T-type calcium channels and human mesangial cell proliferation

Mulgrew, Christopher James

Awarding institution:
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T-type calcium channels and human mesangial cell proliferation

A thesis submitted for the degree of Doctor of Philosophy
by Dr Christopher James Mulgrew

Guy’s, King’s and St Thomas’ School of Medicine
King’s College London
University of London
In memory of my parents,

Patricia and Denis
T-type calcium channels and human mesangial cell proliferation

Abstract

Aberrant proliferation of human mesangial cells (MC) is a critical step in the pathogenesis of mesangioproliferative renal diseases. The T-type calcium channel (T-CaCN) has been proposed to play an important role in the proliferation of a number of non-excitable cell types, but T-CaCN expression and functional significance in MC is not known. The aim of this thesis was to investigate the hypothesis that T-CaCN may play an important role in the proliferation of MC in primary culture.

Expression of mRNA encoding the $\alpha_1$H isoform (CaV3.2 clone) (but not the $\alpha_1$G nor $\alpha_1$I T-CaCN isoforms) was demonstrated in human MC by RT-PCR. Expression of $\alpha_1$H T-CaCN protein was difficult to assess directly due to the lack of a highly sensitive and specific antibody, despite attempts at upregulation of the protein. Electrophysiological studies of MC demonstrated an inward calcium current in a proportion of cells with characteristics consistent with T-CaCN. Culture of MC with the T-CaCN antagonists mibefradil, Ni$^{2+}$ and TTL-1177 resulted in a significant reduction in the growth velocity of MC. This effect was not seen upon incubation with verapamil, an L-type calcium channel antagonist. DNA synthesis in MC treated with each of the T-CaCN antagonists was significantly reduced by up to 50% as shown by BrdU incorporation. This anti-proliferative effect was not associated with direct drug-induced cytotoxicity or apoptosis. FACS analysis of MC incubated with T-CaCN antagonists illustrated an increased proportion of cells remaining in G1 and not progressing into S-phase. Treatment of cultured MC with TTL-1177 resulted in a significant reduction in the signalling protein p-ERK within 30 minutes, an effect not seen with verapamil, suggesting a possible mechanism needing further investigation. MC transfection with siRNA targeting the $\alpha_1$H isoform resulted in significant knockdown of T-CaCN $\alpha_1$H mRNA and a reduction in the growth velocity of cultured MC of approximately 50% compared to control siRNA transfection, with an associated 37% reduction in DNA synthesis.

These results demonstrate evidence for an important role for T-type calcium channels in the proliferation of human mesangial cells, justifying further study in greater detail. This could potentially lead to a novel therapy in the treatment of proliferative renal diseases.
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Abbreviations used

AII  angiotensin II
ACEi  angiotensin converting enzyme inhibitor
ACh  acetylcholine
ARB  angiotensin II receptor blocker
ASO  antisense oligonucleotide
AT₁  angiotensin II type-1 receptor
AT₂  angiotensin II type-2 receptor
ATP  adenosine triphosphate
BAD  Bcl-associated death promoter
[Ca²⁺]i intracellular calcium ion concentration
CaM  calmodulin
CaMK  Ca²⁺-calmodulin-dependent kinase
CCB  calcium channel blocker
cdc2  cell division cycle gene 2
CDK  cyclin-dependent kinase
CRE  cAMP response element
CREB  CRE binding protein
CRF  chronic renal failure
CTGF  connective tissue growth factor
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<td>ECM</td>
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<td>EGF</td>
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<td>Elk</td>
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<td>extracellular signal-regulated kinase</td>
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<td>ESRF</td>
<td>end-stage renal failure</td>
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<td>MAPK</td>
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<td>NDGA</td>
<td>nordihydroguaiaretic acid</td>
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PASMC  pulmonary artery smooth muscle cell
PDGF  platelet-derived growth factor
PI-3K  phosphatidylinositol 3-kinase
PIP₂  phosphatidyl inositol 4,5-diphosphate
p.m.p.  per million population
qPCR  quantitative real-time polymerase chain reaction
ROC  receptor-operated calcium channel
ROCK  Rho-associated kinase
RT-PCR  reverse transcriptase-polymerase chain reaction
RRT  renal replacement therapy
RyR  ryanodine receptor
SERCA  sarco-endoplasmic reticulum Ca²⁺-ATPase
SLE  systemic lupus erythematosus
SMC  smooth muscle cell
SOC  store-operated calcium channel
SR  sarcoplasmic reticulum
SRE  serum response element
T-CaCN  T-type calcium channel
TGF-β  transforming growth factor-β
UKPDS  UK Prospective Diabetes Study
UUO  unilateral ureteric obstruction
VOC  voltage-operated calcium channel
VSMC  vascular smooth muscle cell
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Publications arising from this thesis

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Targeting T-type calcium channels in human mesangial cell proliferation.
T-type calcium channels and human mesangial cell proliferation

1. Introduction

1.1 Scope of this thesis

This experimental thesis relates to the T-type calcium channel (T-CaCN) and its role in the proliferation of human mesangial cells (MC) \textit{in vitro}. It has been suggested that T-CaCN play an important role in the regulation of cell growth in a number of models, although their expression and precise role in MC has not previously been reported. Prior to presenting experimental methodology and results, this introductory chapter will initially focus on the structure and function of the nephron and mechanisms leading to chronic renal failure. This will be followed by an outline of MC proliferation, paying particular attention to mechanisms involved in driving aberrant MC growth and subsequent glomerulosclerosis. There will be a discussion of the role of calcium in cell proliferation and the cell cycle before focussing on the T-CaCN, its structure, functions and mechanisms by which it may be targeted.

1.2 Structure and function of the nephron

1.2.1 The nephron and renal function

Glomerular filtration, tubular secretion and reabsorption take place in the nephron. The nephron (illustrated in Figure 1.1) consists of the filtering unit known as the glomerulus, proximal convoluted tubule, loop of Henle, distal convoluted tubule and collecting duct. A healthy young adult might expect to have around one million nephrons in each kidney. The precise number is established during fetal development and once nephrons are lost, for whatever reason, they cannot be replaced or regenerate. The glomerulus consists of a tuft of capillaries linking afferent and efferent arterioles whose structure is maintained by the mesangium, which in turn consists primarily of mesangial cells and extracellular
mesangial matrix. The glomerular tuft sits in a hollow surrounded by the epithelial cell-lined Bowman’s capsule. The space between the glomerulus and Bowman’s capsule is known as Bowman’s space, and fluid is filtered from the glomerular tuft into this space. The rate of glomerular filtration is an important determinant of the excretion of solutes and water. The glomerular filtration barrier is composed of three distinct layers: (i) the glomerular capillary endothelial cell with its large fenestrations, (ii) the glomerular basement membrane (GBM), and (iii) the epithelial cell or podocyte. The GBM is responsible for maintaining the architectural and structural integrity of the glomerulus. It consists of cords of Type IV collagen interspersed with laminin, nidogen, and the negatively charged heparan sulphate proteoglycans that are largely responsible for the anionic charge barrier of the GBM (Groffen et al, 1999). The epithelial cells are attached to the GBM by discrete finger-like foot processes embedded in the basement membrane.

![Figure 1.1: Structure of the nephron](Image adapted from www.free-ed.net)
1.2.2 The mesangium

The mesangium consists primarily of MC and extracellular matrix (ECM) and is situated between and within glomerular capillary loops in the axial region of the glomerulus (Figure 1.2). MC are similar to smooth muscle cells in their structure and function. They have microfilaments consisting of actin, myosin and alpha-actinin, and respond to a number of vasoactive substances such as Angiotensin II (AII) which is produced by endothelial cells of the afferent arteriole (Kriz et al, 1995). Additionally, the MC itself may release prostaglandins that, along with AII and the inherent contractile properties of the MC, play a role in the regulation of glomerular haemodynamics. This modulation of blood flow through the renal corpuscle is one of a number of important functions of MC in the normal glomerulus, that also include the production of growth factors and of mesangial matrix to provide structural support to the glomerulus.

MC are thought to be involved in immune-mediated glomerular diseases. They are capable of both releasing cytokines such as interleukin-1 (IL-1), IL-6 and epidermal growth factor (EGF), and of proliferating in response to cytokines, including platelet-derived growth factor (PDGF) (Kusner et al, 1991; Moriyama et al, 1995). Subsequent glomerular hypercellularity and expansion of the ECM may lead to the pattern of glomerular injury and renal failure commonly seen in these disorders. Other cells also known to reside within the mesangium and which may contribute up to 15% of the cell population in the mesangium are macrophages and monocytes. These are derived from the bone marrow and are thought to have a primary phagocytic function, although they may also contribute to the localised inflammatory response seen in some glomerular diseases (Schreiner, 1992).

Embryologically, VSMC and MC share a similar developmental source. In response to glomerular injury, evidence suggests both migration of both existing intraglomerular MC and MC from outside the glomerulus. Extraglomerular 'reserve' mesangial cells residing in the juxtaglomerular apparatus were shown by Hugo and colleagues to migrate to and repopulate the rat glomerulus following glomerulolysis by injection of anti-Thy1 antibody (Hugo et al, 1997).
1.3 Chronic Renal Failure

1.3.1 Epidemiology
Chronic renal failure (CRF) is a progressive, irreversible condition which often progresses to end-stage renal failure (ESRF). A diagnosis of ESRF can have a devastating effect on the individual and their family, notwithstanding the significant resources needed to manage this condition. The life expectancy of a patient receiving renal replacement therapy (RRT) is significantly shortened as a result of systemic complications, particularly cardiovascular disease. Much of the data giving an indication of prevalence and causes of RRT come from national or continental registries, with limitations to these data (including variations in completeness of submitted figures) needing to be recognised. However, according to the 11th Annual Report of the UK
Renal Registry (December 2008), the total estimated annual acceptance rate for adults onto RRT in 2007 was 109 patients per million population (p.m.p.). The UK prevalence of RRT at the end of 2007 was 746 patients p.m.p. An annual increase in prevalence of 5.0% since 2000 has been observed (Ansell et al, 2008). Additionally, data from the European Renal Association - European Dialysis and Transplant Association (ERA-EDTA) Registry would suggest that the annual rise in the overall incidence rate of RRT for ESRF may have stabilised in some groups, particularly the elderly (Kramer et al, 2009).

1.3.2 Causes of chronic renal damage
Chronic damage and scarring of glomeruli or the tubulo-interstitial renal compartment, or both, is a pathological finding resulting from a spectrum of diseases and renal insults that cause the syndrome of chronic renal failure. These include autoimmune disease, proliferative diseases including IgA nephropathy and lupus nephritis, diabetes mellitus, hypertension and drug toxicity. Once damage is established, renal function may deteriorate progressively (at variable rates) to ESRF, irrespective of the underlying cause. UK Renal Registry data (December 2008) reports glomerulonephritis (GN) as the underlying renal diagnosis amongst at least 9% and possibly as many as 35% of incident patients with ESRF. A further 20% have ESRF due to diabetic nephropathy. The overall unadjusted five-year survival for incident patients requiring RRT is around 65% for patients between 18-64, falling to around 25% for those over the age of 65. The median age of all incident patients accepted onto RRT in England was 64.1 years (Ansell et al, 2008).

There are over 20 different types of cell in the human kidney and mesangial cells are by no means the most important as proliferation of a number of cell types is implicated in the spectrum of renal diseases seen on renal biopsies. For example, in crescentic glomerulonephritides, while mesangial cell proliferation is seen there is also proliferation of the epithelial cells of Bowman’s capsule leading to the formation of the crescent. A pathological change in podocyte structure and function may present as nephrotic syndrome without any significant change in MC number. As this thesis specifically relates to the expression and function of T-CaCN in human MC, further sections will
focus on MC in renal disease. However, this by no means should imply that pathological changes in other cell types, either within the glomerulus or the vascular or tubulo-interstitial components of the renal parenchyma, are of lesser significance.

1.4 Mesangial cell proliferation and progressive glomerulosclerosis

1.4.1 Mesangial cell proliferation as a cause of renal failure
Aberrant MC proliferation and an increase in extracellular mesangial matrix is a frequent pathological finding in a variety of glomerular renal diseases. IgA nephropathy, the commonest form of GN worldwide, is characterized by proliferation of MC with diffuse deposits of Immunoglobulin A (IgA) in the mesangium. Similar proliferative features are seen in lupus nephritis (renal involvement as part of the auto-immune disease Systemic Lupus Erythematosus (SLE)), post-streptococcal GN and mesangio-capillary GN. A pathological hallmark of diabetic nephropathy is mesangial expansion and progressive glomerulosclerosis (GS). Studies of a rat model of diabetic nephropathy attribute this process to alterations in MC phenotype and MC proliferation, with subsequent up-regulation of genes encoding components necessary for ECM production and mesangial expansion (Young et al, 1995). GS and associated fibrosis of the tubulointerstitial compartment of the kidney are thought to be the final common pathway in the permanent loss of functioning renal tissue. GS is characterized by the replacement of functioning glomerular tissue with increasing deposits of ECM proteins and a concomitant reduction in their breakdown (el Nahas et al, 1997).

1.4.2 Pathogenesis of mesangial cell proliferation and progressive glomerulosclerosis

1.4.2.1 Mechanisms regulating mesangial cell growth
At any stage of glomerulonephritis, MC number depends on a balance between the rates of MC proliferation and MC loss, either via cell apoptosis or necrosis. Turnover of MC in the normal adult human kidney is thought to be very low. Autoradiographic studies of glomerular cell types performed on kidneys of normal Sprague-Dawley rats over 20 years ago illustrated a rate of renewal of cells within the Bowman’s capsule of less than 1% per
day. The majority of these labelled cells were endothelial cells and to a lesser extent mesangial cells (Pabst & Sterzel, 1983). A number of reasons to explain this have been suggested, from the lack of antigen or mitogen exposure, to protection by growth inhibitory factors or receptor down-regulation (Schöcklmann et al, 1999). A disruption of the balance of control of MC proliferation occurs early in the pathological process leading to glomerular injury and progressive GS. Excess MC proliferation and deposition of ECM results in a loss of normal glomerular architecture, with consequent alterations in intraglomerular pressure and haemodynamics, resulting in a reduction in ultrafiltration efficiency and progressive renal impairment (Vleming et al, 1999). MC number may also be regulated by apoptosis, a physiological, ‘programmed’ form of cell death, or by autophagy. The latter is a catabolic process which there is tightly-regulated degradation of a cell’s own components via cellular lysosomal machinery. Studies of experimental glomerulonephritis have demonstrated the process of apoptosis as beneficial in deleting excess MC during spontaneous resolution of mesangial hypercellularity following MC injury in the Thy 1.1 model of nephritis (Baker et al, 1994; Shimizu et al, 1995).

The role of cell cycle regulatory proteins in the regulation of mesangial cell proliferation and apoptosis is now well recognised. Progression through the cell cycle following a mitogenic stimulus relies on specific interactions of a series of positive regulatory cell cycle proteins counterbalanced by negative regulatory inhibitory proteins. Using the Thy 1.1 rat model of mesangial proliferative nephritis, patterns of positive and negative cell cycle regulation may be studied. Upon induction of marked mesangial cell proliferation, increase in cyclins D1 and A were observed, with associated increases in their partner kinases, cyclin-dependant kinase (CDK)-4 and CDK-2 (Shankland et al, 1996). Inhibition of CDK-2 using roscovitine led to a reduction in MC proliferation (Pippin et al, 1997). As might be expected, the cyclin-dependant kinase inhibitor (CDKI) p27Kip1, usually expressed in quiescent MC, is reduced in the proliferative phase of Thy 1.1 nephritis. This is followed by an increase in levels of the previously absent CDKI p21Cip1 during the resolution phase, associated with a decrease in MC proliferation (Griffin et al, 2003). Targeting this fine balance of positive and negative regulatory mechanisms is a potential therapeutic strategy in the treatment of many proliferative diseases.
1.4.2.2 Role of the extracellular matrix (ECM)

A prominent feature of chronic glomerulosclerosis is the replacement of functioning glomerular tissue with ECM due to excess deposition of matrix proteins. Whilst a pathological finding of GS on renal biopsy may be described as evidence of relatively long-standing glomerular disease, changes in matrix synthesis commonly occur soon after a glomerular insult and the onset of glomerular inflammation. A disruption in the balanced interaction between MC and ECM may be responsible for the process of GS. The growth velocity of a mouse MC line increased significantly when grown on films of fibronectin or type I or IV collagen (He et al, 1995). Additionally, in vitro studies of rat MC have shown that type IV collagen and laminin, components of the normal ECM, protected cells from apoptosis induced by serum starvation. This protective effect was not seen with type I collagen or fibronectin (Mooney et al, 1999). These findings support the hypothesis that the balance of MC proliferation and apoptosis is regulated in part by specific interactions with adjacent ECM proteins.

1.4.2.3 Role of cytokines and growth factors

A number of cytokines and growth factors have been implicated in initiating and sustaining MC proliferation, including PDGF, AII and endothelin-1 (ET-1).

