hoc test). The 30 sec and 1 min time points were significantly higher than the phosphorylation in untreated vessels (CNL). Phosphorylation levels at later time points were not significantly different from the level in untreated vessels. P values are difference from untreated vessels (CNL):\* P<0.05; \*\*\* P<0.001; n=4, where n includes all vessels dissected from one animal.

### 3.2.2 Investigation of the time course of $\alpha$ -adducin phosphorylation on Ser 724 in response to endothelin-1

Arteries were incubated for time points from 1 min to 60 min with ET-1 (100nM). In the initial individual time course experiments, vessels were incubated with ET-1 for 2 min, 10 min, 20 min or 60 min. These experiments revealed very little change in phosphorylation levels in response to ET-1 (data not shown). Phosphorylation in response to NA was seen within 30 sec, therefore to determine whether this was the case during ET-1 stimulation, a further time course was carried out in which vessels were incubated for 30 sec, 1 min, 2.5 min or 5 min. Individual time courses (Figures **4.5** A–D) indicate a rapid phosphorylation on Ser 724 within 30 sec in all four cases, with the level of phosphorylation returning to baseline after 20 min. To determine an average phosphorylation time course all results were combined. The average data (Figure 4.6) shows a rapid rise in phosphorylation within 30 sec with the level returning to baseline within 2 min. Consistent with the individual time courses, the only time point with significantly increased phosphorylation when compared with control was 30 sec (p<0.001). In addition, phosphorylation at 30 sec was significantly higher than at all later time points (p<0.001), with phosphorylation at 1 min significantly higher than that at 2.5 min and 5 min (p<0.05).



Figure 4.5 Early time course of  $\alpha$ -adducin phosphorylation in response to endothelin-1. RMSAs were incubated with 100nM ET-1 for 30 sec, 1 min, 2.5 min or 5 min. Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ -adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): actin. Four separate experiments are shown (A–D). Endothelin-1 induced a rapid increase in  $\alpha$ -adducin phosphorylation on Ser 724 in all four experiments.



Figure 4.6 Average a-adducin phosphorylation time course in response to endothelin-1. Data from figures 4.5 and 4.6 were combined to produce a bar chart demonstrating the average  $\alpha$ -adducin phosphorylation time course in response to ET-1. Data were corrected to CNL for each individual experiment and are presented as mean  $\pm$  S.E. Statistical significance was demonstrated by one-way analysis of variance (Tukey post hoc test). Phosphorylation at the 30 sec time point was significantly higher than that in untreated vessels (CNL). P-values are difference from untreated vessels (CNL): P<0.001; n=4, where n includes all vessels dissected from one animal.

### 3.3 The calcium-dependence of vasoconstrictor-induced $\alpha$ -adducin phosphorylation on Ser 724

Adducin has a calmodulin binding domain in its C-terminal MARCKS-related domain and is regulated by calmodulin *in vitro* (Scaramuzzino and Morrow, 1993). Furthermore,  $Ca^{2+}$  and calmodulin are instrumental in vascular smooth muscle contraction; vasoconstrictors induce changes in global  $Ca^{2+}$  levels and these changes regulate force development (Wray et al., 2005). As NA induced changes in the phosphorylation status of  $\alpha$ -adducin with a time course consistent with contraction, it was important to determine whether the phosphorylation was dependent on changes in intracellular  $Ca^{2+}$  levels. Therefore the effect of NA on  $\alpha$ -adducin phosphorylation on Ser 724 in the absence of extracellular  $Ca^{2+}$  was investigated.

# 3.3.1 Investigation of the calcium-dependence of noradrenaline-induced α-adducin phosphorylation on Ser 724

Arteries were incubated for 30 min in the presence or absence of extracellular Ca<sup>2+</sup> before being stimulated with NA for 1 min. Homogenisation was carried out in HEPES buffer in the presence or absence of Ca<sup>2+</sup>; Ca<sup>2+</sup>-free buffer was supplemented with 1mM EGTA. Samples were analysed using the phospho-adducin (Ser 724) antibody. In the presence of extracellular Ca<sup>2+</sup>, NA induced an increase in  $\alpha$ -adducin phosphorylation on Ser 724. However, NA also induced  $\alpha$ -adducin phosphorylation in the absence of extracellular Ca<sup>2+</sup>. There was no significant difference between the level of NA-induced  $\alpha$ -adducin phosphorylation in the presence and absence of extracellular Ca<sup>2+</sup>.



Figure 4.7 Vasoconstrictor-induced a-adducin phosphorylation is independent of calcium influx. RMSAs were incubated for 1 min with  $15\mu M$  NA or 100nM ET in the presence or absence of extracellular calcium. Vessels were homogenised in RIPA buffer and 20ul of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): a-adducin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). **A.** A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. Both NA and ET induced a significant increase in  $\alpha$ -adducin phosphorylation on Ser 724 in both the presence and absence of extracellular calcium. P-values are difference from untreated vessels (CNL): \*\* P < 0.01; n=8, where n includes all vessels dissected from one animal.

# 3.3.2 Investigation of the calcium-dependence of endothelin-induced $\alpha$ -adducin phosphorylation on Ser 724

Arteries were incubated for 30 min in the presence or absence of extracellular Ca<sup>2+</sup> before being stimulated with ET-1 for 1 min. Homogenisation and quantification of  $\alpha$ -adducin phosphorylation was carried out as before. Increased  $\alpha$ -adducin phosphorylation in the presence and absence of extracellular Ca<sup>2+</sup> was detected and there was no significant difference between the level of phosphorylation under normal and extracellular Ca<sup>2+</sup>-free conditions (**Figure 4.7**).

### 3.4 The protein kinase A-dependence of vasoconstrictor-induced $\alpha$ -adducin phosphorylation on Ser 724

There is evidence that adducin is phosphorylated by PKA on Ser 726 (Ser 724 in rats) in erythrocytes (Matsuoka et al., 1996). ET-1 activates cAMP-dependent PKA in coronary smooth muscle (El-Mowafy and White, 1998); therefore the effect of PKA activation and inhibition on  $\alpha$ -adducin phosphorylation on Ser 724 was investigated.

#### 3.4.1 Investigation of the effect of protein kinase A inhibition on endothelin-1induced $\alpha$ -adducin phosphorylation on Ser 724

Vessels were pre-incubated with the PKA inhibitor H89 (10 $\mu$ M) for 30 min, before being treated with ET-1 for 1 min. ET-1 induced a significant increase in  $\alpha$ -adducin phosphorylation on Ser 724 (p<0.05), (**Figure 4.8**). The level of ET-1-induced  $\alpha$ adducin phosphorylation in the presence of H89 was significantly higher than in the absence of H89 (p<0.05); however H89 alone had no effect on phosphorylation.

### 3.4.2 Investigation of the effect of protein kinase A activation on a-adducin phosphorylation on Ser 724

To further investigate the effect of PKA on  $\alpha$ -adducin phosphorylation, two compounds were used to increase PKA activity; IBMX, which inhibits cAMP degradation, and a cell permeable cAMP, dibutyryl cAMP (dcAMP). Vessels were incubated for 60 min with IBMX (50µM) or 10 min with dcAMP in the presence or absence of the PKA inhibitor H89 (1µM). Both IBMX and dcAMP slightly increased the level of phosphorylation on Ser 724; however neither effect was significant (**Figure 4.9**). The presence of H89 had no significant effect on the level of  $\alpha$ -adducin phosphorylation.



Figure 4.8 Endothelin-1 induces  $\alpha$ -adducin phosphorylation on Ser 724 in the presence of the protein kinase A inhibitor H89. RMSAs were incubated with 100nM ET-1 for 1 min in the presence or absence of the PKA inhibitor H89 (10µM). Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ -adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724):  $\alpha$ -adducin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. ET-1 significantly increased  $\alpha$ -adducin phosphorylation on Ser 724 in the presence and absence of H89. P-values are difference from untreated vessels (CNL): \* p<0.05; \*\* p<0.01; n=6, where n includes all vessels dissected from one animal.

ET

ET + H89

H89

0

CNL



Figure 4.9 Activation of protein kinase A has no significant effect on  $\alpha$ -adducin phosphorylation on Ser 724. RMSAs were incubated with 50µM IBMX or dcAMP in the presence or absence of the PKA inhibitor H89 (1µM). Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The  $\alpha$ adducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724):  $\alpha$ -adducin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. Although IBMX and dcAMP appeared to increase  $\alpha$ -adducin phosphorylation on Ser 724 in the presence and absence of H89, these effects were not significant. n=3, where n includes all vessels dissected from one animal. CNL, untreated vessels.

### 3.5 The protein kinase C-dependence of vasoconstrictor-induced $\alpha$ -adducin phosphorylation on Ser 724

The phospho-adducin (Ser 724) antibody was raised against the adducin residue that is phosphorylated by PKC in other cell types; however the site can also be phosphorylated by PKA (Matsuoka et al., 1996). Consequently, a PKC inhibitor, GF109203X (1 $\mu$ M) was used to determine the contribution of PKC to vasoconstrictor-induced  $\alpha$ -adducin phosphorylation. Vessels were pre-incubated with GF109203X for 30 min before being treated with vasoconstrictors according to the protocol.

#### 3.5.1 Investigation of the effect of protein kinase C inhibition on noradrenalineinduced $\alpha$ -adducin phosphorylation on Ser 724

Vessels were treated with NA for 1 min in the presence or absence of GF109203X. NA induced a significant increase in phosphorylation on Ser 724 (p<0.05), in the absence of GF109203X, however in the presence of GF109203X NA failed to induce a significant increase in  $\alpha$ -adducin phosphorylation on Ser 724 (**Figure 4.10A**).

# 3.5.2 Investigation of the effect of conventional protein kinase C inhibition on noradrenaline-induced a-adducin phosphorylation on Ser 724

In order to assess the role of Ca<sup>2+</sup>-dependent PKCs in  $\alpha$ -adducin phosphorylation, vessels were pre-incubated with the conventional PKC inhibitor Go6976 (1µM) for 30 min before treatment with NA for 1 min. NA induced a significant increase in  $\alpha$ -adducin phosphorylation on Ser 724 in the presence (p<0.01) and absence (p<0.05) of Go6976 (**Figure 4.10B**).



Figure 4.10 Noradrenaline-induced a-adducin phosphorylation is dependent on protein kinase C, but not on a conventional protein kinase C. RMSAs were incubated with 15µM NA for 1 min in the presence or absence of the PKC inhibitor GF109203X (1µM) or the conventional PKC inhibitor Go6976 (1µM). Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The aadducin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724):  $\alpha$ -adducin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. NA significantly increased  $\alpha$ -adducin phosphorylation on Ser 724, an effect that was blocked by GF109203X. B. NA significantly increased  $\alpha$ -adducin phosphorylation in the presence of Go6976. P-values are difference from untreated vessels (CNL);\*\* p<0.01; \* p<0.05; n=4, where n includes all vessels dissected from one animal.

# **3.6** Temporal aspects of vasoconstrictor-induced α-adducin phosphorylation and dephosphorylation on Ser 724

In the previous chapter, NA-induced changes in the subcellular distribution of  $\alpha$ adducin were observed after 1 and 5 min. Accordingly the phosphorylation status of  $\alpha$ -adducin was determined at these time points to ascertain any links between subcellular distribution and phosphorylation status.

# 3.6.1 Investigation of the effect of protein kinase C- and Rho-kinase inhibition on noradrenaline-induced α-adducin phosphorylation on Ser 724

As shown earlier in this chapter, treatment with NA for 1 min induces PKC-dependent phosphorylation on Ser 724. There is evidence to suggest that  $\alpha$ -adducin is also regulated by Rho-kinase, therefore experiments were performed in which the effects of PKC and Rho-kinase inhibition on  $\alpha$ -adducin phosphorylation were determined. Vessels were treated for 1 min with NA in the presence or absence of either GF109203X (1µM) or the Rho-kinase inhibitor Y27632 (10µM). There was a significant increase in phosphorylation in response to NA (p<0.01). The NA-induced  $\alpha$ -adducin phosphorylation on Ser 724 was inhibited by the presence of GF109203X (p<0.05), however Y27632 had no effect on the phosphorylation on Ser 724 (**Figure 4.11**).



Figure 4.11 Phosphorylation in response to 1 min noradrenaline treatment can be inhibited by protein kinase C inhibition, but not by Rho-kinase inhibition. RMSAs were incubated with 15µM NA for 1 min in the presence and absence of 1µM GF109203X and 10µM Y27632. Vessels were homogenised in RIPA buffer and 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The actin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): actin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. NA significantly increased  $\alpha$ -adducin phosphorylation on Ser 724, an effect which was blocked by GF109203X, but not Y27632. P-values are difference from untreated vessels (CNL): P<0.05; n=3, where n includes all vessels dissected from one animal.

# 3.6.2 Investigation of the effect of 5 min noradrenaline treatment on a-adducin phosphorylation on Ser 724

The phosphorylation time courses described earlier in this chapter demonstrated a rapid increase in  $\alpha$ -adducin phosphorylation on Ser 724, followed by a reduction in phosphorylation in the majority of cases. Accordingly, 5 min was chosen as a later time point to investigate the dephosphorylation of  $\alpha$ -adducin on Ser 724. Vessels were treated with NA for either 1 or 5 min (**Figure 4.12**). There was a significant increase in phosphorylation after 1 min (p<0.001), with a substantial reduction in the level of phosphorylation after 5 min treatment compared with the level after 1 min treatment (p<0.01). To further investigate  $\alpha$ -adducin dephosphorylation on Ser 724 with regards to dual regulation by PKC and Rho-kinase, vessels were preincubated with Y27632 before treatment with NA for 5 min. This pre-treatment had no effect on  $\alpha$ -adducin dephosphorylation on Ser 724 (**Figure 4.12**).


Figure 4.12 a-adducin is dephosphorylated after 5 min noradrenaline treatment. RMSAs were incubated with  $15\mu$ M NA for 1 min, 5 min or 5 min in the presence of  $10\mu$ M Y27632. Vessels were homogenised in RIPA buffer and  $20\mu$ l of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Ser 724) content was determined by immunoblotting using a polyclonal phospho-adducin (Ser 724) antibody. The actin antibody was used as a loading control. Data were calculated as the ratio of phospho-adducin (Ser 724): actin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows PSer724Adducin levels. NA significantly increased  $\alpha$ -adducin phosphorylation on Ser 724 after 1 min. Following NA treatment for 5 min, the level of phosphorylation on Ser 724 was reduced, both in the presence and absence of Y27632. P-values are difference from untreated vessels (CNL): P<0.05; n=5, where n includes all vessels dissected from one animal.

#### **3.7** The effect of vasoconstrictors on phosphorylation of α-adducin on Thr 445

In addition to their activation of PKC, NA and ET-1 are known to activate Rhokinase. As the previous chapter reported Rho-kinase-dependent redistribution of  $\alpha$ adducin, the effects of NA and ET-1 on  $\alpha$ -adducin phosphorylation on Thr 445 were investigated.

# 3.7.1 Investigation of the effect of noradrenaline and endothelin-1 on phosphorylation of $\alpha$ -adducin on Thr 445

An antibody raised against phospho-adducin Thr 445 was used for immunoblotting with RMSA samples that had been treated with NA for 1 min. Antigen reactivity was very poor. In one blot no detectable difference in phosphorylation on Thr 445 was detected between control and NA-activated samples (**Figure 4.13A**). A band was seen at approximately 60kDa which increased in intensity after NA and ET-1 activation (**Figure 4.13B**). The 60kDa band also increased in intensity following treatment with the serine/threonine phosphatase inhibitor, calyculin (100nM) for 15 min (**Figure 4.13C**). The laboratory-developed polyclonal  $\alpha$ -adducin antibody described in Chapter 3 also detected a band of approximately 60kDa, which may correlate with the band detected with the phospho-adducin (Thr 445) antibody (**Figure 4.13D**). Further investigation into the time course of  $\alpha$ -adducin phosphorylation by Rho-kinase was unsuccessful due to poor antigen reactivity.