Platelet-derived growth factor (PDGF)

PDGF, a 30kDa cationic protein, is synthesised by a number of cells in addition to platelets. These include MC themselves, smooth muscle cells (SMC), endothelial cells and fibroblasts. The PDGF family consists of four members: the comprehensively studied PDGF-A and -B, and the more recently discovered and less well described PDGF-C and -D. Disulfide-bonded homo- or hetero-dimer isoforms of PDGF-A and -B (PDGF-AA, -AB and -BB) bind two structurally related tyrosine kinase receptors – the α- and β-receptor – with resultant dimerisation of the receptor upon binding. Binding to and stimulation of PDGF receptors triggers intracellular signalling cascades resulting in a variety of cellular events, including cell proliferation, reorganisation of the actin cytoskeleton and stimulation of chemotaxis (Diegelmann & Evans, 2004). In addition to being produced by MC, there is considerable evidence supporting the role of PDGF as a mesangial mitogen. PDGF is known to stimulate MC proliferation and accumulation of
extracellular matrix in vitro and in vivo (Floege et al, 1993; Abboud, 1993). Additionally, glomerular expression of both the PDGF-BB dimer and the associated PDGF-β receptor is increased in the anti-Thy1.1 model of nephritis (Iida et al, 1991). Studies of human renal biopsy specimens using in situ hybridisation and immunohistochemistry have demonstrated an increase in glomerular PDGF-β receptor mRNA from patients with mesangioproliferative diseases (Ranieri et al, 2001), and increased PDGF-BB mRNA in specimens from patients with diabetic nephropathy (Langham et al, 2003). A number of therapeutic strategies aimed at reducing or inhibiting PDGF activity in anti-Thy1.1 experimental nephritis have been examined and these will be discussed later in this chapter.

**Angiotensin II (AII)**

AII is the most potent vasoconstrictive agent in the human body, with intra-renal concentrations some 1000-fold higher than in the general circulation. It signals via two receptors: the AII type-1 receptor (AT₁) and the AII type-2 receptor (AT₂), although MC only express the AT₁ receptor. AII plays a critical role in the regulation of the glomerular filtration rate, largely via maintenance of efferent glomerular arteriolar tone. As a result, it is implicated as having a role in the development of GS via preferential constriction of the efferent arteriole leading to increased intraglomerular pressure (Miller et al, 1991). Furthermore, as will be discussed later in this chapter, AII also regulates the expression and function of T-CaCN. In addition to its potent haemodynamic effects, AII may also promote glomerular injury via other mechanisms. AII is known to upregulate the expression of a number of other mitogenic factors, including PDGF, fibroblast growth factor (FGF) and TGF-β (Klahr & Morrissey, 2000). Depending on the intracellular balance between growth factors, AII may promote MC proliferation or hypertrophy. It is also implicated in increasing the expression of a number of extracellular matrix proteins, including fibronectin, laminin and collagens (Mezzano et al, 2001). This increase in ECM deposition is largely mediated by TGF-β. Renal TGF-β expression is increased in normal rats following infusion with AII, and in a rat model of progressive GN with an associated increased ECM deposition (Border & Noble, 1998). AII blockade also results in a reduction in TGF-β expression in progressive glomerulonephritis. Uninephrectomized rats were given a single dose of anti-Thy1.1 monoclonal antibody
and rats treated with the angiotensin converting enzyme inhibitor (ACEi) cilazapril or the AT1 receptor antagonist candesartan for 10 weeks. Treated rats showed a marked reduction in markers of renal damage compared to control groups. Additionally, reduced levels of glomerular protein and mRNA for TGF-β and collagen types I and III were found in the treatment groups. These findings were independent of any effects on blood pressure (Nakamura et al, 1999).

**Endothelin-1 (ET-1)**

The endothelin family consists of three endogenous vasoactive peptides (ET-1, ET-2 and ET-3) that activate two distinct cell surface receptors, known as ETa and ETb. Endothelins may be synthesised by a number of different cells or organs in the body, including lung, brain, liver and renal glomerular and tubular cells. Of the three endothelins expressed in humans, only ET-1 and -3 are expressed in the kidney. ET-1 binds preferentially to the ETa receptor and there is a body of evidence supporting its role in the progression of renal disease. Exogenous administration of ET-1 results in local haemodynamic effects including an increase in afferent and efferent arteriolar pressure with a consequent fall in single nephron glomerular filtration rate (GFR) (Bruzzi et al, 1997). Additionally, ET-1 is known to result in an increase in MC proliferation, upregulation of mRNA for collagen types I, II and IV and laminin, and increased ECM deposition. *In vivo*, studies of glomeruli from rats with streptozocin-induced diabetes mellitus or anti-Thy1.1 nephritis demonstrate an increase in ET-1 gene expression (Nakamura et al, 1995; Yoshimura et al, 1995). Pharmacological targeting of the ETa receptor with the selective antagonist FR-139317 led to a reduction in proteinuria and renal injury in rats that had undergone renal mass reduction (Benigni et al, 1993). It has been suggested that ET-antagonists may have a therapeutic role in clinical renal disease (Hendry & James, 1997).

**1.4.3 Therapeutic strategies to treat mesangial cell proliferation and progressive glomerulosclerosis**

**1.4.3.1 Current therapies**

The treatment of systemic blood pressure is the mainstay of the management of patients
with chronic progressive renal disease (Whelton & Klag, 1989). The development of angiotensin converting enzyme inhibitors (ACEi) and Angiotensin II receptor blockers (ARB) has aided this therapeutic strategy significantly. The cardiovascular benefits, particularly in diabetic patients, of ACE-inhibition (HOPE study investigators, 2000), or a combination of ARB and the direct renin antagonist Aliskiren (Parving et al, 2008), are well recognised, although most cardiovascular trials have tended to exclude patients with moderate to severe renal impairment. One study which examined the effectiveness of the ARB avosentan in patients with overt diabetic nephropathy (mean creatinine 185µmol/l; estimated GFR 33ml/min) did not show a significant difference in primary endpoint outcomes and was terminated early due to an excess of cardiovascular events in the treatment arm (Mann et al, 2010). However, notwithstanding such a limitation, these classes of drugs seem to specifically exert a renoprotective effect in addition to their effect on systemic arterial blood pressure. This is thought to be as a result of their effect on intrarenal haemodynamics in lowering intraglomerular pressure and subsequently reducing proteinuria (Martinez-Maldonado, 2001). Data from the Ramipril Efficacy in Nephropathy (REIN) trial reported a beneficial effect of ramipril in hypertensive patients with heavy proteinuria (>3g/day) in terms of delaying progression of renal disease (The GISEN Group, 1997).

The effects on the rate of renal deterioration of ACEi in patients with a lesser degree of proteinuria, or of ARBs in all groups, are less well established. ACEi have been shown to inhibit MC proliferation in rat Thy-1 glomerulonephritis (Nakamura, 1999). Data from the Modification of Diet in Renal Disease (MDRD) study and data from the UK Prospective Diabetes Study (UKPDS) have illustrated the benefits of tight blood pressure control and blood sugar control in diabetic patients respectively in order to delay progression of renal disease (Klahr et al, 1994; Retnakaran et al, 2006). Additionally, targeting Ras GTPases using Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (‘statins’) may prove useful in inhibiting MC proliferation (Khwaja et al, 2005) in addition to conferring established cardiovascular benefits (Shepherd et al, 1995; Scandinavian Simvastatin Survival Study (4S), 1994). Statins may also have a therapeutic role in the management of proteinuric renal diseases by preventing the development of an albumin-induced pro-inflammatory phenotype in proximal tubular
epithelial cells in response to albumin (Chana et al, 2008).

Specific treatment of glomerular disease will always be determined by the underlying clinical or pathological diagnosis and likely prognosis. Treatment of proliferative lupus nephritis often requires immunosuppression with corticosteroids and cytotoxic drugs such as cyclophosphamide, although newer drugs such as mycophenolate mofetil are increasingly being used for induction of remission (Jayne, 2007). Evidence supporting the use of omega-3 fatty acids in the form of fish oils in IgA nephropathy, in addition to generic measures as detailed earlier, has been described (Donadio et al, 1994), although other smaller studies have shown no significant benefit (Chan & Trachtman, 2006). The use of immunosuppression in IgA nephropathy remains controversial, largely due to the lack of prospective randomised control trial data in this area. However, evidence demonstrating the effectiveness of corticosteroids in reducing proteinuria and preserving renal function in patients with IgAN has been reported (Pozzi et al, 1999), in addition to evidence supporting their long-term effectiveness (Pozzi et al, 2004). There is also evidence to suggest a role for immunosuppression targeting those patients with impaired renal function and/or crescentic disease (Strippoli et al, 2003). Current clinical immunosuppressive regimens have significant side-effects, which may be life-threatening in certain groups such as elderly patients, and have limited efficacy. A number of more specific strategies have been examined experimentally both in vitro and in vivo in an effort to reduce systemic effects.

1.4.3.2 Experimental strategies
These strategies may target any one of the many steps leading to aberrant cell growth, from mitogenic stimulus (e.g. anti-PDGF or anti-PDGF-receptor therapies) to downstream cell signalling cascades (e.g. inhibition of Ras signalling) to inhibition of cell cycle progression (e.g. use of the CDK2 inhibitor roscovitine). These examples will each be outlined briefly.

Various mechanisms of experimental inhibition of PDGF activity have been described. In studies of the anti-Thy1.1 model of nephritis, PDGF activity has been reduced or inhibited. Concomitant reductions in MC proliferation and glomerular injury were seen
upon treatment with anti-PDGF antibodies (Johnson et al, 1992) and with the use of the potent PDGF antagonist trapidil (Razzaque et al, 1995). Similar antiproliferative effects were seen upon targeting PDGF receptor signal transduction (Gilbert et al, 2001) and using PDGF aptamers (Floge et al, 1999).

The Ras family of small GTPases has been examined as a potential therapeutic target in non-malignant, proliferative renal diseases. Ras, a 21 kilodalton protein, may exist in one of four isoforms: Harvey-Ras (H-Ras), Kirsten-Ras splice variants 4A and 4B (Ki(4A)-Ras and Ki(4B)-Ras) and Neural-Ras (N-Ras). Ras proteins act as a molecular switch by cycling between an active GTP-bound state and an inactive GDP-bound state (Wittinghofer, 1998). Whilst in its active state, Ras is responsible for triggering signalling cascades, including those involving downstream effectors such as the Raf serine/threonine kinases, phosphatidylinositol 3-kinase (PI-3K) and Rho GTPases, resulting in cell proliferation, differentiation, cytoskeletal regulation and apoptosis.

Evidence for a role for Ras in the proliferation of renal cells has arisen from in vitro and in vivo studies of renal tubular cells and mesangial cells. The use of antisense oligonucleotides (ASOs) to target Ki- and H-Ras results in a significant reduction in EGF-mediated proliferation of cultured renal fibroblasts (Sharpe et al, 2000). HMG-CoA reductase inhibitors (statins) are inhibitors of Ras prenylation. Statins have been shown to inhibit MC and renal tubular cell proliferation in vitro (Massy et al, 1999; Vrtovsnik et al, 1999). Statins also reduce MC proliferation in vivo in the anti-Thy1.1 model of nephritis (Yoshimura et al, 1999). Both of these effects were independent of any reduction in cholesterol. Further study of anti-Thy1.1 nephritis has illustrated that induction of inflammation was associated with an increase in glomerular Ki- and N-Ras expression, and glomerular injury was reduced upon treatment with the Ras antagonist S-trans-trans-farnesylthiosalicylic acid (FTS) (Clarke et al, 2003). FTS is currently being developed as a therapy for some solid organ malignancies.

Inhibition of specific proteins or protein kinases of the cell cycle leading to cell cycle arrest would theoretically appear to be a therapeutic strategy in the treatment of mesangioproliferative disease. Upon examination of MC proliferation in the Thy1.1
model of glomerulonephritis, Shankland and colleagues demonstrated an increase in immunostaining for cyclin A and its partner kinase CDK2 localising to MC (Shankland et al, 1996). CDK2 is required for G1/S transition and subsequent DNA synthesis in S-phase. Roscovitine is a purine analogue which binds to the adenosine triphosphate (ATP) binding pocket of CDKs, leading to a reduction in the activity of CDK2, CDK5 and cdc2 without altering the levels of CDK protein expressed (Meijer et al, 1997). *In vitro* studies of cultured rat MC have illustrated a concentration-dependent inhibition of FCS-stimulated proliferation upon treatment with roscovitine at concentrations up to 25µM (Pippin et al, 1997). Further *in vivo* studies of the Thy1.1 nephritis model by the same investigators appear to support these findings. The use of specific therapies targeting elements of the cell cycle machinery may therefore be a future strategy in a wide range of proliferative and perhaps also malignant diseases.

It must be noted that, whilst the Thy 1.1 model is widely referenced in experimental MC proliferation, it is an artificial single- or double-hit rat model with significant associated limitations when comparing human MC biology *in vivo.*
1.5 Calcium and cell proliferation

1.5.1 Calcium as an intracellular signal

1.5.1.1 Importance of Ca\(^{2+}\) signalling

Ionized calcium (Ca\(^{2+}\)) is an essential intracellular second-messenger signalling molecule, mediating a variety of cellular events including contraction, differentiation and proliferation (Buonanno & Fields, 1999; Berridge, 1995). The intracellular concentration of free Ca\(^{2+}\) (that is, Ca\(^{2+}\) neither bound nor stored) is very tightly regulated, with a concentration in the cytoplasm some 10,000-fold lower than in extracellular compartment. Mechanisms of action of this diverse cation include binding to and modulation of downstream binding proteins (such as calmodulin).

The importance of cytoplasmic free Ca\(^{2+}\) in the regulation of MC number via a balance of apoptosis and proliferation is described in work by Saleh and colleagues. In their work, MC from 6-10 week old Spague-Dawley rats were isolated and cultured. Thapsigargin, an inhibitor of the endoplasmic Ca\(^{2+}\)-ATPase pump, was used to generate a rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) via release of Ca\(^{2+}\) from intracellular stores and a consequent influx of Ca\(^{2+}\) across the plasma membrane. Treatment of MC with thapsigargin led to an expected rise in [Ca\(^{2+}\)]\(_i\) measured by Fura-2 fluorescence. Pre-treatment of MC with the intracellular Ca\(^{2+}\)-chelator BAPTA-AM prevented any thapsigargin-induced rise in [Ca\(^{2+}\)]. Associated with this rise in [Ca\(^{2+}\)]\(_i\) was an increase in the proportion of apoptotic cells by 12 hours – this was not seen after pre-treatment with BAPTA-AM. Intact, annexin-V negative, thapsigargin-treated MC had a significantly lower [Ca\(^{2+}\)]\(_i\) than apoptotic, annexin-V positive MC. The proliferative effect of PDGF and effects on [Ca\(^{2+}\)]\(_i\) was also assessed. PDGF led to an acute rise in [Ca\(^{2+}\)]\(_i\), induced proliferation of cultured MC, and suppressed basal apoptosis. When MC were pre-incubated with PDGF for 12 hours prior to treatment with thapsigargin, MC proliferative rate returned to basal levels (Saleh et al, 2000). These results illustrate the importance of a rise in [Ca\(^{2+}\)]\(_i\) in both the proliferative and apoptotic signalling cascades. However, the effect on the progression of GS and renal damage of inhibition of Ca\(^{2+}\) entry using antagonists of L-CaCNs has generated controversy. Treatment of rat models of hypertension and chronic renal disease with a variety of L-CaCN antagonists has
demonstrated both accelerated progression of renal disease despite good blood pressure control in some studies (Brunner et al, 1991; Wenzel et al, 1992) versus improved survival with less GS in others (Harris et al, 1987; Dworkin et al, 1993). A recent meta-analysis of four trials examining the therapeutic benefits of ACE-inhibitors and other antihypertensives (including calcium channel blockers) in type 2 diabetics favoured ACE-inhibitors when examining all-cause mortality, acute myocardial infarction, cardiovascular events and stroke, but did not look at renal end points such as time to end-stage renal disease or doubling of serum creatinine (Pahor et al, 2000). Clearly, the effect of a drug on intra-renal haemodynamics can be a confounding variable when it comes to the progression of GS. Whichever results are to be believed, when compared to ACE-inhibitors, L-type calcium channel blockers (CCB) are less effective in preventing GS (Jackson & Johnston, 1988).

Whilst these experiments have studied the effects of L-type CCB in rat models of established renal impairment with or without hypertension, their effects on the rate of mesangial cell proliferation were not assessed. A reduction in $^3$H-thymidine incorporation by human mesangial cells in vitro has been described with verapamil and nifedipine (Schultz & Raij, 1990). Similar results were demonstrated in vivo upon examination of MC from stroke-prone spontaneously hypertensive rats after treatment with manidipine (Fujiwara et al, 1992). As a result of these findings, Orth and colleagues attempted to identify the mechanism involved in a primary culture model of human mesangial cells. The CCB studied (verapamil and nifedipine) partially attenuated the transient increase in free [Ca$^{2+}$], seen with the calcium channel agonist BAY K 8644 and K$^+$-depolarization. These CCB also caused a reduction in markers of cell proliferation. However, further examination of differing CCB and their individual enantiomers raised the possibility that these effects may not have been entirely as a result of actions on L-type calcium channels. Equimolar concentrations of phenylalkamine enantiomers, each differing significantly with respect to their antagonism of L-type Ca$^{2+}$ channels, demonstrated similar inhibition of MC growth (Orth et al, 1996). One must therefore consider the following possibilities: (i) there are other mechanisms of calcium entry important in the regulation of cell proliferation, (ii) L-type CCB may act on other targets to have similar effects, and (iii) other ion channels, in addition to voltage-operated
calcium channels, are involved in a complicated sequence of events initiated by a mitogenic stimulus.

1.5.1.2 Intracellular Ca\(^{2+}\) homeostasis

The cytoplasmic [Ca\(^{2+}\)] can be modulated via a number of mechanisms, as illustrated in Figure 1.3. Cytoplasmic [Ca\(^{2+}\)] may be increased by either influx of Ca\(^{2+}\) from outside the cell or from release of intracellular stores. A series of pumps are responsible for extrusion of Ca\(^{2+}\), returning intracellular concentrations to resting, steady state levels. With the cytoplasmic [Ca\(^{2+}\)] around 10,000-fold lower than in the extra-cellular space coupled with a hyperpolarized resting cell membrane potential, there is a huge electrochemical force favouring influx of Ca\(^{2+}\) ions. Channels on the cell membrane that allow Ca\(^{2+}\) influx include receptor-operated calcium channels (ROC), store-operated...
calcium channels (SOC) and voltage-operated calcium channels (VOC), all of which will be discussed in more detail later in this chapter. ROC are activated following binding of an agonist to an associated membrane receptor and channel opening occurs independently of any changes in membrane potential. SOC also operate independent of cell membrane potential, but open in response to the depletion of intracellular stores of Ca\(^{2+}\), primarily from the endoplasmic reticulum (ER), with signalling being via the ‘calcium influx factor’ (CIF). SOC consist of hetero- or homo-tetrameric subunits that are encoded by the transient receptor potential (TRP) family of genes. These channels are responsible for mediating capacitative calcium entry upon intracellular store depletion. Finally VOC, classically opening and closing in response to changes in membrane potential, provide a third mechanism for Ca\(^{2+}\) influx.

Mitogens or growth factors may bind to cell membrane receptors leading to the formation of diacyl-glycerol (DAG) and inositol 1,4,5-triphosphate (IP\(_3\)), due to the action of phospholipase C (PLC) on phosphatidylinositol 4,5-diphosphate (PIP\(_2\)). Whilst DAG leads to Ca\(^{2+}\) influx via ROC, IP\(_3\) binds to IP\(_3\) receptors (IP\(_3\)R) on the surface of the ER/SR leading to release of Ca\(^{2+}\) from intracellular stores at these sites. Depletion of intracellular stores results in opening of SOC and further Ca\(^{2+}\) influx. Membrane depolarization, perhaps via inhibition of voltage-dependent K\(^+\) channels, leads to opening of VOC.

In order to return the [Ca\(^{2+}\)], to basal levels, free Ca\(^{2+}\) ions may leave the cytoplasm by one of three mechanisms. Ca\(^{2+}\) may be actively sequestered into the ER via a specialized pump on the surface of the ER – the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) - or extruded from the cell altogether via Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase pumps in the plasma membrane. The third mechanism utilises a reverse mode of the Na\(^+\)-Ca\(^{2+}\) exchanger leading to calcium extrusion.

1.5.1.3 Associated downstream signalling mechanisms
Ca\(^{2+}\) signalling in mammalian cells involves a number of downstream mechanisms that lead to and control desired cellular events. Whilst there is a large number of intermediary signalling molecules and cascades described, two mechanisms that are commonly
involved are via calmodulin (CaM) signalling and via the Ras/Raf/MAPK (mitogen-activated protein kinase) pathway.

CaM is a 148aa, highly conserved Ca\textsuperscript{2+} binding protein with a molecular weight of around 16kDa, and can accommodate four Ca\textsuperscript{2+} ions in one of its four helix-loop-helix EF-hand Ca\textsuperscript{2+}-binding motifs (Chin & Means, 2000). The CaM protein is dumbbell-
shaped and upon binding of Ca\(^{2+}\) ions, hydrophobic areas of the CaM protein are exposed leading to interaction with target enzymes. These downstream target enzymes are activated by a variety of methods that include, in the case of calcineurin and Ca\(^{2+}\)-CaM-dependent kinase II (CaMKII), relief of autoinhibition. CaM, its interaction with Ca\(^{2+}\) and subsequent activation of CaMK in quiescent cultures of human MC is associated with induction of the early response gene c-fos (Zeng et al, 2002). Ca\(^{2+}\)/CaM also plays an important role in the regulation of the cell cycle, to be discussed later in this chapter.

The role of members of the Ras family in oncogenesis is highlighted by the finding that in over 10% of human cancers there are point mutations on Ras proteins (Bos, 1989). It is now accepted that in experimental renal disease, common Ras isoforms have crucial roles in cell signalling controlling a plethora of cellular events in a variety of cell types, including human renal fibroblasts and human MC (Sharpe et al, 1999; Sharpe et al, 2000; Khwaaja et al, 2006). As discussed earlier in this chapter, there are three human Ras genes which encode four distinct proteins: H-, N-, and the two splice variants of Kirsten-Ras, Ki(4A)- and Ki(4B)-Ras. These molecules are known as small GTPases as they cycle between an inactive GDP-bound state and an active GTP-bound state, thus functioning as a ‘binary switch’. Cell-surface receptor signals can then be transduced to the cytoplasm (and ultimately the nucleus) via specific effector pathways, especially the MAPK cascade (Raf/MEK/ERK signalling).

Activation of Ras is catalyzed by guanine nucleotide exchange factors (GEFs) and deactivation is driven by GTPase-activating proteins (GAPs). Intracellular Ca\(^{2+}\) concentration is known to be able to regulate Ras activation via indirect methods (Rosen et al, 1994), but may also be able to directly regulate activation via recently identified Ca\(^{2+}\)-regulated GEFs and GAPs (Cullen & Lockyer, 2002). As a result, Ca\(^{2+}\) may determine whether the Ras binary switch is in its ‘on’ or ‘off’ state.

Ca\(^{2+}\)/CaM may also be able to modulate Ras signalling itself. It has been suggested that CaM may define a threshold in the Ras/Raf/MEK/ERK signalling pathway, preventing activation at low doses of growth factor. Additionally, CaM inhibits sustained high ERK1/2 activation after growth factor stimulation, attenuating p21\(^{cip1}\) levels. As a result,
CaM is capable of playing a necessary part in the proliferative cascade mediated by the Ras/Raf/MEK/ERK pathway (Agell et al, 2002).

1.5.2 Calcium and the cell cycle

1.5.2.1 The mammalian cell cycle

The proliferation of mammalian cells is dependent on a highly regulated sequence of events that make up the cell cycle, as illustrated in Figure 1.5 above. The cell cycle consists of two gap phases G\textsubscript{1} and G\textsubscript{2}, S-phase and M-phase. An additional gap phase known as G\textsubscript{0} occurs in some cells that are able to exist in a state of quiescence. Passage through each of these phases is controlled by specific positive or negative regulators, known as cyclin-dependent kinases (CDK) and cyclin-dependent kinase inhibitors (CDKI) respectively. As the name would suggest, each CDK needs to be bound to a partner cyclin protein to have a functional effect. Cell cycle progression is either promoted or arrested depending on the relative expression of CDKs and CDKIs at specific points of the cycle.

Cells that are quiescent may remain in the G\textsubscript{0}-phase of the cell cycle for many months or years. In this phase, minimal mRNA and protein synthesis occurs and only upon mitogen stimulation may cells re-enter the cell cycle into the G\textsubscript{1}-phase. Once in G\textsubscript{1}, cells prepare for DNA synthesis later in the cell cycle by generating the necessary mRNAs and
proteins needed for this process. Cells then encounter the first of two checkpoints – the restriction point, functioning in late-G₁. For cells to be able to pass this point, cyclin D-CDK4(6) complexes need to be activated in order to precipitate a cascade of events which leads to the generation of proteins necessary for progression into S-phase, for example cyclin A and cyclin E. DNA replication occurs during S-phase in preparation for mitosis later in the cycle. Whilst levels of most CDKs remain relatively constant throughout the cycle (Pines, 1993), levels of the cyclins may fluctuate dramatically. Cyclin E peaks around the time of G₁-S transition and diminishes rapidly thereafter, whilst cyclin A levels accumulate during S-phase and throughout G₂, before degradation. Following DNA replication, cells enter G₂-phase containing twice as much DNA in the nucleus compared to G₁. During this gap phase, synthesis of additional mRNAs and proteins occurs in preparation for cell division. There is then a further checkpoint which functions to ensure no errors are present in the duplicated DNA prior to entry into M-phase and mitosis.

1.5.2.2 Ca²⁺, calmodulin and the cell cycle

Calcium ions and calmodulin (CaM) have been implicated as being involved in the re-entry of quiescent cells into the cell cycle and in regulating ‘checkpoints’ between G₁/S-phases and G₂/M-phases (Lu & Means, 1993; Whitaker & Patel, 1990). A number of Ca²⁺/CaM-dependent kinases (CaMK) are known to be responsible for mediating these effects. CaMK consist of a single polypeptide chain and are inactive in the absence of Ca²⁺/CaM due to intrasteric inhibition, whereby the resting conformation of Ca²⁺/CaM inhibits access to the protein substrate. Upon re-entry from quiescence and also at the G₁/S border, CaMK has been shown to phosphorylate the CREB transcription factor and regulate the expression of early response genes such as c-fos (Sheng et al, 1991; Zeng et al, 2002). Inhibitors of CaM have been shown to inhibit DNA synthesis and cell proliferation in synchronised populations of Chinese hamster ovary-K1 (CHO-K1) cells (Hidaka et al, 1981). In these experiments, inhibition of cell proliferation using the CaM antagonist W-7 would support the proposal that CaM is a major mediator of Ca²⁺-dependent cell proliferation. It is important to note, however, that Ca²⁺ and intracellular Ca²⁺ pools may play important roles in cell proliferation independent of CaM, as illustrated by the demonstration of cell cycle arrest of a cell line of smooth muscle cells.
after depletion of the IP₃-sensitive intracellular Ca²⁺ pool with thapsigargin (Short et al, 1993).
### 1.6 Classes of calcium channels

#### 1.6.1 Voltage-operated calcium channels (VOC)

<table>
<thead>
<tr>
<th>Family</th>
<th>Type</th>
<th>$\alpha_1$-subunit</th>
<th>$\text{Ca}_{x,y}$</th>
<th>Distribution</th>
<th>Antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVA</td>
<td>L-type</td>
<td>$\alpha_1S$</td>
<td>$\text{Ca}_{v,1.1}$</td>
<td>Skeletal muscle</td>
<td>All blocked by: Dihydropyridines, Phenylalkamines, Benzothiapine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha_1C$</td>
<td>$\text{Ca}_{v,1.2}$</td>
<td>Cardiac/VSM</td>
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<td></td>
<td></td>
<td>$\alpha_1D$</td>
<td>$\text{Ca}_{v,1.3}$</td>
<td>Endocrine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha_1F$</td>
<td>$\text{Ca}_{v,1.4}$</td>
<td>Retina</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-type</td>
<td>$\alpha_1B$</td>
<td>$\text{Ca}_{v,2.2}$</td>
<td>Neuronal</td>
<td>$\omega$-conotoxin GiVA</td>
</tr>
<tr>
<td></td>
<td>P/Q-type</td>
<td>$\alpha_1A$</td>
<td>$\text{Ca}_{v,2.1}$</td>
<td>Neuronal Urothelium</td>
<td>$\omega$-agatoxin IVA</td>
</tr>
<tr>
<td></td>
<td>R-type</td>
<td>$\alpha_1E$</td>
<td>$\text{Ca}_{v,2.3}$</td>
<td>Neuronal</td>
<td>SNX-482</td>
</tr>
<tr>
<td>LVA</td>
<td>T-type</td>
<td>$\alpha_1G$</td>
<td>$\text{Ca}_{v,3.1}$</td>
<td>Neuronal / renal / VSMC / cardiac</td>
<td>All blocked by: Mibefradil, TTL-1177, Ni$^{2+}$/Cd$^{2+}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha_1H$</td>
<td>$\text{Ca}_{v,3.2}$</td>
<td>VSMC / cardiac</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha_1I$</td>
<td>$\text{Ca}_{v,3.3}$</td>
<td>Neuronal only</td>
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</tr>
</tbody>
</table>

Table 1.1: Classification of voltage-operated calcium channels. HVA = high voltage-activated; LVA = low voltage-activated; VSMC = vascular smooth muscle cell
As their name would suggest, voltage-operated calcium channels open and close, activate and inactivate in response to changes in the membrane potential of the cell in which they are expressed. This would usually mean changes from the resting membrane potential of excitable cells such as neurons or cardiac pacemaker cells, although, some electrically non-excitable cells also express VOC which go on to play important physiological roles. VOC may be broadly classified according to their typical electrophysiological characteristics into high voltage-activated (HVA) and low voltage-activated (LVA) channels. HVA channels typically activate at potentials between −50mV and +10mV, whilst LVA activate at more negative potentials (−70 to −30mV). While this suggests an apparent overlap in activation potentials for LVA and HVA currents, each can also be distinguished by their unique electrophysiological characteristics, with LVA demonstrating rapid inactivation and a typical ‘tail-current’.

As illustrated in table 1.1, the T-type calcium channel is the only member of the LVA group of VOC, whilst HVA channels include L-, N-, P/Q- and R-type channels. The nomenclature of VOC subtypes is often confusing. The most frequent description relates to broad channel type (e.g. L- or T-type). VOC structurally consist of a pore-forming \( \alpha_1 \)-subunit and associated auxiliary subunits (\( \alpha_2 \), \( \beta \) and \( \delta \)). As a result, VOC may be classified more specifically by one of the ten known \( \alpha_1 \)-subunit isoforms (e.g. \( \alpha_1 \)C or \( \alpha_1 \)G). As shown in table 1.1, this nomenclature is often used interchangeably with a more recent system to indicate the ion involved and their voltage sensitivity (e.g. \( \text{Ca}_{\text{v}1.2} \) or \( \text{Ca}_{\text{v}3.1} \)). A number of partially or wholly specific antagonists of VOC have been described, either naturally occurring compounds (e.g. conotoxins) or compounds synthesised to target a specific channel. Each type of VOC will be briefly described in turn.

1.6.1.1 **L-type (HVA)**

L-type calcium channels (L-CaCN) are widely distributed throughout the body and are known to consist of four distinct subtypes – those containing the \( \alpha_1 \)C, \( \alpha_1 \)S, \( \alpha_1 \)D, or \( \alpha_1 \)F pore-forming subunit. The \( \alpha_1 \)C subunit (\( \text{Ca}_{\text{v}1.2} \)) is primarily found in the heart and vascular smooth muscle and is targeted in the treatment of hypertension and cardiac arrhythmias using drugs including nifedipine (a dihydropyridine), verapamil (a
phenylalkamine) or diltiazem (a benzothiapine). Each of these three classes of drugs has
recognised selective potency against all four subunits of the L-type class. $\alpha_1$S subunits
(Ca$_{v}$1.1) are typically found in skeletal muscle whilst $\alpha_1$D subunits (Ca$_{v}$1.3) are primarily
involved in hormone secretion in the endocrine system, including in the anterior pituitary.
Finally, $\alpha_1$F subunits (Ca$_{v}$1.4) are thought to reside exclusively within the retina. A
typical L-type calcium current recording by whole-cell patch clamp studies would be
activated by relatively high depolarizations (-50 to +10mV) and inactivates slowly – hence the name L-type (Long-acting).

1.6.1.2 P-/Q-type (HVA)
P/Q-type calcium channels are primarily found in neuronal tissue, although they have
also been reported in the vascular smooth muscle cells of renal resistance vessels in the
rat and in SMC from the human umbilical artery (Hansen et al, 2000; Salemme et al,
2007). The pore-forming unit is the $\alpha_1$A subunit (Ca$_{v}$2.1), and a specific blocker of these
channels is $\omega$-agatoxin IVA – a 48 amino acid peptide isolated from the venom of the
American funnel web spider (Mintz et al, 1992). Other channel antagonists that are
partially specific for P/Q-type channels but also have varying effects on N- and L-CaCNs
include $\omega$-agatoxin III (also from the funnel web spider) and $\omega$-grammotoxin SIA isolated
from tarantula venom.

1.6.1.3 N-type (HVA)
The $\alpha_1$B subunit (Ca$_{v}$2.2) gives the N-type calcium channel its unique physiological
characteristics. N-type channels are exclusively found in neurons and were distinguished
from other high voltage-activated calcium channels (L-type) in chick dorsal root ganglion
(DRG) neurons by Nowycky et al over 20 years ago. A large conductance channel
(typically ~20pS) was named an L-CaCN and a channel of intermediate size (around
13pS) was named the N-type channel (Nowycky et al, 1985). The same authors also
identified the T-CaCN as having a low Ba$^{2+}$ conductance of ~8pS. A highly-selective
blocker of N-type channels in a variety of species is the 27 amino acid peptide $\omega$-
conotoxin GiVA, isolated from the Conus geographus sea snail (Olivera et al, 1994). A
clinical trial of a synthetic version of $\omega$-conotoxin MVIIA (another highly selective
naturally occurring N-type antagonist, isolated from the Conus magus snail) is underway
for the treatment of severe pain, although its mediocre specificity for N-type channels has led to concerns about side-effects (Penn & Paice, 2000).

1.6.1.4 R-type (HVA)

R-type voltage-operated calcium channels consist of a transmembrane $\alpha_1E$ subunit (Ca$_{2.3}$) in addition to the auxiliary subunits found in all VOC. R-type channels are typically found in neurons and are blocked by the partially selective toxin SNX-482 isolated from *Hysterocrates gigas*, although this toxin may also inhibit L-CaCN.