**Figure 4.13 Phosphorylation of a-adducin on Thr 445.** Vessels were treated as described below and homogenised in RIPA buffer. 20µl of lysate from each sample was loaded onto a 9% SDS polyacrylamide gel. The phospho-adducin (Thr 445) content was determined by immunoblotting using a polyclonal phospho-adducin (Thr 445) antibody. A. Vessels were treated with 1µM NA for 1 min. The antibody detected a band of approximately 120kDa, however no difference in phosphorylation on Thr 445 was observed between untreated (CNL) and NA-activated samples. B. Using the same antibody, a band of approximately 60kDa was detected which increased in intensity after stimulation of vessels for 1 min with either 15µM NA or 100nM ET. C. Vessels were treated with 100nM calyculin for 15 min before homogenisation. The 60kDa band also increased in intensity after calyculin treatment. D. The laboratory-developed  $\alpha$ -adducin antibody also detected a band of approximately 60kDa, which may correlate with the band seen with the phospho-adducin Thr 445 antibody. For all experiments n=1.

#### **3.8** The subcellular distribution of α-adducin phosphorylated on Ser 724

As NA- and ET-1-induced phosphorylation of  $\alpha$ -adducin in RMSA have been demonstrated in this study, it was also important to determine the subcellular localisation of the phosphorylated protein and thereby identify any association with the actin cytoskeleton.

### 3.8.1 Investigation of the effect of noradrenaline on the subcellular distribution of *a*-adducin phosphorylated on Ser 724

Vessels were treated with NA for 1 min to induce phosphorylation on Ser 724, before being subjected to differential centrifugation to separate subcellular components. Lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and probed with the phospho-adducin (Ser 724) adducin antibody. Approximately 17.4% of  $\alpha$ -adducin phosphorylated on Ser 724 (pSer724adducin) was present in the Triton X-100 soluble membrane fraction, with 35.8% in the cytosolic fraction and 46.9% in the Triton X-100 insoluble cytoskeleton (**Figure 4.14**).



Figure 4.14 Phosphorylated a-adducin is present mainly in the cytosolic and cytoskeletal fractions. RMSA extracts were treated with NA before being subjected to differential centrifugation to separate subcellular component to form cytosolic, Triton X-100-soluble and cytoskeletal fractions.  $30\mu$ l of each fraction was loaded onto a 9% SDS polyacrylamide gel and samples were subjected to immunoblotting using the phospho-adducin (Ser 724) antibody. The sum of antigen detected in the three fractions was taken as total protein (100%) and the amount of antigen in each fraction was calculated as a proportion of this total. Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative immunoblot is shown. B. Chart shows percentage of total antigen present in each fraction. A mean of 46.9% of antigen was present in the cytoskeletal fraction with 35.8% in the cytosolic fraction and 17.4% in the Triton X-100 soluble fraction. P-values are difference from Triton X-100-soluble: p<0.01; n=4, where n includes all vessels dissected from one animal.

## **3.8.2** Localisation of $\alpha$ -adducin phosphorylated on Ser 724 in cultured vascular smooth muscle cells

Cultured aortic smooth muscle cells were serum-starved for 24 hours before being treated with NA and stained with the phospho-adducin (Ser 724) antibody in order to visualise the subcellular distribution of the phosphorylated protein. Representative images are shown in **Figure 4.15**. An increase in antigen reactivity was observed following NA treatment. In treated cells, staining illustrates the distribution of phospho-adducin (green) throughout smooth muscle cells. Similar to the  $\alpha$ -adducin and spectrin staining described in the previous chapter, filament like structures can be seen (indicated by arrows) in several places, implying association of phospho-adducin with the actin cytoskeleton. In addition, there are areas of stronger staining indicating clustering of phospho-adducin in certain areas of the cell.

# 3.8.3 Localisation of a-adducin phosphorylated on Ser 724 in relation to filamentous actin in cultured vascular smooth muscle cells

In order to visualise the localisation of phospho-adducin (Ser 724) in relation to actin filaments, cultured aortic smooth muscle cells were serum-starved for 24 hours before being treated with NA and stained with the phospho-adducin (Ser 724) antibody and phalloidin stain. Representative images are shown in **Figure 4.16**. Again specific patches of phospho-adducin staining can be seen, however the phosphorylated protein does not seem to associate particularly with actin filaments (red).

For both figures described above, matched IgG controls will be required in order to confirm the specificity of the adducin signal.

### A. Unstimulated cells



### **B.** NA-stimulated cells



Figure 4.15 Phospho-adducin (Ser 724) staining increased in response to noradrenaline treatment in vascular smooth muscle cells. Cells were serum-starved for 24 hours before being treated with NA for 1 min, fixed with 4% paraformaldehyde and stained with the phospho-adducin (Ser 724) antibody (green) and DAPI (blue). Increased staining can be seen throughout the NA-treated cells. Filamentous staining can be seen in some parts of the cell, as indicated by arrows. Pictures shown are representative of three experiments.

Adducin	Phalloidin	Combined
Adducin	Phalloidin	Combined • • • •
Adducin	Phalloidin	Combined • m *
Adducin	Phalloidin	Combined • • • •

Figure 4.16 Phospho-adducin (Ser 724) staining is not localised with actin filaments in vascular smooth muscle cells. Smooth muscle cells were serum-starved for 24 hours before being treated with NA for 1 min, fixed with 4% paraformaldehyde and stained with the phospho-adducin (Ser 724) antibody (green) and phalloidin (red). Patches of phospho-adducin staining, seen throughout the NA-treated cells, do not appear to localise to actin filaments. Pictures shown are representative of three experiments.

#### **4.0 Discussion**

A number of proteins involved in vasoconstrictor-induced smooth muscle contraction are thought to be important for phosphorylation and subsequent regulation of adducin in other cell types. Therefore it was of interest to investigate the possibility that such proteins phosphorylate adducin in smooth muscle and whether this regulation may be involved in agonist-stimulated contraction.

The previous chapter reported the presence of both  $\alpha$ - and  $\gamma$ -adducin in RMSA. Although a number of adducin's serine residues are phosphorylated in vitro (Matsuoka et al., 1996), only Ser 726 has been shown to alter adducin's function in vivo (Matsuoka et al., 1998). Accordingly, the equivalent residue in rat (Ser 724) was chosen for investigation in RMSA. A polyclonal antibody raised against the peptide corresponding to amino acids 656-668 of the phosphorylated  $\gamma$ -isoform was used. The antibody should detect phosphorylated Ser 662 of  $\gamma$ -adducin, phosphorylated Ser 724 of  $\alpha$ -adducin, and phosphorylated 713 of  $\beta$ -adducin. In the current study, two vasoconstrictors, NA and ET-1, induced a rapid increase in  $\alpha$ -adducin phosphorylation on Ser 724. Although  $\alpha$ -adducin phosphorylation was observed in response to vasoconstrictor activation, phosphorylation of  $\gamma$ -adducin could not be detected. It has been shown that  $\gamma$ -adducin is phosphorylated in a PKC-dependent manner in a number of cell lines (Imamdi et al., 2004). In these studies both the  $\alpha$ and  $\gamma$ -isoforms were phosphorylated on Ser 724 in response to the same treatment, therefore NA- and ET-1-induced  $\gamma$ -adducin phosphorylation may be expected in the current study. The absence of a  $\gamma$ -adducin band during western blotting with this polyclonal antibody could be due to a genuine lack of  $\gamma$ -adducin phosphorylation in response to the vasoconstrictors used. Similarly, poor antigen reactivity could be the cause. However, a lack of  $\gamma$ -adducin phosphorylation could strengthen the argument raised in Chapter 3 regarding the stability of  $\alpha$ -adducin in the absence of other adducin isoforms. The fact that  $\gamma$ -adducin phosphorylation was not detected under conditions in which  $\alpha$ -adducin was phosphorylated in RMSA may further support the idea that  $\alpha$ -adducin is either functioning as a homodimer/tetramer or is being stabilised through interactions with another membrane component, and is therefore functioning independently of the  $\gamma$ -isoform. Alternatively, if  $\alpha$ -adducin and  $\gamma$ -adducin form heterodimers, phosphorylation on one isoform, namely  $\alpha$ -adducin, may be sufficient to regulate the entire heterodimer. As described in the previous chapter, a peptide block, in which the nitrocellulose membrane is probed with the antigen used to produce the antibody prior to western blotting with the adducin antibody, would help to confirm the specificity of the phospho-adducin (Ser 724) antibody.