1.6.1.5 T-type (LVA)

The low voltage-activated (LVA) T-CaCN can be identified as having an $\alpha_1$-subunit of one of three distinct isoforms: $\alpha_1G$ (Ca$_{3.1}$), $\alpha_1H$ (Ca$_{3.2}$) or $\alpha_1I$ (Ca$_{3.3}$). The latter of these three, $\alpha_1I$, is expressed almost exclusively in the central nervous system, whereas $\alpha_1G$ and $\alpha_1H$ are widely distributed throughout many tissues of the body. The development of the selective antagonist mibefradil by Hoffmann-La Roche in the early 1990’s greatly advanced the study of these channels. Polyvalent cations such as nickel (Ni$^{2+}$) and cadmium (Cd$^{2+}$) are known to be potent antagonists of T-CaCN, with additional antagonists being developed with increasing specificity, including the compound TTL-1177.

1.6.2 Store-operated calcium channels (SOC)

As discussed earlier, cytoplasmic [Ca$^{2+}$] may be increased by either release from intracellular stores or via influx of Ca$^{2+}$ across the cell membrane. Emptying of intracellular Ca$^{2+}$ stores leads to activation of Ca$^{2+}$ influx via SOC – also known as capacitative Ca$^{2+}$ entry. Influx via SOC is the major mode of Ca$^{2+}$ entry in electrically non-excitabile cells, with SOC being found in cells of all eukaryotes (Parekh and Putney, 2005). The predominant store of intracellular Ca$^{2+}$ is in the endoplasmic reticulum (ER) and Ca$^{2+}$ is essential to maintaining the functional integrity of the ER. Persistent depletion of Ca$^{2+}$ from the ER may lead to errors such as protein misfolding or inappropriate apoptosis. The key property of SOC is their activation in response to store depletion, thus facilitating the rapid refilling of intracellular Ca$^{2+}$ stores. A number of distinct SOC currents have been described, the best characterized being the Ca$^{2+}$ release-
activating Ca\(^{2+}\) current (CRAC). However, the signalling mechanisms linking store Ca\(^{2+}\) depletion to opening of membrane CRAC channels are unclear.

Ca\(^{2+}\) channels with properties consistent with SOC have been demonstrated in human MC. Work by Rong and colleagues demonstrated a voltage-independent, low single-channel conductance channel that was activated in response to thapsigargin-induced depletion of intracellular Ca\(^{2+}\) (Rong et al, 2000). Treatment of human MC with epidermal growth factor (EGF) has been shown to activate an intracellular signalling mechanism involving tyrosine kinase and protein kinase C (PKC) resulting in activation of SOC (Rong & Sansom, 2001). It has been suggested that this may play an important role in cell proliferation and differentiation. SOC are thought to be encoded by the transient receptor potential channel (TRPC) family of genes. Recent work has demonstrated the presence of TRPC1 and TRPC4 genes in mouse mesangial cells, with knockdown of TRPC4 expression using ASO resulting in an 83% reduction in SOC activity (Wang et al, 2004). Comparable results have not yet been demonstrated in human MC.

1.6.3 **Receptor operated calcium channels (ROC)**

ROC are likely to be closely related to SOC, both in their functional and pharmacological properties and also as part of the TRPC family. Since the initial hypothesis that activation of receptors may lead to calcium entry into SMC independent of membrane depolarisation in the 1970s (Bolton, 1979), ROC have been described in a number of SMC types. ROC are activated by agonists acting on a range of G-protein-coupled receptors which include ATP, ACh and noradrenaline. It is also thought that considerable interaction occurs between ROC, SOC and VOC in the regulation of Ca\(^{2+}\) entry.
1.7 T-type calcium channels (T-CaCN)

1.7.1 T-CaCN genes and protein structure

Following the description of a novel inward calcium current in fertilized starfish eggs in the mid-1970’s (Hagiwara et al, 1975), now known to be via the T-type calcium channel, this ‘low-threshold’ transient calcium current has been widely described in a variety of tissues (Yunker, 2003 (review); See table 3.1). Advances in molecular biology led to the identification in the late 1990’s of three distinct T-CaCN isoforms: α₁G, α₁H and α₁I (Perez-Reyes et al, 1998; Cribbs et al, 1998; Lee et al, 1999). These isoforms are also known as Caᵥ3.1, Caᵥ3.2 and Caᵥ3.3 respectively.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Gene</th>
<th>Genbank No.</th>
<th>Chromosome</th>
<th>cDNA length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>CACNA1G</td>
<td>AF134986</td>
<td>17q22</td>
<td>7648</td>
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<tr>
<td>α₁H</td>
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<tr>
<td>α₁I</td>
<td>CACNA1I</td>
<td>AF129133</td>
<td>22q13.1</td>
<td>6740</td>
</tr>
</tbody>
</table>

Table 1.2: Genomic location and size of human T-type calcium channel genes

Each α₁-isofrom has a cDNA sequence of around 7000 base pairs, which is transcribed into a large transmembrane protein of around 2500aa in length. This protein forms the channel pore consisting of four repeated transmembrane domains (I-IV), each containing six transmembrane segments (S1-S6) (Figure1.6). There is a high degree of sequence homology between LVA and HVA α₁-isofroms in the transmembrane regions. Segment 4 (S4) is the voltage sensor of activation of these voltage-activated channels as it contains positively-charged amino acid residues at every 3-4 positions. These may undergo a conformational change and migrate in response to changes in the electrical field leading to opening of the pore. Membrane depolarization and the subsequent electrical field imposed leads to outward movement of this segment, thus driving channel opening. In contrast, LVA α₁-subunits are less than 60% identical to each other and less than 20% identical to those forming HVA channels. This is due to the great diversity in the linkage segments, especially the intracellular loops.
Between domains II and III there is a large intracellular loop that has been shown to be important in channel regulation by, for example, Ca\(^{2+}\)-calmodulin-dependant kinase II (CAMKII) (Welsby et al, 2003). Other intracellular molecules known to interact with LVA channels leading to modulation of channel activity include G-proteins, which have binding sites on the I-II intracellular loop, with varying affinity between isoforms. \(\alpha_1\)H subunits, but not \(\alpha_1\)G or \(\alpha_1\)I, contain a G-protein binding site (QXXER) in this region (Yunker & McEnery, 2003). Four shorter extracellular loops, situated between S5 and S6 of each domain and known as the ‘P-loop’, are thought to be involved in channel opening. In HVA channels, four glutamate residues in each loop maintain a selective negatively-charged pore region. In LVA, one of these glutamate residues is replaced by aspartate in the P-loop, maintaining the ring of negative charge but altering the current properties. LVA channels can demonstrate the current characteristics of HVA by mutating LVA channels and replacing the aspartate for glutamate (Talavera et al, 2001).

![Figure 1.6: Structure of the T-type calcium channel \(\alpha_1\)-subunit](see text above for description)

### 1.7.2 Distribution and functions of T-CaCN

T-CaCN are expressed in a variety of tissues throughout the body, including neuronal tissue, cardiovascular tissue, smooth muscle, the kidney and various endocrine organs and tissues. T-CaCN play diverse roles, which include mediation of neuronal rebound
burst firing and generation of neuronal pacemaker activity (Perez-Reyes, 2003). They are also likely to be involved in the regulation of contraction of smooth muscle in vascular, bronchial, gastrointestinal and uterine smooth muscle (Akaike et al, 1989; Janssen, 1997; Xiong et al, 1993; Young et al, 1993), and regulation of hormone release such as pituitary secretions (Matteson & Armstrong, 1986). It has been proposed that a steady influx of calcium necessary to mediate many of these functions may be mediated by T-CaCN via a ‘window current’ in non-excitable cells, near to the resting membrane potential, as described in section 1.7.3 (Cohen, 1988). Evidence of a role for T-CaCN in tissue development and proliferation will be described in section 1.9 later in this chapter.

1.7.3 Regulation of T-CaCN expression and function

T-CaCN expression may be up- or down-regulated, and functionally modulated, by one or more of a number of signalling mechanisms (Yunker, 2003). Local and systemic factors that have been identified as contributors to this regulatory process include AII, aldosterone, ET-1 and Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII). There is additional evidence of the modulatory effects of other local molecules, including G-protein subunits and HVA L-CaCN auxiliary subunits.

Whole-cell patch clamp studies of bovine adrenal glomerulosa cells performed almost 20 years ago investigated calcium currents following stimulation of aldosterone secretion with AII (Cohen et al, 1988). Two distinct calcium channels were identified, now known to be T-type and L-type. Additionally, high K\(^{+}\) and AII increase T-CaCN current, and cells were shown to be highly sensitive to small changes in extracellular K\(^{+}\), maintaining a steady Ca\(^{2+}\) influx via T-CaCN adequate to stimulate aldosterone secretion. It was on the basis of these experiments that Cohen and colleagues proposed the idea of the ‘window current’ of Ca\(^{2+}\) influx via T-CaCN. The investigators demonstrated T-CaCN activation at the resting membrane potential of \(-80\) to \(-50\) mV to a much greater extent than the L-type current, thus implying the presence of a steady-state inward Ca\(^{2+}\) current via T-CaCN at these voltages – results similar to those described in other cell lines (Cohen et al, 1988; Cohen et al, 1987). A typical ‘window-current’ is illustrated in figure 1.7.
Figure 1.7: Typical T-CaCN activation-inactivation kinetics in glomerulosa cells, demonstrating proposed ‘window-current’. Steady state activation (blue squares) and inactivation (red triangles) curves demonstrating typical T-CaCN kinetics in adrenal glomerulosa cells. The inactivation curve demonstrates the typical tail current exhibited by T-CaCN. As the curves overlap, the area shaded green illustrates the voltage range in which there should be steady-state current through T-CaCN (the ‘window-current’). (Adapted from Cohen et al, 1988)

Another potent vasoconstrictor, ET-1, has also been shown to influence T-type current. When ET-1, a 21aa peptide, was added to cultured neonatal rat ventricular myocytes the T-type current was enhanced in a dose-dependent manner, with no effect on L-type current. This enhancement of T-current was inhibited by staurosporine – a protein kinase C inhibitor – identifying a potential signalling pathway involved (Furukawa et al, 1992).

Whilst G-proteins and other kinases are known to target and regulate HVA channels (Catterall, 2000), evidence of the direct regulation of LVA T-CaCN has historically been sparse. Using chimeric channel constructs generated from wild-type α1G and α1H channels, the regulation of T-CaCN by CAMKII has been described. In these chimeric models, the intracellular amino acid linker between domains II and III was exchanged between α1G and α1H molecules. Replacement of the HII-III linker with the corresponding GII-III linker abolished CAMKII-induced up-regulation of α1H, whilst the
reverse circumstances resulted in $\alpha_1G$ channels gaining CAMKII-dependent regulation in the presence of the H$_{II}$-III sequence. Investigation of potential substrate sites on the H$_{II}$-III linker identified a unique single serine residue ($\text{Ser}^{1198}$), which is phosphorylated by CAMKII and critical to channel regulation (Welsby et al, 2003).
1.8 T-type calcium channel antagonists

Although T-CaCN were first recorded over 30 years ago (Hagiwara et al, 1975), the lack of specific T-CaCN inhibitors has delayed research in this field. Additionally, there exists significant heterogeneity between cell type and individual T-CaCN α1-subunits with regard to their voltage-dependent kinetics and sensitivities to various antagonists. However, a number of organic and inorganic compounds have been identified as being capable of attenuating the T-CaCN current with varying degrees of specificity. A recent review by Yunker listed over 40 compounds from a broad spectrum of drug classes as having such an effect in a variety of animal cell models (Yunker, 2003). A few of these compounds have been shown to have antiproliferative effects both in vitro and in vivo. These include mibefradil, TTL-1177, nickel (and some other polyvalent cations) and the phenylalkamine verapamil. Many others (such as phenytoin, propofol and nifedipine) are in use clinically for various indications, with the targeting of T-CaCN not thought to be of primary importance in their mechanism of action. This does, of course, raise questions over the specificity of these drugs as antagonists of the T-CaCN, given their multiple roles and other targets, although support comes from the inorganic ion nickel which is known to be relatively specific for T-CaCN and behaves in a similar way to mibefradil and TTL-1177 in other experimental models. However, for the purpose of our experiments, T-CaCN antagonists which had been widely used in other published studies of cell proliferation were used, while accepting the significant limitations caused by the uncertainty over some of their molecular targets.

1.8.1 Mibefradil

![Chemical structure of mibefradil](image)

Figure 1.8: Chemical structure of mibefradil
Mibefradil is a non-dihydropyridine calcium channel antagonist. First developed by pharmaceutical company F. Hoffmann-La-Roche and initially known as Ro 40-5967 (its full description being (1S,2S)-2-[2[[3-(2-benzimidazolylpropyl)methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride)), mibefradil has significantly advanced the study of T-type calcium currents in a variety of cell types, allowing more detailed examination of the physiological functions of T-type calcium channels.

1.8.2 TTL-1177

Following drug design and production specifically aimed at blocking Ca\(^{2+}\) entry via T-CaCN, TTL-1177 has since been shown to inhibit the proliferation of prostate cancer cells both in vitro and in a mouse model (Haverstick et al, 2000). Immunodeficient mice inoculated with the PC-1 prostate cancer line before treatment with TTL-1177 lived significantly longer and displayed no evidence of drug toxicity. At present, this novel compound is some way from assessment in human clinical trials. Further refinement of this drug is ongoing, in addition to the development of additional compounds that inhibit Ca\(^{2+}\) influx via T-CaCN and interfere with cell cycle progression (McCalmont et al, 2004).

1.8.3 Nickel (Ni\(^{2+}\)) and polyvalent cations

Although not used clinically, inorganic divalent and trivalent cations were amongst the first chemicals used to identify and block T-type calcium currents (Hagiwara et al, 1975; Llinas & Yarom, 1981; Carbone & Lux, 1984). Ni\(^{2+}\) preferentially inhibits T-CaCN over
L-CaCN and furthermore may help identify specific T-CaCN subunits. Ni$^{2+}$ preferentially inhibits currents conducted by the $\alpha_1$H isoform by a factor of ten compared to those conducted by $\alpha_1$G or $\alpha_1$I upon examination of two distinct expression systems (*Xenopus* oocytes and human embryonic kidney (HEK)-293 cells) (Lee et al, 1999). Significant heterogeneity would appear to exist between endogenous T-CaCN as illustrated by the observed variability in the concentrations of individual cations needed to half-maximally inhibit T-CaCN currents. For example, the profile of smooth muscle cells (Akaike et al, 1989) differs from that recorded from skeletal muscle cells (Satoh et al, 1991) or cortical neurons (Ye & Akaike, 1993). It is highly probable that variability in sensitivity between different species also occurs.

### 1.8.4 Verapamil

![Chemical structure of verapamil](image)

Verapamil is typically recognised as being a high voltage-activated, L-type calcium channel antagonist, and is commonly used in the treatment of hypertension, angina and supraventricular or junctional tachycardias. It is significantly more potent against HVA than LVA currents, although this varies between tissue type. However, half-maximal inhibition of T-CaCN currents has been demonstrated in rat aortic smooth muscle cells using a concentration of 70$\mu$M, compared to an IC$_{50}$ of 0.9$\mu$M for HVA-type currents (Akaike et al, 1989). Whilst this dose needed to inhibit T-CaCN would be impractical clinically in view of likely side effects, it provides another experimental tool for the investigation of T-CaCN in *in vitro* cell systems.
1.9 Evidence of a role for T-type calcium channels in cell proliferation

Several lines of evidence link T-CaCN with cell proliferation. An association has been established between the rate of vascular smooth muscle cell (VSMC) proliferation and T-CaCN current density, first demonstrated by work published in 1989 by Akaike following studies of primary cultures of rat aorta smooth muscle cells (SMC) (Akaike et al, 1989). Further work by Kuga demonstrated an association between expression of L- or T-type currents and the cell cycle. Again studying primary cultures of rat aortic SMC, the authors used immunocytochemical analysis to determine the cell cycle phase of individual cells, and identified the type of Ca\(^{2+}\)-channel current expressed in each of these cells using the whole-cell voltage-clamp method (Kuga et al, 1996). Cells examined were found to express both L- and T-currents, but the temporal expression of each current was determined by cell cycle phase. Quiescent cells in G\(_0\)-phase expressed only L-type current. T-type current was expressed in 37% of cells in G\(_1\)-phase (in addition to L-type), whilst in S-phase 90% of cells expressed a T-type current. During mitosis, (M-phase) there were once again only L-type currents measured. In addition to this evidence linking proliferation to T-current expression, an increase in T-current was shown to be associated with the duration of cell culture, with T-current declining when cells reached confluence and their proliferative rate slowed.

Work by Guo and colleagues strengthened this proposed association between T-type current expression and cell cycle phase. Studying primary cultures of ventricular myocytes isolated from 1-day-old Wistar rats and using whole cell voltage-clamping and BrdU-labelling, 27% of cells at day 5 of culture expressed a T-type current. However, 95% of cells at day 5 which labelled positive for BrdU, indicating cells in S-phase, expressed a T-type current, compared to only 19% of the non-labelled cells (Guo et al, 1998). This neonatal ventricular myocyte proliferation, shown by BrdU-labelling, diminished over the 15-day culture period and was paralleled by a progressive loss in T-type current. As such, it would appear that the activity of T-CaCN plays an important role in normal human embryonic and rat neonatal development. However, at least in
cardiac myocytes and possibly in other cell types, the T-current then diminishes to a very low level in adulthood unless the cell is stimulated to re-enter the cell cycle.