The time course of NA- and ET-1-induced  $\alpha$ -adducin phosphorylation varied across experiments. Differing phosphorylation peaks and variation in the fold change in phosphorylation were recorded between experiments carried out in individual animals. In order to bring any activated proteins back down to baseline levels, all vessels were equilibrated for 30 min before being treated; however variation in the level of basal phosphorylation on Ser 724 was still evident in many cases. This variation may account for observed differences in the fold-change of phosphorylation following stimulation. The variation in basal phosphorylation observed in the current study is in keeping with results in HeLa cells in which there was a proportion of adducin phosphorylated under control conditions (Pariser 2005). In addition, fractionation data in the previous chapter point to a role for adducin in the constant turnover or 'treadmilling' of actin filaments. For phosphorylation to be involved in this process, adducin would need to be continually switching between phosphorylation states, therefore at any given time there would be a proportion of phosphorylated adducin present in the cell.

Despite the variation in the vasoconstrictor-induced phosphorylation time courses, it was clear that  $\alpha$ -adducin phosphorylation increased in response to both vasoconstrictors and diminished after the initial peak in all cases. In some experiments a second peak in phosphorylation was recorded, raising the possibility of a biphasic response. Indeed, p38 mitogen-activated protein kinase (MAPK) is activated by ET-1 in a biphasic manner in RMSA (Ohanian et al., 2001). A study in adipocytes reported little or no increase in adducin phosphorylation in response to 5 min insulin treatment, despite association between PKC $\lambda$  and adducin (Laustsen et al., 2001). If the phosphorylation was in keeping with the time course in the current study it is possible that the phosphorylation peak was missed in the study of Laustsen et al., due to the fact that earlier time points were not tested.

The two vasoconstrictors chosen here have different activation profiles in terms of the PKC isoforms they activate, and this is reflected in the phosphorylation time course reported. Both  $\alpha_1$ -adrenoceptors and ET-1 receptors couple to phospholipase C (PLC), resulting in breakdown of PIP<sub>2</sub> and production of DAG (Mueed et al., 2005). However, their effects on the activation of PKC isoforms in RMSA differ. For example, NA significantly increases particulate PKC $\alpha$  in RMSA, but only slightly increases particulate levels of PKC $\varepsilon$ , whereas ET-1 increases particulate PKC $\alpha$  and  $\varepsilon$  to a similar extent (Mueed et al., 2005). Depending on which PKC isoform/isoforms are involved in  $\alpha$ -adducin phosphorylation in the current study, these differences in

PKC isoform activation may contribute to the variation in phosphorylation time courses between NA- and ET-1-stimulated vessels. It has been suggested that activation of multiple PKC isoforms leads to alternate functions with different isoforms being directed to the subcellular compartment in which their target protein resides (Mineo et al., 1998; Rybin et al., 1999; Sampson et al., 2007). The distinct activation profiles of NA and ET-1 in terms of the PKC isoforms activated may lead to differing adducin functions, further contributing to the variation between vasoconstrictor-induced  $\alpha$ -adducin phosphorylation time courses.

The NA-induced  $\alpha$ -adducin phosphorylation described in this chapter was independent of Ca<sup>2+</sup> influx. In agreement with this data, PMA-induced release of adducin from the membrane skeleton was unaffected by EGTA-AM in platelets, and occurred in the absence of fluctuations in intracellular Ca<sup>2+</sup>, leading to the suggestion that the process is Ca<sup>2+</sup> independent (Barkalow et al., 2003). It should also be noted that calmodulin binding activity may be more active in β-adducin than  $\alpha$ -adducin (Matsuoka et al., 1996), which could account for the lack of Ca<sup>2+</sup>-dependence in the current study, as very little evidence of the presence of β-adducin in RMSA was found (Chapter 3). However, the fact that the conventional PKC inhibitor Go6976 did not affect NA-induced  $\alpha$ -adducin phosphorylation, and the fact that phosphorylation was independent of Ca<sup>2+</sup> influx, may indicate that the signalling pathway involving adducin is independent to that which activates the rise in intracellular Ca<sup>2+</sup> and cross bridge cycling. However, intracellular stores were not emptied in these experiments; therefore they only reflect adducin's dependence on Ca<sup>2+</sup> influx and not on the release of Ca<sup>2+</sup> from intracellular stores. Further experiments in which Ca<sup>2+</sup> stores are

emptied will be required in order to fully determine the  $Ca^{2+}$ -dependence of  $\alpha$ -adducin phosphorylation.

The conventional PKC inhibitor did not affect vasoconstrictor-induced α-adducin phosphorylation, suggesting a role for novel PKCs in vasoconstrictor-induced aadducin phosphorylation. In addition to their differences in  $Ca^{2+}$ -dependency, conventional and novel PKC isoforms are activated within a slightly different time frame in relation to changes in intracellular  $Ca^{2+}$  (Nelson et al., 2008). The time course experiments in the current study demonstrate elevated NA- and ET-1-induced  $\alpha$ -adducin phosphorylation which is sustained for several minutes in some cases and novel PKCs can persistently associate with the membrane, providing further evidence for novel PKC involvement. Indeed adducing associates with PKC<sub>l</sub> in insulin-treated adipocytes (Laustsen et al., 2001), and overexpression of PKCS in MDCK cells induces phosphorylation of adducin on Ser 726 (Chen et al., 2007a). Moreover ET-1, which induced phosphorylation of adducin on Ser 724 in the current study, induces translocation of PKCS to the plasma membrane in RMSA smooth muscle cells (Nelson et al., 2008). However, NA fails to induce any translocation of PKCS in RMSA (Mueed et al., 2005); therefore the particular isoform/isoforms involved in NA- and ET-1-induced  $\alpha$ -adducin phosphorylation will require further investigation.

The fact that  $\alpha$ -adducin phosphorylation occurs in the absence of extracellular Ca<sup>2+</sup> does not eliminate the possibility that this signalling pathway is involved in contraction. Although a rise in intracellular Ca<sup>2+</sup> is important for contraction of smooth muscle cells, there is evidence that calcium influx is not essential for the smooth muscle to initiate a depolarisation in response to NA (Nilsson et al., 1998). In

addition, a significant component of ET-1–induced contraction of coronary smooth muscle is  $Ca^{2+}$  antagonist-insensitive and involves activation and translocation of  $Ca^{2+}$ -independent PKC $\epsilon$  (McNair et al., 2004). Therefore it is still of interest to determine adducin's involvement in smooth muscle cell contraction; this will be investigated further in Chapter 5.

Interestingly, in the current study, a large proportion of pSer724adducin was present in the cytoskeletal fraction, despite the theory that phosphorylation of  $\alpha$ -adducin releases it from the cytoskeleton (Barkalow et al., 2003; Gilligan et al., 2002). The data presented here are in agreement with a study carried out in the renal epithelial cell line LLC-PK1, in which cisplatin induced a PKC-dependent increase in phosphorylation of the serine residue 726 of both the  $\alpha$ - and  $\gamma$ -adducin isoforms, which were primarily observed in the cytoskeletal fraction (Imamdi et al., 2004). The high percentage of pSer724adducin remaining in the cytoskeletal fraction in the current study may be due to increased exposure to phosphatases in the cytosol i.e. upon translocation from the cytoskeleton adducin is immediately dephosphorylated, therefore the level of pSer724adducin detected in the cytosol is lower than expected. The phosphatase involved in dephosphorylation on the Ser 724 site has not been investigated here, however it is known that myosin phosphatase dephosphorylates  $\alpha$ adducin in vitro and in MDCK cells (Kimura et al., 1998). Based on in vitro data, one hypothesis is that phosphorylation of MLCP induces its redistribution to a cytosolic position where it is active (Hartshorne et al., 1998). If this is the case, adducin and MLCP would be in closer proximity after removal of adducin from the cytoskeleton, thereby mediating adducin dephosphorylation. However, it should be noted that there is no significant difference between the level of pSer724adducin in the cytosolic and cytoskeletal fractions. This is in contrast to the distribution of  $\alpha$ -adducin in resting vessels (Chapter 3) where a significantly higher level of  $\alpha$ -adducin was associated with the cytoskeleton. Taken together, these data indicate that the pool of  $\alpha$ -adducin in the cytosol is more heavily phosphorylated than that in the cytoskeleton, implying an increase in the level of phosphorylated adducin in the cytosolic fraction upon stimulation with NA.