Xu and colleagues studied atrial myocytes from adult Wistar-Froth rats that had undergone subcutaneous implantation of GH3 cells, a cell line isolated from a rat pituitary tumour, causing increased serum growth hormone (GH) levels. In control animals, atrial myocyte T-currents contribute only a tiny fraction of the overall inward Ca$^{2+}$ current. However, in atrial cells from tumour-bearing rats, there was a 2-fold increase in T-current density two weeks after inoculation, although heart weight at this point was unchanged. This increase in T-current preceded the increase in heart weight seen at later time points, with a 3-fold increase in T-current at 8 weeks corresponding to a 2.5-fold increase in heart weight and a 1.5-fold increase in cell size (Xu & Best, 1990). These findings would suggest that in cells stimulated to re-enter the cell cycle, an increase in T-current precedes and may be an initiating factor in cell proliferation or hypertrophy. Finally, neonatal rat cardiac myocytes exposed to high glucose concentrations resulted in increased cell proliferation and an increase in cells in S-phase; both effects were reversed by the T-CaCN antagonists Ni$^{2+}$ and mibefradil. Cells treated with high glucose had elevated mRNA levels for $\alpha_1G$ and $\alpha_1H$ T-CaCN and a higher Ca$^{2+}$ influx upon depolarization by KCl; again Ni$^{2+}$ attenuated this latter effect (Li et al, 2005).

Therapeutic interventions to modulate cell proliferation by targeting T-CaCN have been reported in both animal models and cultures of human cells. Wang et al demonstrated the inhibition of PDGF-stimulated proliferation of C3H/10T1/2 mouse fibroblasts (which express only T-type VOC) following treatment with nordihydroguaiaretic acid (NDGA) – an inhibitor of VOC (Wang et al, 1993). Mibefradil has been shown to inhibit the proliferation of human peripheral blood mononuclear cells (Lijnen et al, 1999) and also cultured human astrocytoma (U87-MG) and neuroblastoma (N1E-115) cells, leading to interest in a potential therapy for solid tumours of this kind. Cells over-expressing the $\alpha_1H$ T-CaCN subunit demonstrated a doubling of proliferative rate, whilst transfection of an antisense oligonucleotide (ASO) against $\alpha_1G$ mRNA led to a 45% reduction in proliferative rate at day 4. Additionally, an increase in expression of Cyclin D1, a marker
for non-proliferating cells, was seen in association with a reduced rate of proliferation as a result of serum starvation or treatment with mibebradil (Panner et al, 2005).

MC are recognised as being similar in structure and function to vascular smooth muscle cells (VSMC). Schmitt et al suggested a possible role of the T-CaCN in 1995 when reporting parallel in vivo and in vitro experiments studying VSMC proliferation. In their in vivo studies, male Wistar-Kyoto rats underwent balloon injury to the left common carotid artery. Rats were divided into treatment groups receiving mibebradil, amlodipine or verapamil at a range of concentrations, with the left and right (as control vessel) carotid arteries being harvested and examined at day 14. Mibebradil significantly reduced the area of neointima formation by 48%, whilst the L-type calcium channel blockers amlodipine and verapamil had no effect despite similar reductions in systemic blood pressure in all groups. Parallel experiments examining primary cultures of Wistar-Kyoto rat aortic SMC showed a 50% reduction in cell number at day 3 when cells stimulated with 10% fetal calf serum were treated with mibebradil compared to controls. Verapamil had no effect on SMC growth velocity (Schmitt et al, 1995).

Recent work by Brooks and colleagues has demonstrated a link between T-CaCN expression and function and VSMC proliferation. In their studies, rat A10 VSMC and COS-7 cells were stably transfected with human $\alpha_1$H cDNA. Following $\alpha_1$H over-expression, the proliferative rate of A10 cells increased 2.3-fold and that of COS-7 cells increased 4-fold compared to vector controls (Brooks et al, 1999). Further data from the same group has demonstrated that stable over-expression of T-type calcium channel isoforms in HEK-293 cells accelerates cell proliferation (Wang et al, 2002). Similarly, Rodman et al have recently reported data illustrating a role for T-CaCN in the control of human pulmonary artery smooth muscle cell (PASMC) proliferation. Primary cultures of human PASMC expressed T- and L-type $Ca^{2+}$ currents that were inhibited by mibebradil and nifedipine respectively, and mRNA for $\alpha_1$G and $\alpha_1$H T-CaCN subunits were demonstrated by RT-PCR. Small-interfering RNA (siRNA) knockdown of the $\alpha_1$G (Ca3.1) gene resulted in a significant reduction in PASMC growth velocity, whilst FACS analysis showed that treatment of cells with mibebradil resulted in a reduction of progression into S-phase of the cell cycle (Rodman et al, 2005).
In the light of these findings, of the similarities between VSMC and MC, and of the lack of published work detailing the expression and function of T-CaCN in MC, we have studied the expression of T-CaCN in human mesangial cells in primary culture and examined the effects of T-CaCN antagonists and siRNA on human mesangial cell proliferation *in vitro*.

### 1.10 Aims of the project

- To demonstrate T-type calcium channel expression in quiescent and proliferating human mesangial cells (MC) at the mRNA and protein level
- To determine the anti-proliferative effects of T-type calcium channel antagonists, and to examine cell death by apoptosis or necrosis
- To examine the effect of siRNA on T-CaCN expression and function, and on mesangial cell proliferation and apoptosis
- To examine the effects of T-type calcium channel antagonists on cell cycle progression
- To examine the relationship between T-CaCN and cell cycle regulatory proteins
2. Materials and Methods

2.1 Cell Model

2.1.1 Choice of human mesangial cells
Cryopreserved normal human mesangial cells (MC) at passage 3 were obtained from Clonetics (Cat. No. CC-2559) and stored in liquid nitrogen. MC are pre-characterized by Clonetics as staining positive for the mesangial cell markers fibronectin and alpha-smooth muscle actin (α-SMA) and negative for cytokeratin 19 (a marker of epithelial cells) and von Willebrand factor (Factor VIII) antigen (an endothelial cell marker). Further characterisation in our laboratory included confirmation of α-SMA staining.

Alternative cell lines or human mesangial cells from other sources were not used in the experiments reported in this thesis. This of course brings limitations that must be considered when reviewing the results reported and these are discussed in section 3.5.

2.1.2 Primary culture of human mesangial cells
Human MC were cultured in Techne incubators at 37°C with 95% O₂ and 5% CO₂ using tissue culture flasks and dishes from Falcon (Marathon Laboratory supplies, London UK). Prior to first use, the cells were rapidly thawed at 37°C, suspended in medium and plated into 75cm² tissue culture flasks. The standard growth medium used was RPMI-1640 medium (Gibco) supplemented with insulin-transferrin-sodium selenite (5µg/ml), penicillin (100IU/ml), streptomycin (100µg/ml), amphotericin (2.5µg/ml) and fetal calf serum (FCS) (Sigma) to give a final concentration of 10% FCS (RPMI-1640/10%FCS). Upon passaging or seeding into experimental conditions, adherent MC were washed twice in phosphate-buffered saline (PBS) (Sigma) followed by being incubated at 37°C for five minutes with 1ml of 1X Trypsin-EDTA (Life-Technologies, Paisley, UK). Released cells were re-suspended in RPMI-1640/10%FCS and re-plated onto culture dishes as appropriate. For routine subculture, cells were split 1:2 to 1:4 when confluent and propagated in 75cm² tissue culture flasks. All experiments were carried out between
passages 5-10. After passage 11, cells tended to grow much more slowly and also changed their phenotype with the production of increased mesangial matrix. As a result, these cells were deemed unsuitable for study.

2.2 Electrophysiological measurements

MC at passage 4-5 were trypsinised as described earlier and plated into 6-well plates in standard growth medium. After 24 hours, cells were approximately 50% confluent and ready for electrophysiological study. Whole cell patch-clamp electrophysiological studies were performed by Dr Linda McLatchie at the Rayne Institute, St Thomas’ Hospital. T-CaCN currents were measured using whole-cell voltage-clamping with an Axopatch 200A controlled by pClamp software (v6.0). Currents were recorded at room temperature (~22°C) via 1-2MΩ pipettes filled with a Cs-rich pipette solution containing (in mM): CsCl 120, MgCl₂ 5, CaCl₂ 0.5, Na₂ATP 5, Na₃GTP 0.3, EGTA 12, HEPES 10, pH 7.2, in combination with a bath solution containing (in mM): NaCl 138 HEPES 10, glucose 20, CaCl₂ 5, pH 7.35. T-CaCN currents were recorded from a holding potential of −90mV during 200msec steps to −20mV. HEK cells over-expressing α₁G or α₁H T-CaCN isoforms were used in Dr McLatchie’s laboratory as positive controls and using similar methodology to that used when examining MC.

2.3 Reverse transcriptase – polymerase chain reaction (RT-PCR)

In order to determine which T-CaCN isoforms were transcribed in primary cultures of MC, semi-quantitative RT-PCR was performed. Sterile techniques and plastics were used for all procedures involving handling of RNA. Water was made RNAse-free by treatment with 0.1% diethylpyrocarbonate (DEPC) overnight. The DEPC was then degraded by autoclaving. This water was used to make up all solutions not supplied in kits.
2.3.1 Total cellular mRNA isolation

MC were cultured in 75cm² tissue culture flasks in low (0.2% FCS) or standard (10% FCS) RPMI-1640 growth medium until such time cells were 80-90% confluent (approximately 8 days for 0.2% FCS and 5 days for 10% FCS). Growth medium was removed and cells were washed once with PBS. Total RNA was isolated using the Qiagen RNeasy Mini-Kit® (Qiagen Ltd, Crawley, UK) following the manufacturers instructions. Cells were lysed directly in the culture dish by addition of a lysis buffer/β-mercaptoethanol solution, followed by disruption of the cell membranes by pipetting. Cells were then homogenised by loading into a Qiagen QIA-Shredder column and subsequently centrifuged for two minutes at 14,000rpm. An equal volume of 70% ethanol was added to the homogenised lysate prior to its transfer into an RNeasy mini-column. After a series of rinses in wash buffer, total mRNA was eluted with 40μl of RNase-free water. Once eluted, the total amount of RNA isolated was quantified using a spectrophotometer. Absorbance was measured at 260nm and 280nm giving a ratio. An absorbance of 1 unit at 260nm (A260) corresponds to 40μg of RNA per ml whilst absorbance at 280nm (A280) is indicative of the amount of DNA present. The A260/A280 ratio of all samples was between 1.5-2.0, confirming the purity of the isolated RNA. Isolated RNA was stored at -80°C until used in experiments.

2.3.2 Primer design

RT-PCR primer pairs were designed to specifically target the T-CaCN α₁-subunit genes being studied using the Primer 3 web-based primer design program (MIT, Boston, MA, USA). Of the many primer sequences suggested, those that were selected needed to be non-palindromic, at least 50% G-C rich, of 17-21 base pairs in length and with melting temperatures as close as possible to its pair. The primer sequence used to detect the α₁H subunit was the same as that used by Haverstick and colleagues in their study of T-CaCN in a human prostate cancer cell line (Haverstick & Gray, 2000). Potential sequences were then put into the BLAST program to ensure specificity to the gene of interest. Primers designed against the housekeeping gene β-Actin were also used to confirm equal loading of mRNA. The sequences used are listed in Table 2.1, along with the gene GenBank accession number and sequence melting temperatures (Tₘ(°C)).
2.3.3 RT-PCR

In order to minimise pipetting errors or contamination, which may occur when carrying out reverse transcription (to generate cDNA) and DNA amplification steps separately, both steps were performed concurrently in the same tube using the Promega’s Access RT-PCR System (Promega, Southampton, UK) following the manufacturer’s instructions. The kit uses AMV reverse transcriptase (AMV RT) from Avian Myeloblastosis Virus for first strand DNA synthesis, and the thermostable Tfl DNA polymerase from *Thermus flavus* for second strand cDNA synthesis and DNA amplification. An advantage of the Access RT-PCR system is that it includes a single buffer that permits activity of both polymerases, negating the need for buffer additions between the reverse transcription and DNA amplification steps. As a result, the amount of cDNA available for amplification is maximised whilst reducing the risk of contamination.

In all RT-PCR reactions, 0.5µg of total RNA was used with 50µM of T-CaCN primer. Due to the relative abundance of transcribed β-Actin in MC compared to transcribed T-CaCN isoforms, the concentration of β-Actin primers used in reactions was reduced 10-fold to 5µM. The annealing temperature for each reaction was calculated to be 4-5°C below the melting temperature of each primer (provided by Amersham, the primer manufacturer) and averaged between the forward and reverse primers. Therefore, for

<table>
<thead>
<tr>
<th>Gene (GenBank Number)</th>
<th>Primer base sequence (5'-3')</th>
<th>Product Length (base pairs)</th>
<th>$T_m$(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1G (AF134985)</td>
<td>Fwd: CTCTTCCGAGTCTCCACAGG</td>
<td>233</td>
<td>59.98</td>
</tr>
<tr>
<td></td>
<td>Rev: TCCAGCTCAGCTCTAGCTC</td>
<td></td>
<td>60.00</td>
</tr>
<tr>
<td>CACNA1H (AF051946)</td>
<td>Fwd: TCGAGGAGGACTTCCACAAG</td>
<td>176</td>
<td>64.30</td>
</tr>
<tr>
<td></td>
<td>Rev: TGCATCCAGGAATGGTGAG</td>
<td></td>
<td>64.90</td>
</tr>
<tr>
<td>CACNA1I (AF393329)</td>
<td>Fwd: GCTGGAGGGACAGTGATATT</td>
<td>152</td>
<td>60.00</td>
</tr>
<tr>
<td></td>
<td>Rev: GACAACAGGAAGGCTGCTC</td>
<td></td>
<td>60.00</td>
</tr>
<tr>
<td>β-Actin (BC013835)</td>
<td>Fwd: CCCACACTGTGCCCATC</td>
<td>594</td>
<td>64.00</td>
</tr>
<tr>
<td></td>
<td>Rev: TGATCCACATCTGCTGGAAG</td>
<td></td>
<td>63.90</td>
</tr>
</tbody>
</table>

Table 2.1: Sequences for T-CaCN α₁-isofrom and β-Actin RT-PCR primers
CACNA1G the annealing temperature used was 59°C and for CACNA1H and CACNA1I it was 56°C. The optimum Mg²⁺ concentration was determined by a series of 40 cycle RT-PCR reactions with increasing Mg²⁺ concentrations between 25µM and 75µM. The optimum concentration was found to be 50µM and this was used in all further experiments.

The RT-PCR reaction protocol used is illustrated below:

**First strand cDNA synthesis**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48°C for 48mins</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>1</td>
<td>94°C for 2mins</td>
<td>AMV RT inactivation RNA/cDNA/Primer denaturation</td>
</tr>
</tbody>
</table>

**Second strand cDNA synthesis and PCR amplification**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-40</td>
<td>94°C for 30sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>56-59°C for 1min</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>68°C for 2mins</td>
<td>Extension</td>
</tr>
<tr>
<td>1</td>
<td>68°C for 7mins</td>
<td>Final extension</td>
</tr>
<tr>
<td>1</td>
<td>4°C</td>
<td>Soak</td>
</tr>
</tbody>
</table>

**Figure 2.1: RT-PCR protocol**

For initial detection of T-CaCN isoform mRNA, 40 cycles of PCR was used for maximal amplification. However, a semi-quantitative approach was necessary in order to assess the effects of siRNA transfection on mRNA levels. This was achieved by determining the exponential phase of the amplification reaction. The cycle number was varied between 20 and 40 cycles, with cycle numbers 27-32 being demonstrated to be in the exponential phase for CACNA1H amplification (as shown in chapter 5, figure 5.2). 10% of the reaction products were then analysed on a 1.5% agarose gel containing 1µl/100ml ethidium bromide. TAE buffer containing 1µl/100ml ethidium bromide was used for the electrophoresis.
2.4 Calcium channel inhibitors

The calcium channel inhibitors mibefradil, verapamil and Ni\(^{2+}\) ions (as nickel chloride) (all Sigma), were made up as stock solutions at a concentration of 10mM in water before being filter-sterilised and stored at 4°C until use. Fresh stock solutions of Mibefradil, Ni\(^{2+}\) ions and Verapamil were made up every 2-3 months. TTL-1177 was made up to a solution of 10mM in 100% ethanol and stored at -20°C (Ref). TTL-1177 was a gift from Dr Lloyd Gray, University of Virginia, Charlottesville, USA.

2.5 Assessment of cell proliferation

2.5.1 MTS assay

Cell proliferation was measured using Promega’s CellTiter 96\(^{®}\) Aqueous Non-Radioactive Cell Proliferation Assay. This assay is based on the cellular conversion of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)) into a formazan product. The formazan absorbs light at a wavelength of 490nm, which can be read on a 96 well tissue culture plate reader. The recorded absorbance is recognised as being directly proportional to the number of living cells present in the culture (Cory et al, 1991). The optimal number of MC to be plated in each well of a 96 well plate was determined by seeding plates with cells at increasing density from 2500 to 10000 cell per well, in triplicate. RPMI-1640 culture medium contained 10% FCS and cell number was measured daily for three days shown in figure 2.2). A seeding density of 5000 cells per well gave a curve over 24 to 72 hours which appeared to demonstrate peak growth velocity at 24-28 hours, plateauing by 72 hours. This density was used in all MTS assays studying the effects of calcium channel antagonists.
Figure 2.2: Assessment of optimal plating density for MTS proliferation assays. A plating density of 5000 mesangial cells per well (cpw) cultured in standard growth medium over three days produced a typical sigmoidal growth curve whilst using the fewest possible cells.