In a number of different tissues/cells including rat hippocampus, human erythrocytes, human embryonic kidney (HEK) cells and MDCK cells, reactivity with a Ser 726 phospho-adducin antibody increased in response to PMA, but not forskolin, leading to the conclusion that Ser 726 is an in vivo phosphorylation site for PKC or other PMAactivated kinases but not for cAMP-dependent protein kinase (Matsuoka et al., 1998). The data presented here concur with these findings; rather than inhibiting ET-1induced a-adducin phosphorylation, the PKA inhibitor H89 further increased phosphorylation on Ser 724 in the presence of ET-1. Although initially this indicated that PKA may have an inhibitory effect on ET-1-induced adducin phosphorylation, increasing intracellular levels of PKA with IBMX or dcAMP did not induce a significant increase in phosphorylation. It should be noted here that the increase in phosphorylation in response to IBMX and dcAMP, although not significant, was higher than that induced significantly by ET-1 in previous figures. This makes clear the importance of further investigation into the involvement of PKA in this system, however the data presented here do not provide conclusive evidence for a role for PKA in the ET-1-induced phosphorylation of  $\alpha$ -adducin.

It has been suggested previously that adducin is dually regulated by PKC and Rhokinase (Fukata et al., 1999) and we know that vasoconstrictors activate both kinases in smooth muscle. In the current study, NA-induced phosphorylation of  $\alpha$ -adducin on Ser 724 is inhibited by GF109203X, but not by Y27632, demonstrating regulation by PKC, but not Rho-kinase. In Chapter 3 it was shown that the initial change in  $\alpha$ adducin distribution is regulated in the same way as phosphorylation - NA treatment for 1 min induces dissociation of  $\alpha$ -adducin from the cytoskeleton, an effect which is dependent upon PKC, but not Rho-kinase. As both processes are PKC, but not Rhokinase dependent this may suggest that the trigger for the translocation of  $\alpha$ -adducin away from the cytoskeleton is its phosphorylation on Ser 724. Indeed, the phosphorylation status of adducin has been shown to determine its distribution in a number of cell types including platelets (Gilligan et al., 2002), MDCK cells (Matsuoka et al., 1998) and erythrocytes (Kalfa et al., 2006). Adducin is thought to act as an actin capping protein and the data presented in the current chapter along with the fractionation data in Chapter 3 support a model in which adducin is associated with the cytoskeleton in resting vessels, capping actin filaments to prevent elongation. Upon activation, it is phosphorylated and dissociates from the cytoskeleton in order to allow rearrangement of the filaments, a process which is known to be important in smooth muscle function.

Although the initial phase of  $\alpha$ -adducin translocation described in Chapter 3 was Rhokinase-independent, the second phase was Rho-kinase-dependent. The data presented in this chapter show  $\alpha$ -adducin dephosphorylation on Ser 724, in response to NA treatment at 5 min. This occurs within the same time frame as the second phase of NA-induced  $\alpha$ -adducin translocation. Interestingly, both the rise and fall in phosphorylation on Ser 724 were independent of Rho-kinase. There have been contrasting reports about whether Rho-kinase inhibition affects the phosphorylation status of adducin on Ser 724. In guinea pig cochlea, preincubation with Y27632 for 30 min inhibited adducin phosphorylation at both Thr 445 and Ser 726 (Zhang et al., 2003). However, in agreement with the data presented in the current study, Y27632 did not affect phosphorylation of  $\alpha$ -adducin on Ser 726 in platelets (Tamaru et al., 2005). Furthermore, in organotypic cultures of mouse retinas, the Rho-kinase inhibitor H-1152P (1µM) suppressed the Rho-kinase-dependent phosphorylation of adducin without affecting the protein kinase A/C-mediated phosphorylation events (Tura et al., 2009). The fact that the NA-induced phosphorylation on Ser 724 described in the current chapter is Rho-kinase-independent, in contrast to the second phase of NA-induced  $\alpha$ -adducin redistribution shown in Chapter 3, uncouples the distribution of  $\alpha$ -adducin from its state of phosphorylation on Ser 724. This raises the possibility that phosphorylation at a second site is required for additional control of adducin's distribution. As NA-induced reassociation of a-adducin with the cytoskeleton is dependent upon Rho-kinase (Chapter 3); phosphorylation at one of the threonine sites phosphorylated by Rho-kinase in other cell types could be involved. Indeed it has been shown in vitro that phosphorylation of adducin by Rho-kinase on Thr 445 enhances its interaction with actin filaments (Kimura et al., 1998; Tamaru et al., 2005).

In the current study  $\alpha$ -adducin phosphorylation on Thr 445 was investigated using two different phospho-adducin (Thr 445) antibodies. However, due to poor antigen reactivity conclusive evidence for phosphorylation at this site could not be provided. As there are a number of potential Rho-kinase phosphorylation sites on  $\alpha$ -adducin, it

is of course possible that Rho-kinase phosphorylates another residue such as Thr 480 although there is less evidence in the literature for phosphorylation at this site. One of the phospho-adducin (Thr 445) antibodies did detect a band of 60kDa which was regulated by both vasoconstrictors and calyculin, possibly an adducin cleavage product. In untreated retinas, a strong band of 74kDa, thought to be a caspase-3-dependent cleavage product of adducin, was detected using a phospho-adducin (Thr 445) antibody. The band at 74kDa and several weaker bands of intermediate and smaller size were expressed at approximately 50% lower levels in retinas treated with the Rho-kinase inhibitor H-1152P (Tura et al., 2009). Unfortunately, in the current study the signal was not consistent enough to study this pathway any further.

In the absence of an antibody to detect  $\alpha$ -adducin phosphorylation on Thr 445, the time course of any phosphorylation which may occur at this site, and indeed the relationship between PKC- and Rho-kinase-dependent phosphorylation could not be investigated. To speculate; as PKC and Rho-kinase are both activated by vasoconstrictors,  $\alpha$ -adducin could be phosphorylated by both kinases initially. In idea. platelet activation with support of this 1µM 9,11-epithio-11,12methanothromboxane A<sub>2</sub> (STA<sub>2</sub>), induced a rapid increase in phosphorylation of  $\alpha$ adducin on both Thr 445 and Ser 726 (Tamaru et al., 2005). However as here, Rhokinase activity is not required for the initial dissociation of  $\alpha$ -adducin from the cytoskeleton, it could be that there are three different phosphorylation 'states', unphosphorylated, phosphorylated on Ser 724 and phosphorylated on Thr 445. The net increase in phosphorylation on Ser 724 in response to NA and ET-1 represents the overall phosphorylation state of adducin in the entire vessel. However, it is likely that pools of adducin are present in all phosphorylation states in different parts of the cell.

Indeed, the phospho-adducin (Ser 724) staining presented here in smooth muscle cells, illustrates the pools of phosphorylated protein in different parts of the cell, in contrast to the more uniform  $\alpha$ -adducin staining presented in Chapter 3. As different areas within the cell have different needs in terms of filamentous networks it will be of interest to view the whereabouts of adducin in different phosphorylation states, and to study the condition of the surrounding filaments. It is highly likely that these data do not tell the entire story and therefore more detailed examination of adducin phosphorylation on a cellular level is warranted.