An assessment of the validity of the MTS assay in accurately assessing cell number was performed. Cells were plated into a 96-well plate at a range of cell numbers and then immediately counted using the MTS assay. A linear correlation was demonstrated between the two methods, as shown in figure 2.3.

Figure 2.3: Validation of MTS assay. MC were plated at a range of concentrations (2500 to 10000 cells per well) (x-axis). Plated cells were then immediately counted using the MTS assay giving an absorbance value equivalent to viable cell number (y-axis).

After serum-deprivation in 0.2% FCS for 48 hours, cells were seeded into 96-well plates at a density of 5000 cells per well and were incubated with T-CaCN inhibitors (i) mibefradil (0-15µM), (ii) TTL-1177 (0-15µM), (iii) Ni^{2+} (0-400µM) or (iv) L-type antagonist verapamil (0-200µM), all made up in RPMI-1640/10%FCS. MC proliferation
was stimulated by 10% FCS. Cell number was assessed on the day of seeding and at intervals of 24 hours up to 72 hours.

2.5.2 BrdU incorporation
DNA synthesis was assessed by Bromodeoxyuridine (BrdU) incorporation. BrdU is taken up into the genomic material of proliferating cells as they pass through S-phase and DNA replication takes place. Cells were plated onto 35mm culture dishes in RPMI-1640/10%FCS for 24 hours. Following one wash with PBS, cells were incubated in 0.2% FCS for 48 hours. RPMI-1640/10%FCS +/- drugs at a range of concentrations were then added for a further 48 hours. BrdU (at a final concentration of 10µM) was added to each dish for the final 16 hours. Cells were then washed with PBS, fixed for 45 minutes (in 3 volume 50mM glycine pH 2 and 7 volume ethanol), and then washed again with PBS. Cells were subsequently incubated in 4M hydrochloric acid for 10 minutes, washed three times in PBS and then blocked in a solution containing 5% goat serum and 0.05% Tween in PBS for 15 minutes. Monoclonal anti-BrdU primary antibody at a dilution of 1:100 in blocking solution was then added and cells were incubated at 4°C overnight. Cells were then washed with PBS the following morning before being incubated with TRITC-conjugated anti-mouse IgG secondary antibody at a dilution of 1:50 for 30 minutes at room temperature in the dark. Cell nuclei were then counterstained with Hoechst 33342 at a concentration of 10µg/ml for 15 minutes at room temperature in the dark. Cells were then visualised with a fluorescent microscope. BrdU positive cells would stain nuclei red whilst all cell nuclei would stain blue. A total of around 150 cells were counted from at least three different randomly chosen fields. The experiments were repeated three times.
2.6 Assessment of apoptosis and cytotoxicity

2.6.1 Detection of apoptosis
MC were plated onto 35mm culture dishes in RPMI-1640/10%FCS for 24 hours. Following one wash with PBS, cells were incubated in 0.2% FCS for 48 hours. RPMI-1640/10%FCS +/- drugs at a range of concentrations were then added for a further 24 hours. At this point, cell nuclei were stained with Hoechst 33342 at a final concentration of 10µg/ml and incubated at room temperature for 10 minutes in the dark. Propidium iodide (PI) was then added to the medium for a further 5 minutes in the dark at a final concentration of 5µg/ml. Cells were then visualised with a fluorescent microscope. Hoechst 33342 freely enters all cells and stains nuclei blue. PI is a highly polar dye that is impermeable to cells with an intact membrane, only entering necrotic or late apoptotic cells and staining these cells red. Early apoptotic cell nuclei could then be identified as showing signs of chromatin condensation and nuclear fragmentation.

Staurosporin (at a final concentration of 1µM) was added to one culture plate for two hours prior to the addition of Hoechst 33342 and PI as a positive control. A total of around 150 cells were counted from at least three different randomly chosen fields. The experiments were repeated three times.

2.6.2 Detection of cytotoxicity
Drug-induced cytotoxicity was assessed by quantification of lactate dehydrogenase (LDH) from cultured cells. Cells were seeded at a density of 1x10^4 cells per well into a 96-well plate. 24 hours later, cells were washed once with PBS and medium replaced with phenol red-free RPMI-1640 medium supplemented with 5% FCS +/- drugs. LDH activity was then assessed a further 24 hours later using the Promega Cytotox 96 Non-radioactive Cytotoxicity Assay according to the manufacturers instructions. Cytotoxicity was calculated as a percentage of maximum LDH release determined by addition of lysis buffer to positive control untreated cells 45 minutes prior to reading the assay.
2.7 Cell cycle analysis using flow cytometry

The position of MC within the cell cycle at a given point in time was established using flow cytometry. Cells were plated onto 60mm culture dishes in RPMI-1640/10%FCS for 24 hours. Following one wash with PBS, cells were incubated in 0.2% FCS for 48 hours. RPMI-1640/10%FCS +/- drugs at a range of concentrations were then added for a further 48 hours. BrdU (at a final concentration of 10µM) was added to each dish for the final 24 hours of culture. Nocodazole (Sigma) was added to the culture medium at a range of concentrations for the final 16 hours. Nocodazole was added in order to bring about cell cycle arrest at G2/M phase, thus facilitating the study of events at the G1/S phase border. It is an antimitotic agent that disrupts microtubules by binding to beta-tubulin and preventing formation of one of the two interchain disulfide linkages, inhibiting microtubule dynamics, causing disruption of mitotic spindle function and fragmentation of the Golgi complex. As illustrated in figure 2.4, the proportion of cells held in G2/M increased from around 6% with no nocodazole added to 20% with 80nM nocodazole. Above this concentration there was no significant change in cell cycle phase. Therefore, a final concentration of 80nM of nocodazole was used in all further experiments.

![Figure 2.4: Alteration in cell cycle phase of cultured human MC upon incubation with nocodazole. Nocodazole at a range of concentrations was added to culture medium for the final 16 hours of treatment prior to flow cytometric analysis as described in section 2.7. (n=1)](image-url)
MC were trypsinised and removed as detailed earlier. Cells were centrifuged at 1100rpm for 5 minutes, washed once with PBS, centrifuged once more before being fixed in 1ml ice-cold 70% ethanol and transferred to a FACS tube. Cells were then either analysed immediately or stored for up to two weeks at 4°C prior to analysis. Fixed MC were centrifuged at 2000rpm for 5 minutes, ethanol was removed and cells were re-suspended in 0.5ml 4M hydrochloric acid before being incubated at 37°C for 10 minutes. The reaction was then stopped with 3ml PBS, cells were centrifuged once more (2000rpm, 5 minutes) before addition of 100µl of primary monoclonal anti-BrdU antibody at a dilution of 1 in 5 in a solution of PBS, 0.5% Tween 20 and 1% FCS. Cells were vortexed briefly and incubated at room temperature for 60 minutes. 3ml PBS was then added to nuclei before further centrifugation (2000rpm, 5 minutes). A volume of 100µl of anti-mouse FITC-labelled secondary antibody was then added to nuclei at a dilution of 1 in 10 in a solution of PBS, 0.5% Tween 20 and 1% FCS. Again, cells were briefly vortexed and incubated for 30 minutes in the dark. After addition of 3ml PBS, centrifugation and a further wash, PBS was removed and 1ml of staining solution was added to each tube. The staining solution consisted of 50µg/ml PI in PBS. PI binds to DNA and cell cycle phase can thereby be determined since G2 cells have twice the amount of DNA as G1 cells, with S phase cells containing an intermediate amount. Cells were vortexed and incubated in the dark for up to 30 minutes until analysis in a flow cytometer.

2.8 Cell synchronisation

Two methods were used in an attempt to synchronise populations of human MC – serum-deprivation and the double thymidine block technique.

For serum-deprivation experiments, MC were cultured, trypsinised and plated into 60mm dishes as described previously. Once approximately 40-50% confluent, cells were washed in PBS and fresh medium added – either containing 10% FCS (standard culture medium) or 0.2% FCS (serum-deprivation). After 72 hours, cells were washed with PBS.
once more and medium containing 10% FCS was added to all plates, with protein lysates isolated immediately, at 7 hours and at 24 hours.

For thymidine release experiments, MC were similarly cultured, trypsinised and plated into 60mm dishes. Once cells were 40-50% confluent, medium was removed and replaced with standard growth medium (10% FCS) containing 2mM thymidine (Sigma). Cells were then incubated for 12 hours. Cells were then washed three times with PBS to remove thymidine and standard growth medium replaced for a further 12 hours. Cells then received a second exposure of 2mM thymidine in standard growth medium for a further 12 hours. Following this, cells were washed a further three times with PBS and ‘released’ by addition of fresh standard growth medium. Nocodazole was added at a concentration of 80ng/ml at the time of release from the second thymidine exposure.

2.9 Western blotting

2.9.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The effects of various experimental conditions on cell signalling proteins were examined using Western blotting. Cultured cells to be examined were washed three times in PBS then lysed by the addition of ice-cold PBSTDS lysis buffer (150μl for a 35mm culture dish, 300μl for a 60mm dish or 500μl for a 100mm dish). Cellular material was stood on ice for five minutes before being collected with a cell scraper and the total lysate transferred to a microcentrifuge tube on ice for 45 minutes. This was then centrifuged at 10,000rpm at 4°C for 4 minutes to remove nuclear material. The supernatant was harvested and either frozen at -80°C or assayed immediately for protein content using Pierce bicinchoninic acid protein assay reagents (Pierce, Chester, UK).

In each experiment, 5-20μg of total lysate was analysed. Equal amounts of total protein were used and volumes were normalised using PBSTDS. An equal volume of 2X Western sample buffer (WSB) containing 10% 2-mercaptoethanol was added to each tube and all protein samples were denatured by heating tubes to 100°C for three minutes
before returning to ice prior to gel loading. The samples were then loaded on a 6, 10 or 12% SDS polyacrylamide resolving gel (depending on protein size). Biotinylated (Amersham, Bucks, UK) and colour molecular weight markers were also loaded and the gel was run at a constant voltage of 200V until the dye had run from the bottom of the gel.

Following electrophoresis, gels were soaked in Towbin transfer buffer for 3-5 minutes before being blotted onto nitrocellulose membranes (Hybond C, Amersham, Bucks, UK) at 100V for 45 minutes. Membranes were then either dried and stored at room temperature or blocked and detected as described below.

### 2.9.2 Immunodetection

Nitrocellulose membranes were blocked for one hour in either 5% dried skimmed milk/PBST (for non-phospho-specific primary antibodies) or 5% bovine serum albumin (BSA)/TBST (for phospho-specific primary antibodies). Primary antibody was diluted in blocking solution, added to the membrane in a heat-sealed bag and rocked for 1 hour at room temperature or at 4°C overnight. Membranes were then washed three times for 10 minutes in PBST or TBST, then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:1000 in PBST or TBST in a heat-sealed bag for 45 minutes at room temperature. After a further three washes in PBST / TBST to remove excess secondary antibody, the membrane was developed with Amersham chemiluminescence ECL system and exposed to film for between 15 seconds and 10 minutes.
2.10 Small-interfering RNA (siRNA) transfection

2.10.1 siRNA design

siRNA sequences were ordered from Qiagen to be specifically targeted against the Ca\(_{\text{v}}\)3.2 gene (GenBank No.: AF051946). Two pairs of siRNA molecules (named siRNA-1 and siRNA-2) were used in preliminary experiments using Vero cells and human MC, before finding a pair that successfully and reliably knocked down \(\alpha_1\)H (Ca\(_{\text{v}}\)3.2) mRNA expression (siRNA-3), described in Table 2.2 below.

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>CAC CAG GAA CAT CTC CAC CAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>r(CCA GGA ACA UCU CCA CCA A)dTdT</td>
</tr>
<tr>
<td>Antisense</td>
<td>r(UUG GUG GAG AUG UUC CUG G)dTdG</td>
</tr>
</tbody>
</table>

Table 2.2: Sequences for T-CaN \(\alpha_1\)H siRNA molecules (named siRNA-3)

2.10.2 Transfection protocol

MC were trypsinised and plated in standard culture medium into 96-well plates at a density of 2500 cells/well for proliferation assays, or into 60mm dishes at an equivalent density for total RNA isolation experiments. Transfection of siRNA took place after 18-24 hours at which point the cells were approximately 30-50% confluent. For each well of a 96-well plate, 10µl of Ca\(_{\text{v}}\)3.2 siRNA as a 20µM aqueous stock solution was diluted in 160µl Optimem I serum-free medium. 6µl of Oligofectamine transfection reagent was diluted in 24µl Optimem I and allowed to stand for 5-10 minutes. The diluted siRNA and diluted oligofectamine were then added together and allowed to stand for 15-20 minutes at room temperature. Plated cells were then washed once with Optimem I, then 40µl fresh Optimem I was added to each well followed by 10µl of the siRNA complex solution to the relevant well, giving a final siRNA concentration of 200nM. A negative control siRNA sequence (Silencer #1, Ambion) was similarly transfected to a control group of MC. All cells were then incubated at 37\(^\circ\)C for 24 hours to allow entry of the siRNA complexes into the cells prior to addition of 50µl 2X growth medium to all wells. For 60mm dishes, reagent volumes were adjusted accordingly.
2.10.3  **Assessment of cell number**

The effect of \( \alpha_1 \)H siRNA transfection on MC proliferation was assessed using the MTS assay and BrdU incorporation method as described earlier. Cell number was assessed at day 0, 2, 3 and 6. BrdU incorporation was assessed at day 3.

2.10.4  **Total RNA isolation**

Total cellular RNA was isolated at days 3 and 6 as described in chapter 2.3.1 earlier. Once isolated, RNA was stored at -80°C prior to RT-PCR.

2.10.5  **Protein lysate isolation**

MC were transfected with siRNA as detailed above and incubated at 37°C for 24 hours. Cellular protein lysate was then isolated from plates prior to addition of 2x growth medium, then at 3, 6, 12 and 24 hours after addition of 2x growth medium as described earlier.

2.11  **Statistical analysis**

Statistical analysis of cell proliferation, apoptosis, BrdU incorporation and cytotoxicity was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni’s post-test comparing all sets of variables. Results are expressed as mean ± SEM and were taken to be significant if \( p \leq 0.05 \).
2.12 Materials, solutions and buffers

All materials, chemicals and biochemicals were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated.

2.12.1 Cell culture medium
All culture mediums were obtained from Gibco, BRL, Life Technologies (Paisley, UK). Foetal calf serum (FCS) and goat serum were obtained from Sigma (Poole, Dorset, UK).

2.12.2 Buffers and solutions

*Phosphate buffered saline (PBS):* tablets obtained from Sigma, Dorset, UK

*PBST:* 1x PBS, 0.1% tween 20 (Bio-Rad, Herts, UK)

*PBSTDS lysis buffer:* 1x PBS, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, leupeptin 0.5µg/ml, pepstatin 1.0µg/ml, EDTA 1.0mM, PMSF 0.2mM

*TAE Buffer (50x) per 500ml:* 121g Tris base, 28.6ml glacial acetic acid, 14.6g EDTA

*Towbin buffer (1000ml):* 200ml methanol, 14.4g glycine, 3.03g Tris base

*Tris buffered saline (TBS):* 50mM Tris-HCl (pH 7.5), 150mM NaCl

*TBST:* 1x TBS, 0.1% tween 20 (Bio-Rad, Herts, UK)

*Western sample buffer (WSB) (6x concentrate):* 0.5M Tris (pH 6.8), 0.35M SDS, 30% glycerol, 0.6M dithiothreitol, 0.175M bromophenol blue (Bio-Rad, Herts, UK)

2.12.3 Antibodies

*Antibodies obtained from Sigma:*
Monoclonal anti-BrdU antibody (B-2531)
FITC-conjugated anti-mouse IgG secondary antibody (F-2012)
TRITC-conjugated anti-mouse IgG secondary antibody (T-7657)

*Antibodies obtained from Santa Cruz, Autogen Bioclear:*
Polyclonal anti-α1H T-CaCN goat IgG antibody – N-18 (sc-16261)
Polyclonal anti-α1H T-CaCN goat IgG antibody – C-20 (sc-16263)
Monoclonal anti-cyclin A mouse IgE antibody (sc-239)
Monoclonal anti-cyclin E mouse IgG1 antibody (sc-25303)
*Antibodies obtained from Amersham:*

Horse Radish Peroxidase (HRP)-conjugated anti-mouse IgG antibody (NA-931)

*Antibodies obtained from New England Biolabs, USA:*

Anti-phospho-Akt (ser 473) antibody (9271S)
Anti-Akt antibody (9272)
Anti-phospho-44/42 MAPK (E10) monoclonal antibody (9106S)
Anti-phospho-p38 antibody (921LS)
3. Expression of T-type calcium channel isoforms in human mesangial cells

3.1 T-type calcium channel expression in mesangial cells

Despite the molecular structure of T-type calcium channels being identified and cloned in the late 1990’s, there remains only limited knowledge of the expression and functional significance of T-CaCN in non-excitable cells such as MC. As described in Chapter 1, an inward calcium current mediated via T-CaCN is important in a number of physiological processes including neuronal firing, hormone secretion, cell differentiation, fertilization and smooth muscle contraction. The heterogeneity of T-CaCN as a result of the three channel isoforms gives rise to varied cellular functions. T-CaCN have been described in vascular smooth muscle cells (VSMC) (Akaike et al, 1989) and their role in the control of cell proliferation has been reported (Schmitt et al, 1995; Rodman et al, 2005). This chapter will describe efforts to demonstrate the expression of T-CaCN in primary cultures of human MC at the mRNA and protein level and demonstrate a calcium current mediated via the T-CaCN.