As PKC-dependent responses occur after 1 min and Rho-kinase-dependent responses occur after 5 min, this suggests that any phosphorylation by Rho-kinase would occur subsequent to the PKC-dependent phosphorylation. The data presented in this and the previous chapter support a sequence of events in which  $\alpha$ -adducin is phosphorylated by PKC on Ser 724, followed by its dephosphorylation on this site and its phosphorylation by Rho-kinase on one of the Rho-kinase phosphorylation sites (Thr 445 and 480). To further enhance the predicted model, the data on spectrin distribution described in the previous chapter should be taken into account. The NAinduced increase in cytoskeletal spectrin was blocked by both PKC- and Rho-kinase inhibition, thus may be coupled with the phosphorylation described in the current chapter. Indeed inhibition of the phosphorylation on Ser 724 has also been shown to alter the cellular distribution of spectrin in MDCK cells. Cells expressing an unphosphorylatable S716A/S726A mutant a-adducin exhibited increased levels of cytoplasmic spectrin (Matsuoka et al., 1998). Moreover, PKC-induced adducin phosphorylation inhibits its activity in promoting spectrin-actin complexes (Abdi and Bennett, 2008). With regards to the function of this signalling pathway, the data supports the following model in which  $\alpha$ -adducin and spectrin play a role in the formation of actin filaments:

- 1. Vasoconstrictors induce activation of PKC which phosphorylates  $\alpha$ -adducin, triggering its movement away from the cytoskeleton allowing elongation of actin filaments.
- 2. Adducin is then dephosphorylated at the PKC site and phosphorylated by Rhokinase triggering its movement back towards the cytoskeleton along with additional spectrin in order to stabilise the filamentous actin network.

The time course of actin filament formation and the role of  $\alpha$ -adducin and spectrin in this process will be further investigated in Chapter 5.

# Chapter 5: Noradrenaline-induced filamentous actin formation is regulated by $\alpha$ -adducin in rat mesenteric small arteries

#### **1.0 Introduction**

Reorganisation of the actin cytoskeleton and regulation of the F: G-actin ratio is crucial for many cellular functions including migration, changes in morphology, vesicular trafficking and remodelling of vessels (Gerthoffer, 2007; Heerkens et al., 2007; Lanzetti, 2007). As described in Chapter 1, vascular smooth muscle contains a substantial pool of G-actin, which is reduced upon an increase in intraluminal pressure (Cipolla et al., 2002), and the F: G-actin ratio is regulated by a number of actin-associated proteins, such as adducin and spectrin. The previous two chapters reported vasoconstrictor-induced changes in the phosphorylation status and subcellular distribution of  $\alpha$ -adducin and spectrin, and it was suggested that these changes may be involved in cytoskeletal regulation and thereby vascular smooth muscle contraction. This chapter further investigates the involvement of  $\alpha$ -adducin and spectrin in vascular smooth muscle function.

#### 2.0 Methods

The protocols for the experimental techniques used to gain the results described in this chapter are detailed in section 2.1 of Chapter 2.

#### **3.0 Results**

### **3.1 Investigation of the effect of vasoconstrictors on the F: G-actin ratio in rat** mesenteric small arteries

 $\alpha$ -adducin and spectrin are involved in cytoskeletal regulation in a number of cell types and it is known that NA induces F-actin formation in RMSA (Srinivasan et al., 2008). As the previous chapters described regulation of  $\alpha$ -adducin and spectrin by NA, the effect of NA treatment on the F: G-actin ratio in RMSAs was investigated.

# 3.1.1 Effect of 1 min noradrenaline treatment on the F: G-actin ratio in rat mesenteric small arteries

 $\alpha$ -adducin phosphorylation and dissociation from the cytoskeleton in response to treatment with NA was observed within 1 min. Accordingly, vessels were stimulated with NA (15µM) for 1 min before being subjected to fractionation to separate the F and G-actin components. NA treatment for 1 min had very little effect on the F: G-actin ratio (1.03 a.u. in resting vessels vs 1 a.u. in NA-treated vessels, ns **Figure 5.1**).

# 3.1.2 Effect of 5 min noradrenaline treatment on the F: G-actin ratio in rat mesenteric small arteries

Changes in the subcellular distribution of  $\alpha$ -adducin and spectrin were also observed following 5 min NA treatment, therefore vessels were then treated with NA for 5 min before separating F and G-actin fractions. A representative western blot is shown in **Figure 5.2A**. Densitometric data are shown in **Figure 5.2B**. NA treatment for 5 min induced a significant increase in the F: G-actin ratio (1 a.u. in resting vessels vs 2.59 a.u. in NA-treated vessels, p<0.001).

## 3.1.3 Effect of protein kinase inhibition on noradrenaline-induced changes in the F: G-actin ratio in rat mesenteric small arteries

The previous chapters showed regulation of  $\alpha$ -adducin by PKC and Rho-kinase. To determine the role of PKC and Rho-kinase in the NA-induced increase in the F: G-actin ratio, vessels were pre-incubated with either GF109203X (1µM) or Y27632 (10µM), before treating with NA for 5 min (**Figure 5.2**). NA treatment failed to induce an increase in the F: G-actin ratio in the presence of GF109203X (1 a.u. in untreated vessels vs 1.23 a.u. in NA-treated vessels pre-treated with GF109203X, ns) or in the presence of Y27632 (1 a.u. in untreated vessels vs 1.11 a.u. in NA-treated vessels pre-treated with Y27632, ns).





Figure 5.1 Noradrenaline treatment for 1 min has very little effect on the F: G actin ratio in rat mesenteric small arteries. RMSAs were incubated with  $15\mu$ M NA for 1 min. Vessels were homogenised in F-actin stabilisation buffer and F and G actin fractions were separated using ultracentrifugation of both F and G actin fractions were loaded onto a 9% SDS-polyacrylamide gel and membranes were probed with a G-actin antibody. Data were calculated as the ratio of F actin: G actin and corrected to CNL for each individual experiment. Data are presented as mean  $\pm$  S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative blot is shown. B. Chart shows F: G actin ratios in untreated (CNL) and NA-treated vessels. Very little change was seen in the F: G actin ratio following NA treatment for 1 min: ns; n=5, where n includes all vessels dissected from one animal.



Figure 5.2 Noradrenaline treatment for 5 min increased the F: G actin ratio, an effect that could be inhibited by both GF109203X and Y27632. RMSAs were incubated with  $15\mu$ M NA for 5 min in the presence or absence of either the PKC inhibitor GF109203X ( $1\mu$ M) or the Rho-kinase inhibitor Y27632 ( $10\mu$ M). Vessels were homogenised in F-actin stabilisation buffer and F and G actin fractions were separated using ultracentrifugation.  $40\mu$ l of both F and G actin fractions were loaded onto a 9% SDS-polyacrylamide gel and membranes were probed with a G-actin antibody. Data were calculated as the ratio of F actin: G actin and corrected to CNL for each individual experiment. Data are presented as mean ± S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test). A. A representative blot is shown. B. Chart shows F: G actin ratios in each treatment group. The F: G actin ratio increased in response to NA treatment. This increase was attenuated by pre-treatment with GF109203X or Y27632. P-values are difference from untreated vessels (CNL): p<0.01; n=3, where n includes all vessels dissected from one animal.

# 3.2 The effect of $\alpha$ -adducin neutralisation on noradrenaline-induced F-actin formation and contraction in rat mesenteric small arteries

The data above suggest that signalling pathways involving  $\alpha$ -adducin may be important for NA-induced F-actin formation in RMSA. In the absence of adducin inhibitors, the  $\alpha$ -adducin antibody was transfected into intact RMSA using Chariot transfection reagent to neutralise endogenous protein and further determine the role of  $\alpha$ -adducin in F-actin formation.

# 3.2.1 Effect of α-adducin neutralisation on noradrenaline-induced F-actin formation in rat mesenteric small arteries

Following  $\alpha$ -adducin or rabbit serum transfection, vessels were treated with NA for 5 min before centrifugation to separate F and G-actin components. In vessels transfected with rabbit serum, NA induced an increase in filamentous actin, significantly increasing the F: G-actin ratio (0.36 in resting vessels vs 0.63 in NA-treated vessels, p<0.05). However, in vessels transfected with  $\alpha$ -adducin antibody NA had no effect on the F: G-actin ratio (0.32 in resting vessels vs 0.32 in  $\alpha$ -adducin transfected vessels, ns) (**Figure 5.3**).