3.2 T-type calcium channel isoform mRNA expression in human mesangial cells

In order to determine which T-CaCN isoforms are expressed in human MCs, reverse-transcriptase polymerase chain reaction (RT-PCR) was used to examine isoform mRNA species. Using this technique, low levels of mRNA could be identified and individual isoforms distinguished. Primers were designed and the RT-PCR protocol was performed as described in Chapter 2. 40 cycles of PCR were performed. The presence of a band for the housekeeping gene β-Actin acted as a control to ensure equal RNA loading.

The results of an RT-PCR experiment examining T-CaCN isoform expression in primary cultures of human MC are shown in Figure 3.1. Two separate RNA isolates taken from
examples of cultures of proliferating human MC (labelled MC1 and MC2) expressed the $\alpha_1H$ T-CaCN isoform. The $\alpha_1G$ T-CaCN isoform was not expressed in either isolate. Total cellular mRNA isolates from HEK-293 cells overexpressing $\alpha_1H$ and $\alpha_1G$ isoforms are shown as positive controls. RT-PCR did not demonstrate the presence of the $\alpha_1I$ isoform in human MC. However, a positive control for the $\alpha_1I$ T-CaCN isoform was not available at the time of our experiments and therefore this result cannot absolutely prove its absence in human MC.

![Figure 3.1: Detection of T-type calcium channel isoforms in primary cultures of human MC.](image)

The reaction was carried out in the plateau phase of amplification using 40 cycles of PCR. Results from two separate MC RNA isolates are illustrated (MC1 and MC2). The upper band in each lane represents the RT-PCR amplification product of the housekeeping gene $\beta$-Actin whilst the lower bands represent the amplification products of the T-type channel isoforms indicated ($\alpha_1G$ in the upper panel, $\alpha_1H$ in the centre panel and $\alpha_1I$ in the lower panel). mRNA for $\alpha_1H$ was detected whilst $\alpha_1G$ nor $\alpha_1I$ mRNA were not detected. The pictures shown are representative results of three RT-PCR experiments. (Product sizes: $\alpha_1G$, 233bp; $\alpha_1H$, 176bp).
3.3 T-type calcium channel protein expression in human mesangial cells

In an attempt to demonstrate T-type channel protein expression in cultured human MC, two commercially available polyclonal antibodies were purchased from Santa Cruz Biochemicals. Both were affinity-purified goat polyclonal antibodies; one being raised against an epitope consisting of the first 18 amino acid residues at the N-terminus of the human T-type channel α_{1}H protein (N-18) and the other raised against the final 20 residues at the C-terminus (C-20). Cells grown in RPMI-1640 medium supplemented with 10%FCS were lysed and SDS-PAGE performed as described in Chapter 2. Each antibody was used at a range of concentrations but no band could be detected which would be of the appropriate size to represent the T-type channel α_{1}H protein (approximately 250kDa). Total protein lysate isolated from HEK-293 cells stably over-expressing the α_{1}H protein was used as a positive control in all experiments.

In view of these negative results, attempts were made to upregulate the expression of the T-type channel α_{1}H protein by co-incubation of proliferating human MC with compounds thought to influence T-CaCN expression and function. Proliferating human MC were therefore serum starved in 0.2% FCS for 48 hours as described in chapter 2, prior to incubation for 24 and 48 hours with either 10% FCS, AII (100nM or 200nM) or PDGF (10ng/ml or 20ng/ml). Again, despite repeated attempts to demonstrate the T-type channel α_{1}H protein using the two antibodies available, this was not possible in our experiments.

As illustrated in figure 3.2 below, the T-type channel current found in primary cultures of MC is small. Consequently, the difficulties in demonstrating T-CaCN protein expression in primary cultures of human MC may not be particularly surprising, although the inability of the antibodies to detect the positive control would indicate the antibodies were ineffective. The current amplitude was found to peak at around 3pA whilst the single channel current of a T-type channel is thought to be around 0.1pA. This would mean that there are only around 30 active copies of this individual T-type channel.
isoform expressed at any one time in any one cell. Although this would represent only a small fraction of the inward calcium current, it is likely to be allowing an almost continual steady influx of Ca\(^{2+}\) at or near to the resting membrane potential.

### 3.4 T-type calcium current in human mesangial cells

It has been possible to demonstrate a calcium current consistent with that mediated via a T-type channel in MC as shown in Figure 3.2. This small inward current was observed in 3 of 11 cells. The small size of this current made it difficult to measure and characterize, but its voltage activation and inactivation kinetics are consistent with a voltage-gated time-dependant low voltage-activated inward current such as the T-type calcium current. The control element of these experiments studied HEK cells over-expressing \(\alpha_1\)G or \(\alpha_1\)H T-CaCN isoforms, with results shown in the lower panel of figure 3.2. As the amount of expression of \(\alpha_1\)G and \(\alpha_1\)H varies between the HEK cultures and cell to cell, in order to make their time-courses comparable the peak currents have been normalised. As a result, the current calibration bar on the lower panel is an approximation of \(\sim 200\)pA. The time-course of the current demonstrated in MC strongly suggests that the T-CaCN current in MC is carried via the \(\alpha_1\)H isoform, when viewing alongside the normalised time points from control cells. This is consistent with the RT-PCR findings.

As previously discussed, the inward calcium ion flux mediated via T-type channel contributes only a small fraction of the total Ca\(^{2+}\) influx from the extracellular space needed to allow the cell to perform essential functions such as contraction or proliferation. The majority of studies describing characteristics of T-type channel have been either in excitable cells, such as the early reported descriptions in nervous tissue and cardiac pacemaker cells (Carbone & Lux, 1984; Nilius et al, 1985), in cells maximally expressing T-CaCN at a certain point in development, disorder or disease (Guo et al, 1998; Del Toro et al, 2003; Mariot et al, 2002) or using cells over-expressing one or more T-type channel isoform following transfection with cDNA of the particular channel to be studied (Chemin et al, 2000; Gray et al, 2004). This has allowed investigators to
accurately establish typical activation and inactivation kinetics for the T-type channel. As a result, the expression of a T-type channel current is understandably difficult to demonstrate in all cells of primary cultures of MC. The relative abundance of other calcium ion currents, including the L-type calcium current, and the apparent low level of expression of the T-type channel in cultured MC can often make it difficult to identify the typical T-type current characteristics consistently.

Figure 3.2: An example of an inward calcium current recorded from human MC. Panel A illustrates an inward current consistent with a T-type calcium current recorded from primary cultures of MC in response to depolarisation to –20mV from –90mV. Similar small time-dependant currents were seen in 3 of 11 cells tested. Mean current amplitude was 0.1±0.1 pA/pF. Cell capacitance was 33±9 pF and hence absolute current magnitude was typically very small and close to the limit of detection. Panel B shows $\alpha_1$H and $\alpha_1$G currents recorded from HEK cells stably over-expressing both types of channel using a similar protocol to that used when examining mesangial cells. The currents evoked were scaled to the same maximum value and have been plotted on the same horizontal (time) scale. To aid interpretation, the traces from the lower figure have been superimposed on the upper figure measured from human MC, suggesting that the MC current identified most closely resembles the $\alpha_1$H current.
3.5 Discussion

The results presented in this chapter report for the first time the expression of T-type calcium channels in primary cultures of human mesangial cells. The α₁H (Ca_{v}3.2) isoform is the only T-CaCN isoform expressed in human MC, demonstrated at the mRNA level. Patch-clamp electrophysiology studies recorded a small inward current which would be consistent with a T-type calcium current. However, it was not possible to consistently measure this current in all cells studied. Finally, attempts at demonstrating T-CaCN protein using Western blotting techniques were unsuccessful. There were probably a number of reasons for this, each to be discussed shortly.

Firstly, it must be acknowledged that a limitation to the data presented in this chapter is that cells from a single source only were used. The rationale behind this decision was to attempt to standardise experiments performed over a long time period as much as possible by studying only primary cultures of human mesangial cells. The commercially available cells used in these experiments were from adult donors only and had been assessed as to their purity before being supplied. It is conceivable that a tiny number of other cell types may have contaminated one or more vials. However, cells such as tubular or glomerular epithelial cells are more likely to stop proliferating due to contact inhibition once cultured as described here, while primary cultures of human mesangial cells do not exhibit such inhibition and continue to proliferate, forming ‘hillocks’ in primary culture. Cells such as those used in this study had previously been widely used in our laboratory and as a result a wealth of experience in their handling was established. However, it is accepted that in retrospect, further staining of each vial of cells used, beyond that routinely performed, would have added to the robustness of the data presented, giving evidence of reproducibility between each purchased vial and subsequent experiments.

An alternative source of human mesangial cells to be grown in primary culture could have been sought from nephrectomy specimens. The process of isolation of human MC from this source is similar to that employed and described commercially with similar assessments of staining and characteristics. Such a source of cells would have provided
alternative evidence to either support or contradict the results presented following use of commercially purchased cells. However, the supply of nephrectomy specimens from which to isolate cells was limited and erratic, despite close liaison with the local urology department.

An immortalised cell line of MC could potentially have been used. A large number of such cell lines are available, allowing an almost endless supply of cells with similar characteristics. It is unclear, however, if the expression of T-CaCN channel isoforms varies between lines and as such how relevant such experiments would be to human pathophysiology. SV 40 transformed human mesangial cells would provide the potential for reproducible, consistent results and are widely used. They are characterised as retaining the phenotypical characterisation of human MC (that is, their typical morphological characteristics in primary culture, α-actinin expression, the production of ECM and staining negatively for cytokeratin and endothelial cell markers). It has also been shown that such cells retain the typical MAP-kinase / ERK activity seen in non-transformed cells (McMahon et al, 2000). However, in order to be more confident that any findings were important in human disease, experiments would need to be repeated with non-transformed human MC, either from nephrectomy specimens or from a commercial source. There is also the potential once more for variable expression of T-CaCN isoforms in transformed versus non-transformed cells.

Since the single-channel characterisation of the T-type calcium current in the early 1980’s, there has been a rapid increase in the number of published studies investigating the T-CaCN. Over the past 10 years, there have been an average of around 70 papers per year reporting studies of T-CaCN in neurons and a similar number concentrating on heart and VSM preparations combined (Nilius et al, 2006). There has also been a lower but steady interest in cancer cells and non-vascular smooth muscle cells. Human MC would fall into this latter category of non-excitatable cells. Reports linking T-CaCN expression with the proliferation of malignant cells and non-excitatable cells have recently been gathering momentum. Table 3.1 below lists a selection of tissues in which a possible role for T-CaCN in cell growth has been proposed. A comparison is made between the
reported expression of each at the mRNA, protein and functional electrophysiological level.

<table>
<thead>
<tr>
<th>Cell / Cell line</th>
<th>Current measured?</th>
<th>mRNA expression demonstrated?</th>
<th>Protein expression demonstrated?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TUMOURS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>No</td>
<td>Yes (α₁H)</td>
<td>No</td>
</tr>
<tr>
<td>Brain</td>
<td>No</td>
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<td>Yes (α₁G &amp; α₁H)</td>
</tr>
<tr>
<td>- Neuroblastoma N1E-115</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>- Glioma U87MG</td>
<td>No</td>
<td>Yes (α₁G &amp; α₁H)</td>
<td>Yes (α₁G &amp; α₁H)</td>
</tr>
<tr>
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<td>Yes (α₁G, H &amp; I)</td>
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<td>No</td>
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<td>Yes (α₁G &amp; α₁H)</td>
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<td><strong>NON-EXCITABLE CELLS</strong></td>
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<tr>
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<tr>
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</tr>
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</tr>
<tr>
<td>- urethra</td>
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<td>No</td>
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</tr>
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</tr>
<tr>
<td>- canine bronchial</td>
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<td>No</td>
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</tr>
<tr>
<td>Neonatal rat ventricular myocyte</td>
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</table>


The inward current measured in human MC illustrated in figure 3.2 was not consistently measured in all cells – a finding which was not entirely surprising. Variability in observed currents have been reported in primary culture preparations of rat and human VSMC (Rodman et al, 2005; Quignard et al, 1996) – recognised as a ‘cousin’ of the MC. In general, it has been difficult to measure a T-type current in primary cultures of non-
transformed VSMC. Reasons for such problems are likely to include the co-expression of a much more prevalent L-type calcium current allied to the small single-channel conductance of the T-CaCN, variability in T-CaCN current expression throughout the cell cycle, and the quality of the cell preparation. There is little reason to doubt that each of these difficulties may apply to our studies of MC. Furthermore, as a T-type current could not be measured from all cells, any effects of T-CaCN antagonists could not be robustly investigated.

Cells expressing T-CaCN demonstrate unique electrophysiological and often functional characteristics depending on the T-CaCN isoform(s) expressed. Human MC express the $\alpha_1$H (Ca$\text{v}^3.2$) T-CaCN only, as illustrated in figure 3.1. mRNA for neither the $\alpha_1$G (Ca$\text{v}^3.1$) nor the $\alpha_1$I (Ca$\text{v}^3.3$) were demonstrated by RT-PCR. This finding is consistent with isoform expression of other cell types, in particular VSMC and malignant cells. Such cells tend to express either the $\alpha_1$G or $\alpha_1$H isoform alone, or both together. As outlined in chapter 1, expression and function of these isoforms may be up- or down-regulated by a number of molecules or chemical messengers (for example, G-proteins or AII). Whilst expression of the $\alpha_1$I isoform has been reported in VSM, it is predominantly expressed in the central nervous system, particularly the caudate nucleus and amygdala (Perez-Reyes et al, 1998).

From the data reported in this chapter, it is difficult to make an assessment of the relative expression of the $\alpha_1$H isoform at any given point in the cell cycle. The cultured human MC used in these experiments were unsynchronized and as a result any isolated mRNA would conceivably be from cells at various points in the cell cycle. An increase in the proliferative rate of VSMC is associated with an increase in $\alpha_1$H expression (Kuga et al, 1998). However, limitations of this assessment must be considered, including the relative success of achieving quiescence after serum-starvation and the possibility that other channels and signalling molecules may be up- or down-regulated as a result (for example L-CaCN).

Whilst a calcium current consistent with one carried via T-CaCN has been demonstrated in human MC, in addition to mRNA encoding the $\alpha_1$H isoform, expression of T-CaCN
protein was not demonstrated. Consequently, the degree to which T-CaCN genes are translated into functional channel proteins cannot yet be established. There are a number of possible explanations for these difficulties.

Firstly, the lack of a highly sensitive and specific commercially available antibody raised against individual T-CaCN isoform epitopes is a significant contributing factor. No sample blots illustrating successful testing of either of the polyclonal antibodies used were available from the manufacturer. Discussions with our research collaborators have revealed similar problems in their studies of T-CaCN expression in VSMC (Shattock et al, personal communication). To date, a monoclonal antibody raised against a T-CaCN isoform is not available, nor have there been reports of the successful use of such an antibody. The successful use of polyclonal T-CaCN antibodies in order to identify T-type channel protein expression has been reported by a few authors. Cell lines of two human brain tumours were shown to demonstrate protein expression using a commercial polyclonal antibody (Panner et al, 2005). The expression of Ca_{v}3.1 protein was demonstrated in primary cultures of pulmonary artery SMC whilst the Ca_{v}3.2 antibody was ineffective at detecting protein (Rodman et al, 2005). Additionally, an antibody to detect Ca_{v}3.2 (\alpha_{1}H) channel protein was used by Wolfe et al in examining G-protein function in HEK-293 cells stably overexpressing the \alpha_{1}H subunit (Wolfe et al, 2003).

Secondly, it is likely that at a given point in time, the proportion of total Ca^{2+} influx entering the MC via T-CaCN is very low. T-CaCN carry only a tiny fraction of the Ca^{2+} entering a cell and as such these channels are likely to be expressed in low numbers. Whilst a basal number of copies of T-CaCN protein may well be present much of the time – possibly allowing a basal level of Ca^{2+} entry via ‘calcium spikes’ at or close to the resting cell membrane potential (the ‘window current’) – the number of channel copies may increase at certain points in the cell cycle. Again, to fully investigate this hypothesis one would need a synchronised population of cells in addition to a reliable antibody.

Finally, at present it is not possible to establish to what degree post-transcriptional modification of T-CaCN may have on their function in human MC. As outlined in
chapter 1, a number of signalling molecules and hormones are capable of regulating and modifying T-CaCN expression and function, including AII and aldosterone.

In summary, the expression of the Ca\textsubscript{v}3.2 (\(\alpha_1\)H) T-CaCN has been demonstrated in proliferating human MC at the mRNA level and possibly also at the electrophysiological level. Detection of protein expression was not possible in these experiments, despite attempts at upregulation of channel expression using PDGF and Angiotensin II. The absence of detection of positive control samples in the Western blots would suggest the antibodies are ineffective in this setting.
4. Pharmacological agents targeting T-type calcium channels in mesangial cells

4.1 Experimental use of calcium channel inhibitors

The effect of inhibition of the T-CaCN on human MC proliferation was investigated using three distinct T-type calcium channel antagonists – mibefradil, TTL-1177 and the divalent cation Ni$^{2+}$. The L-type calcium channel antagonist verapamil was used as a comparison control. Each individual compound was used at a range of concentrations around the published EC$_{50}$ for inhibition of both the T-type current and proliferation of a variety of cell models (Yunker, 2003).