Figure 5.3 Neutralisation of a-adducin using Chariot transfection reagent inhibits noradrenaline-induced actin filament formation. RMSAs were incubated with either  $\alpha$ -adducin antibody, to neutralise  $\alpha$ -adducin, or rabbit serum, as a control, for 3 hours before being treated with 15 $\mu$ M NA for 5 min. Vessels were homogenised in Factin stabilisation buffer and F and G actin fractions were separated using ultracentrifugation. 40 $\mu$ l of both F and G actin fractions were loaded onto a 9% SDSpolyacrylamide gel and probed with a G-actin antibody. Data are expressed as mean  $\pm$  S.E. and statistical significance was determined by paired t-test. A. A representative blot is shown. B. Chart shows F: G actin ratios in  $\alpha$ -adducin-neutralised and control (rabbit IgG) vessels in the absence and presence of 15 $\mu$ M NA. In vessels treated with rabbit serum, NA induced an increase in the F: G actin ratio, however following neutralisation of  $\alpha$ -adducin, NA failed to induce an increase in the F: G actin ratio. P-values are difference from untreated vessels (CNL): P<0.05; n=6, where n includes all vessels dissected from one animal.

# 3.2.2 Effect of $\alpha$ -adducin neutralisation on contraction in rat mesenteric small arteries

In order to determine the effect of  $\alpha$ -adducin neutralisation on contraction, contractile responses of small arteries transfected with either  $\alpha$ -adducin or rabbit serum were measured using pressure myography after addition of either 50 mM KPSS or cumulative concentrations of NA (0.6, 3, and 15 $\mu$ M) at 2 min intervals, as described previously (Clarke et al., 2007).

Following agonist treatment, arteries were incubated in calcium free PSS containing 2mM EGTA and passive lumen diameter was recorded. To normalise responses for differences in starting vessel diameter the data were expressed as % of passive lumen diameter. A dual-chamber organ bath was used, allowing a control artery (rabbit serum) and a test artery ( $\alpha$ -adducin) to be studied simultaneously. Delivery of  $\alpha$ -adducin antibody did not alter the early (<30 sec) or sustained (>2 min) contractile response to NA or the response to non-receptor induced contraction (potassium-induced membrane depolarisation) compared with a paired artery that had received rabbit serum (**Figure 5.4**).



Figure 5.4 Neutralisation of  $\alpha$ -adducin has no effect on noradrenaline-induced contraction in rat mesenteric small arteries. Lumen diameter was recorded from arteries mounted in a pressure myograph following delivery of  $4\mu g \alpha$ -adducin antibody or rabbit serum. A. Vessels were stimulated with 50mM K<sup>+</sup> (KPSS) for 2 min. **B&C:** Vessels were stimulated with NA as indicated. B. The maximum decrease in lumen diameter occurring before 30 sec or C. lumen diameter at 2 min post addition of NA was recorded. Data are expressed as a percentage of the maximum passive lumen diameter (%Ca<sup>++</sup> free); n=4, where n is one vessel.

## **3.3** The effect of spectrin neutralisation on F-actin formation and contraction in rat mesenteric small arteries

It has been shown here that  $\alpha$ -adducin is important for NA-induced increases in Factin, and the previous chapters have shown interactions between  $\alpha$ -adducin and spectrin and overlap in their regulatory signalling pathways. Accordingly we investigated the effect of neutralising endogenous spectrin on the F: G-actin ratio and contraction.

# 3.3.1 Effect of spectrin neutralisation on noradrenaline-induced F-actin formation in rat mesenteric small arteries

Following  $\alpha$ -adducin or rabbit serum transfection, vessels were treated with NA for 5 min before centrifugation to separate F and G-actin components. In vessels transfected with rabbit serum, NA induced an increase in F-actin, significantly increasing the F: G-actin ratio (0.36 in resting vessels vs 0.63 in NA-treated vessels, p<0.05) (**Figure 5.5**). NA also induced a significant increase in the F: G-actin ratio in spectrin-transfected vessels (0.44 in resting vessels vs 0.88 in NA-treated vessels, p < 0.05).

# 3.3.2 Effect of spectrin neutralisation on noradrenaline-induced contraction in rat mesenteric small arteries

Arteries were transfected with either rabbit serum or spectrin antibody and treated as described earlier. Delivery of spectrin antibody did not alter the early (<30 sec) or sustained (>2 min) contractile response to NA or the response to non-receptor induced contraction (potassium-induced membrane depolarisation) compared with a paired artery that had received rabbit serum (**Figure 5.6**).



Vessels transfected with spectrin antibody



Figure 5.5 Neutralisation of spectrin using Chariot transfection reagent has no effect on noradrenaline-induced actin filament formation. RMSAs were incubated with either spectrin antibody, to neutralise spectrin, or rabbit serum, as a control, for 3 hours before being treated with  $15\mu$ M NA for 5 min. Vessels were homogenised in *F*-actin stabilisation buffer and *F* and *G* actin fractions were separated using ultracentrifugation.  $40\mu$ l of both *F* and *G* actin fractions were loaded onto a 9% SDS-polyacrylamide gel and membranes were probed with a G-actin antibody. Data are expressed as mean  $\pm$  S.E. and statistical significance was determined by paired t-test. A. A representative blot is shown. B. Chart shows F: G actin ratios in spectrin-neutralised and control (rabbit IgG) vessels in the absence and presence of  $15\mu$ M NA. NA induced an increase in the F: G actin ratio, both in vessels which were incubated with rabbit serum and in those incubated with spectrin antibody. P-values are difference from untreated vessels (CNL): P<0.05; n=3, where n includes all vessels dissected from one animal.



Figure 5.6 Neutralisation of spectrin has no effect on noradrenaline-induced contraction in rat mesenteric small arteries. Lumen diameter was recorded from arteries mounted in a pressure myograph following delivery of  $4\mu g$  spectrin antibody or rabbit serum. A. Arteries were stimulated with 50mM K<sup>+</sup> (KPSS) for 2 min. B&C: Arteries were stimulated with NA as indicated. B. The maximum decrease in lumen diameter occurring before 30 sec or C. lumen diameter at 2 min post addition of NA was recorded. Data are expressed as a percentage of the maximum passive lumen diameter (%Ca<sup>++</sup> free); n=4, where n is one vessel.

#### 4.0 Discussion

The dynamic turnover and subsequent functions of the actin cytoskeleton are well established in the erythrocyte, as is the contribution of adducin and spectrin to these functions. Nucleated cells were once thought to contain a static actin cytoskeleton which provided support to the plasma membrane (Gascard and Mohandas, 2000); however they are now known to contain a similar dynamic actin cytoskeleton to that in erythrocytes, rearrangement of which is essential for a number of major cellular functions.

In canine carotid smooth muscle strips, an increased F: G-actin ratio can be seen as early as 1 min after stimulation with NA (Tang and Tan, 2003a). Contrastingly, in the current study NA stimulation of RMSA for 1 min induced very little change in the F:G-actin ratio. However, individual systems and particular cellular functions require cytoskeletal changes within different time frames. Locomoting keratinocytes move their body length in about 1 min, which requires complete turnover of the entire actin network within that time; in dramatic contrast, pure actin filaments are intrinsically stable, undergoing only slow subunit exchange (Pollard and Borisy, 2003). It follows then that actin regulatory proteins must account for this difference. Thus, the explanation for the lack of change in the F:G-actin ratio at the 1 min time point may be that the relevant actin binding proteins, including adducin, are being redistributed in order to allow filament formation i.e. cytoskeletal changes are occurring at this time point, but the changes have not yet resulted in a net increase in F-actin. In agreement with this theory are the data describing NA-induced  $\alpha$ -adducin phosphorylation and redistribution within 1 min, presented in Chapters 3 and 4. It is possible then that adducin caps actin filaments in resting vessels and requires time to

translocate before a net increase in F-actin is seen. The translocation of  $\alpha$ -adducin and most probably a number of other actin binding proteins presumably occurs in order for actin filament formation to commence. Indeed, a number of signalling proteins are known to be activated within this time frame in smooth muscle; NA induces a rapid increase in Src kinase activity within 30 to 45 sec (Ohanian et al., 2001), activation of Hsp kinase activity within 1 to 2 min (Srinivasan et al., 2008), and phosphorylation of PYK2 and paxillin within 1 min (Ohanian et al., 2005), all of which are associated with subsequent changes in the F:G-actin ratio.

The data presented in section 3.1.2 describe a net increase in filamentous actin in response to 5 min NA treatment, strengthening the model described above in which adducin uncaps actin filaments in order for further filament formation to occur. Furthermore, in the current study the NA-induced increase in F-actin could be blocked by pre-treatment with GF109203X or Y27632, compounds that inhibited phosphorylation and redistribution of  $\alpha$ -adducin and redistribution of spectrin in the previous chapters. Moreover, neutralisation of  $\alpha$ -adducin in RMSA inhibited the NA-induced increase in the F:G-actin ratio, confirming the involvement of  $\alpha$ -adducin in filamentous actin formation in this system. In agreement with the data presented here, Rho-kinase is required for F-actin formation in aortic smooth muscle cells (Sauzeau et al., 2001) and human saphenous vein smooth muscle cells (Cario-Toumaniantz et al., 2002).

The fact that the F:G-actin ratio increased in response to 5 min NA treatment, also supports the model described in Chapter 3 in which association of  $\alpha$ -adducin and spectrin with the cytoskeleton in response to NA treatment for 5 min occurred in order

to support a newly formed actin filamentous network in the cell. Indeed, both adducin and spectrin have been implicated in formation and stabilisation of membranes in other cell types (Abdi and Bennett, 2008; Anong et al., 2009; Baines, 2009) and evidence has been provided for involvement of spectrin in resistance to mechanical stresses, a function that may be important in smooth muscle during contraction. Thus, following NA-induced actin filament formation,  $\alpha$ -adducin and spectrin may localise with the actin cytoskeleton to link the newly formed filaments with the plasma membrane in order to form the stable network that has been described in other cell types.

Inhibition of spectrin had no effect on NA-induced actin filament formation. This may be because  $\alpha$ -adducin is acting independently of spectrin in regulating the actin cytoskeleton. However, due to the lack of a positive control to confirm the downregulation of spectrin in these experiments, it may be that the antibody is ineffective in neutralising spectrin. Considering how closely adducin and spectrin are involved in other cells, and the fact that NA regulates both proteins in this system, we would expect neutralisation of spectrin to affect NA-induced filament formation, thus incomplete neutralisation of spectrin in these experiments seems likely.

There is a great deal of evidence supporting a role for cytoskeletal rearrangement in general, and actin filament formation in particular, in vascular smooth muscle contraction (An et al., 2002; Cipolla et al., 2002; Mauss et al., 1989; Mehta and Gunst, 1999; Nunes, 2002; Ohanian et al., 2005; Srinivasan et al., 2008; Tang and Tan, 2003a; Tang and Tan, 2003b). In addition, Rho-kinase and PKC are known to play a role in the calcium sensitisation which mediates smooth muscle contraction

(Altmann et al., 2003; Asano and Nomura, 2003; Buus et al., 1998; Damron et al., 2002; Fujihara et al., 1997; Ghisdal et al., 2003; Lee et al., 1999; Matrougui et al., 2001). The fact that  $\alpha$ -adducin is clearly regulated by PKC and Rho-kinase in RMSA, and that inhibition of  $\alpha$ -adducin inhibits actin filament formation, may suggest that  $\alpha$ adducin could be involved in vascular smooth muscle contraction. However, despite the inhibition of actin filament formation, neutralisation of  $\alpha$ -adducin failed to inhibit either K<sup>+</sup>- or NA-induced contraction. Although NA-induced F-actin formation has been demonstrated here, it should be noted that there is evidence to suggest that different actin isoforms perform distinct cellular functions. y-actin, the least abundant actin isoform in vascular smooth muscle, is the most dynamically remodelled by  $\alpha$ adrenergic receptor stimulation, suggesting that the non-muscle actin cytoskeleton is more dynamic than the contractile cytoskeleton (Kim et al., 2008a). It is possible therefore that  $\alpha$ -adducin is involved in reorganisation of the dynamic cytoskeleton that is less involved in contraction, explaining the lack of effect of adducin neutralisation on contraction, despite inhibition of filament formation. In support of this idea, the Milan strain of rats, which carry an adducin mutation, exhibit normal contractile responses to NA (Rizzoni et al., 2009).

As the cytoskeletal changes described in this thesis do not appear to be involved in smooth muscle contraction, another possibility is that changes occur as a method of regulating membrane proteins and their delivery to the membrane, thereby determining composition of the membrane, as described in Chapter 1. One particular function in which the regulation of membrane proteins may be important in smooth muscle is transmission of force to the extracellular matrix which leads to tissue remodelling, which may occur as a result of persistent functional responses (Bakker et
al., 2004). Indeed, evidence exists that increased stress fibre formation is important for transmission of force to the extracellular matrix (Wang et al., 2006). In small arteries acute remodelling of the arterial wall occurs in response to increased intraluminal pressure and contractile agonists, allowing adaptation of vascular diameter and wall thickness to local flow and pressure (Martinez-Lemus et al., 2009). The role of the actin cytoskeleton in these responses is still not fully understood, however changes in F-actin may spatially coordinate cell receptors and intracellular signalling mediators, thereby permitting upstream effectors, such as integrins and focal adhesion kinase, to couple with their intracellular targets (Chapados et al., 2006). Indeed,  $\alpha$ -adducin and its acute phosphorylation by Rho-kinase are substantially involved in coronary constrictive remodelling and vasospastic responses, as shown by upregulation of phospho-adducin Thr 445 at coronary lesions (Morishige et al., 2001). Thus, the NA-induced,  $\alpha$ -adducin-dependent cytoskeletal changes reported in this chapter may be involved in such processes in RMSA and eventually lead to remodelling following persistent activation of vessels by NA. However, the involvement of adducin in these mechanisms will require further investigation.

Although adducin and spectrin and their regulation of the F: G actin ratio do not appear to be involved in contraction, a number of other functions require cytoskeletal rearrangement in smooth muscle, and different pools of remodelling actin are involved in distinctive functions, therefore the role of  $\alpha$ -adducin-dependent filamentous actin formation will require further investigation.

## **Summary and Conclusions**

Rearrangement of the actin cytoskeleton is crucial for many cellular functions, and actin binding proteins are essential in regulating such rearrangement. The current study has demonstrated the presence of  $\alpha$  and  $\gamma$ -adducin in rat mesenteric small arteries. In addition, the presence of spectrin has been established and an interaction between spectrin and  $\alpha$ -adducin has been illustrated in resting vessels. Both proteins were also associated with the actin cytoskeleton in resting vessels.

In response to acute NA stimulation,  $\alpha$ -adducin was phosphorylated on Ser 724 and dissociated from the actin cytoskeleton and spectrin, processes that were dependent on PKC. Following slightly prolonged treatment with NA,  $\alpha$ -adducin was dephosphorylated on Ser 724 and reassociated with the actin cytoskeleton in a Rho-kinase-dependent manner. In addition, it increased its association with spectrin, concurrent with an increase in the proportion of spectrin associated with the cytoskeleton, which was also dependent on PKC and Rho-kinase. As there is no evidence to suggest that spectrin is regulated by either of these kinases, this raises the possibility that early dissociation of adducin is necessary for later recruitment of spectrin. In addition, an increase in filamentous actin was observed following prolonged NA treatment, an effect which could be inhibited by PKC inhibition, Rho-kinase inhibition and neutralisation of  $\alpha$ -adducin, implicating adducin and its dissociation and reassociation with the cytoskeleton in actin filament formation.

These results along with previous data in other cell types illustrate a role for adducin as an actin capping protein in resting vessels, which also associates with spectrin in order to stabilise the membrane structure. It is suggested that the function of vasoconstrictor-induced phosphorylation of  $\alpha$ -adducin by PKC and concurrent dissociation from the cytoskeleton is to uncap filaments, allowing rearrangement of the actin cytoskeleton. It is further suggested that following filament formation, most likely mediated by a number of actin binding proteins,  $\alpha$ -adducin becomes phosphorylated by Rho-kinase and re-associates with the actin cytoskeleton in order to recruit extra spectrin molecules to the cytoskeleton, thereby stabilising the filamentous actin network.

Many studies have demonstrated a need for actin filament formation in vascular smooth muscle function. Here, evidence is provided for involvement of  $\alpha$ -adducin and spectrin in rearrangement of the actin cytoskeleton in vascular smooth muscle tissue. It will now be of interest to study this signalling pathway in further detail and to ascertain the involvement of other actin binding proteins in the process, and indeed to determine the cellular functions that  $\alpha$ -adducin-induced rearrangement of the actin cytoskeleton helps to regulate.

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