Each drug was made up to the relevant concentration in standard growth medium (RPMI-1640/10%FCS). In all experiments, growth medium with or without added drugs was left unaltered throughout its course. Medium was not changed at any point during the experiments, with one exception. Owing to the natural colour of nickel chloride in a solution of sterile water (pale green), medium was changed in all wells of the 96-well proliferation assay plates immediately prior to reading an individual plate each day. The medium was removed from all wells, fresh medium added, then MTS reagent immediately added as described in Chapter 2. If left unchanged, wells containing nickel chloride in solution turned dark brown on addition of MTS reagent, giving results that were inconsistent with the cell number present.
4.2 Effects of calcium channel inhibitors on proliferation of human mesangial cells

4.2.1 Effects on cell number
Cultured MC were serum-starved in RPMI-1640 medium supplemented with 0.2% FCS for 48 hours to induce quiescence before being plated into 96-well plates at a density of 5000 cells per well. Calcium channel antagonists were then added to wells in triplicate at a range of concentrations. Cell proliferation was stimulated using standard growth medium (RPMI-1640 / 10% FCS). Cell number was assessed at every 24 hours using Promega’s CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay as described in chapter 2.

Figure 4.1 illustrates the cell number curves following co-incubation of MC with mibefradil at a range of concentrations over a period of 72 hours. Mibefradil has a dose-dependent inhibitory effect on cell growth velocity. 7.5µM mibefradil had no effect on cell growth velocity compared to vehicle treated cells. 10µM mibefradil had a moderate effect, whilst treatment with 12.5µM completely inhibited the expected increase in cell number over the experimental period. Treatment with 15µM mibefradil caused a progressive fall in cell number over the 72 hours. Figure 4.2 illustrates a non-linear regression dose-response curve for mibefradil following treatment of MC with concentrations of drug up to 40µM. From this, an EC₅₀ for mibefradil treatment of proliferating human MC can be estimated at 10.3µM (n=12).
Figure 4.1: Cell number curves illustrating modulation of human MC proliferation by mibefradil. Cells were incubated in growth medium supplemented with 10% FCS in the presence of increasing concentrations of mibefradil. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 4 repeated experiments. **p<0.001 when compared to vehicle-treated cells at day 3.

Figure 4.2: Modulation of human MC proliferation after 72 hours by mibefradil. Cells were incubated and cell number assessed as described in figure 4.1. The number of cells plated in each treatment group at the start of the experiment is illustrated by the blue dotted line. The EC\textsubscript{50} for mibefradil is illustrated by a red dotted line on the non-linear regression dose-response curve and can be estimated at 10.3±0.1µM. n=3 in each experiment, with results shown from 4 repeated experiments. r\textsuperscript{2}=0.9758.
Illustrated in Figure 4.3 are cell growth curves of human MC following co-incubation with TTL-1177 at a range of concentrations over 72 hours. TTL-1177 has a dose-dependent effect on cell proliferation. Concentrations up to and including 10µM had no effect on cell growth velocity. Treatment of MC with 15µM TTL-1177 resulted in cell number remaining virtually unchanged throughout the 72-hour experimental time-course. Figure 4.4 shows the dose-response curve of the treatment of MC with TTL-1177 at concentrations up to 40µM. An EC$_{50}$ for TTL-1177 can be estimated as 13.4µM (n=9).

The cell growth curves in figure 4.5 illustrate results of co-incubation of human MC with Ni$_2^+$ (as nickel chloride solution) at a range of concentrations over 72 hours. The divalent cation Ni$_2^+$ is shown to have a dose-dependent effect on MC proliferation. Total cell number appeared to remain unchanged over the 72 hour period after treatment with 400µM Ni$_2^+$. Figure 4.6 shows a linear regression curve of the treatment of MC with Ni$_2^+$ at concentrations up to 400µM. From this, the EC$_{50}$ for Ni$_2^+$ in proliferating MC was crudely estimated as between 100-200µM (n=9).
**Figure 4.3:** Cell number curves illustrating modulation of human MC proliferation by TTL-1177. Cells were incubated in growth medium supplemented with 10% FCS in the presence of increasing concentrations of TTL-1177. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. *p<0.05, **p<0.001 when compared to vehicle-treated cells at day 3.

**Figure 4.4:** Modulation of human MC proliferation after 72 hours by TTL-1177. Cells were incubated and cell number assessed as described in figure 4.3. The number of cells plated in each treatment group at the start of the experiment is illustrated by the blue dotted line. The EC$_{50}$ for TTL-1177 is illustrated by a red dotted line on the non-linear regression dose-response curve and can be estimated at 13.4±1.6µM. n=3 in each experiment, with results shown from 3 repeated experiments. $r^2=0.9895$. 
Figure 4.5: Cell number curves illustrating modulation of human MC proliferation by Ni$^{2+}$ (nickel chloride).

Cells were incubated in growth medium supplemented with 10% FCS in the presence of increasing concentrations of Ni$^{2+}$. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. **p<0.001 when compared to vehicle-treated cells at day 3.

Figure 4.6: Modulation of human MC proliferation after 72 hours by Ni$^{2+}$ (nickel chloride). Cells were incubated and cell number assessed as described in figure 4.5. The number of cells plated in each treatment group at the start of the experiment is illustrated by the blue dotted line. The EC$_{50}$ for Ni$^{2+}$ is illustrated by a red dotted line on the linear regression dose-response curve and can be crudely estimated at around 160µM. n=3 in each experiment, with results shown from 3 repeated experiments. $r^2$=0.8525.
Cell growth curves illustrating the results of co-incubation of human MC with the L-type calcium channel antagonist verapamil are shown in figures 4.7 and 4.8. As shown in figure 4.7, treatment of MC with verapamil at concentrations up to 20µM had no effect on cell growth velocity over 72 hours. This is the concentration range for L-type calcium channel inhibition. There was no significant difference in total cell number between any of the treatment groups at 72 hours. Cell growth curves following treatment with 50-400µM of verapamil are shown in figure 4.8. 50µM verapamil has no effect on MC proliferation, whilst treatment with 100µM verapamil almost entirely inhibits the expected increase in cell number over 72 hours. Treatment with 200µM and 400µM resulted in a rapid reduction in cell number with very few cells recorded as viable at 72 hours. From figure 4.9, an EC$_{50}$ for verapamil can be estimated at 75µM (n=9). However, the value of this figure needs to be questioned. It is possible that verapamil is not anti-proliferative at all, as cell number is largely unchanged between 5-50µM while beyond this cell number drops off sharply, probably due to toxicity and cell death.

![Graph](image)

**Figure 4.7:** Cell number curves illustrating absence of modulation of human MC proliferation by verapamil at concentrations up to 20µM. Cells were incubated in growth medium supplemented with 10% FCS in the presence of increasing concentrations of verapamil. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. p>0.05 comparing all groups to vehicle-treated controls at day 3.
Figure 4.8: Cell number curves illustrating modulation of human MC proliferation by verapamil at concentrations between 50–400 µM. Cells were incubated in growth medium supplemented with 10% FCS in the presence of increasing concentrations of verapamil. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. **p<0.001 when compared to vehicle-treated cells at day 3.

Figure 4.9: Modulation of human MC proliferation after 72 hours by verapamil. Cells were incubated and cell number assessed as described in figure 4.5. The number of cells plated in each treatment group at the start of the experiment is illustrated by the blue dotted line. The EC₅₀ for verapamil is illustrated by a red dotted line on the linear regression dose-response curve and can be estimated at 75 µM. n=3 in each experiment, with results shown from 3 repeated experiments. r²=0.9074.
4.2.2 Effects on DNA synthesis

The effect of co-incubation of human MC with T- and L-type calcium channel antagonists for 48 hours on DNA replication was assessed by a double-staining technique combining BrdU incorporation and nuclear staining with Hoechst 33342. BrdU was added to the growth medium of each experimental dish 16 hours prior to cell fixation. It acts as a thymidine analogue and is incorporated into the cellular DNA in place of thymidine during S-phase of the cell cycle. Following fixation, the incorporated BrdU can be detected using a specific monoclonal antibody, itself then detected by a TRITC-labelled secondary antibody. Hoechst 33342 is used to counterstain all cell nuclei. As an illustration, figure 4.10 shows the results of BrdU incorporation in proliferating human MC in the absence of T-CaCN inhibitors, with the all viable cell nuclei in the left panel staining blue with Hoechst 33342, while in the right-hand panel below those MC which are proliferating and have passed through S-phase and therefore incorporated BrdU are counted as positive cells staining red.

![Figure 4.10: Hoechst 33342 staining and BrdU incorporation in cultured human MC.](image)

Representative examples of proliferating primary cultures of human MC following fluorescent staining techniques are shown. (Scale bar = 100µm).

It can be argued that the EC$_{50}$ for each of the inhibitors that have been identified from the proliferation assays shown in section 4.1 should have been used in the BrdU experiments. This is acknowledged; however, the experiments reported in this section studying the effects on DNA replication of T-CaCN inhibitors again across a range of concentrations
covering the EC$_{50}$ from the MTS proliferation assays were designed to reinforce the data presented in section 4.1, in addition to being able to demonstrate a dose-dependent effect across a concentration range.

Figure 4.11 shows the effect on DNA replication of co-incubation of human MC with increasing concentrations of mibebradil. Mibebradil has a dose-dependent inhibitory effect on DNA replication. 10µM mibebradil reduced the number of BrdU positive cells, although this did not reach statistical significance when compared to untreated cells. Treatment with 12.5µM mibebradil reduced the percentage of cells staining positive for BrdU from 55.7±2.5% in untreated cells to 35.6±0.3% (p<0.01). Treatment with 15µM mibebradil resulted in a further reduction in BrdU-positive cells to 27.7±3.3% (p<0.001) - a 50% reduction.

The effects of treatment of human MC with TTL-1177 on DNA replication are shown in figure 4.12, illustrating a dose-dependent effect. Following co-incubation with 10µM TTL-1177, there was a reduction in the proportion of BrdU-positive cells from 55.7±2.5% in untreated cells to 37.3±4.4% in the treatment group (p<0.001). Co-incubation with 15µM led to a further fall in the percentage of BrdU-labelled cells to 25.3± 3.5% (p<0.001).
Figure 4.11: BrdU incorporation in human MC after treatment with mibefradil. The total percentage of BrdU-positive cells is plotted after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either none or increasing concentrations of mibefradil. Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. *p<0.01, **p<0.001 when compared to untreated control cells.

Figure 4.12: BrdU incorporation in human MC after treatment with TTL-1177. The total percentage of BrdU-positive cells is plotted after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either none or increasing concentrations of TTL-1177. Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. *p<0.01, **p<0.001 when compared to untreated control cells.
Figure 4.13 shows the modulation of DNA replication of proliferating human MC following co-incubation with nickel chloride (Ni\(^{2+}\)) for 48 hours. Treatment with 200µM Ni\(^{2+}\) resulted in a significant reduction in the percentage of BrdU-labelled cells, from 71.1±3.6% to 29.8±3.7%. Treatment with 400µM Ni\(^{2+}\) resulted in a further reduction to 7.5±1.1%.

Figure 4.14 illustrates the effect of treatment of MC with verapamil at on BrdU incorporation. Treatment with 20µM verapamil demonstrated a reduction in the proportion of BrdU-labelled cells compared to untreated cells, from 71.0±2.5% in the untreated group to 64.6±1.3%. However, this failed to reach statistical significance (p>0.05). Treatment with verapamil at 200µM produced a marked reduction in BrdU-labelled cells, falling to 2.3±1.5%. Examination under both the light microscope and the fluorescent microscope demonstrated cells that appeared to be necrosing or dead.

Assays of apoptosis and cytotoxicity were subsequently performed in all treatment groups (with results described in sections 4.3 and 4.4).
Figure 4.13: BrdU incorporation in human MC after treatment with nickel chloride (Ni\(^{2+}\)). The total percentage of BrdU-positive cells is plotted after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either none or increasing concentrations of Ni\(^{2+}\). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. **p<0.001 when compared to untreated control cells.

Figure 4.14: BrdU incorporation in human MC after treatment with verapamil. The total percentage of BrdU-positive cells is plotted after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either none or increasing concentrations of verapamil. Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. **p<0.001 when compared to untreated control cells.
4.3 Apoptotic and cytotoxic effects of calcium channel inhibitors

4.3.1 Assessment of apoptosis

To assess for any potential pro-apoptotic effects of the T-type channel inhibitors being examined, nuclear staining with Hoechst 33342 was used. MC were cultured in 35mm dishes, were serum-starved in 0.2% FCS for 48 hours before being stimulated with RPMI-1640/10%FCS and treated with T-type channel inhibitors as described earlier. After 24 hours of treatment Hoechst 33342 was added to the medium for 10 minutes, followed by propidium iodide (PI) for a further 5 minutes. Cells were then visualised by fluorescent microscopy.

Apoptotic cells show characteristic features of chromatin condensation and fragmentation. ‘Late’ apoptotic or early necrotic cells would stain positively for PI due to disruption of the cell membrane.

Control cells in growth medium only and those treated with test compounds (mibefradil and TTL-1177 up to 15µM, Ni\(^{2+}\) up to 400µM and verapamil up to 200µM) were examined. Around 150 cell nuclei in 5 separate fields for each condition were examined. Treatment with mibefradil, TTL-1177 or Ni\(^{2+}\) produced no significant increase in the proportion of nuclei staining positive for PI. Treatment with verapamil at concentrations up to and including 100µM also showed no significant increase in PI staining, with less than 1% of cells staining positive for PI in each group. Treatment with verapamil at 200µM led to a significant increase in PI staining. However, the number of cells per field of view was very low due to apparent cell death.
MC stained with Hoechst 33342 following treatment are shown in figure 4.15. Cells treated with staurosporin, an agent that induces apoptosis, is shown as a control panel. Staurosporin-treated cells show condensed, intensely staining chromatin in 36.3±3.8% of cells (n=3).

Figure 4.15: Primary cultures of MC stained with Hoechst 33342. Intact, non-apoptotic nuclei are seen in the left panel. Characteristic apoptotic changes of nuclear fragmentation and chromatin condensation are seen in the right panel after treatment with staurosporin (at a final concentration of 1µM).

The results of Hoechst 33342 staining of MC nuclei after treatment with mibefradil or TTL-1177 are illustrated in figures 4.16 and 4.17. Exposure of cultured cells to staurosporin produced an expected increase in apoptotic cells (as shown in figure 4.15). There was no change in the number of apoptotic cells 24 hours after treatment of 10% FCS-stimulated cells with either mibefradil (up to 15µM) or TTL-1177 (up to 12.5µM). There did appear to be a slight increase in the percentage of apoptotic cells after treatment with TTL-1177 at 15µM, although this was not statistically significant.
**Figure 4.16: Effect of mibefradil on apoptosis of human MC.** Cells were grown on 35mm dishes until 50-70% confluent, serum-depleted for 48 hours, then treated with mibefradil at a range of concentrations for 24 hours. Apoptosis was assessed using Hoechst 33342 staining. Error bars are SEM. n=3, with 5 fields being viewed at each experiment. ***p<0.001 compared to data from all other treatment conditions.

**Figure 4.17: Effect of TTL-1177 on apoptosis of human MC.** Cells were grown on 35mm dishes until 50-70% confluent, serum-depleted for 48 hours, then treated with TTL-1177 at a range of concentrations for 24 hours. Apoptosis was assessed using Hoechst 33342 staining. Error bars are SEM. n=3, with 5 fields being viewed at each experiment. ***p<0.001 compared to data from all other treatment conditions.
The results of Hoechst 33342 nuclear staining after treatment of human MC with nickel chloride and verapamil are illustrated in figures 4.18 and 4.19.

After 24 hours treatment with 200µM Ni\(^{2+}\), there was a significant increase in apoptotic figures visualised, appearing to be maximal at 200µM and not increasing further at 400µM.

Treatment with verapamil at 20µM induced no increase in apoptosis at 24 hours, reflecting the absence of any alteration in cell growth velocity in this treatment group. Treatment of MC with 200µM verapamil resulted in a high degree of apoptosis (to almost 20% at 24 hours) and apparent cell necrosis. Visualised cell number was markedly reduced in this treatment group, concurring with the results of the cell number assay shown in figure 4.8.
Figure 4.18: Effect of nickel chloride (Ni\textsuperscript{2+}) on apoptosis of human MC. Cells were grown on 35mm dishes until 50-70% confluent, serum-depleted for 48 hours, then treated with Ni\textsuperscript{2+} at a range of concentrations for 24 hours. Apoptosis was assessed using Hoechst 33342 staining. Error bars are SEM. n=3, with 5 fields being viewed at each experiment. **p<0.01 compared to data from untreated control cells.

Figure 4.19: Effect of verapamil on apoptosis of human MC. Cells were grown on 35mm dishes until 50-70% confluent, serum-depleted for 48 hours, then treated with verapamil at a range of concentrations for 24 hours. Apoptosis was assessed using Hoechst 33342 staining. Error bars are SEM. n=3, with 5 fields being viewed at each experiment. **p<0.01 compared to data from untreated control cells.
4.3.2 Assessment of cytotoxicity

The possibility of any potential direct cytotoxic effects of the calcium channel antagonists being studied and thus contributing to the reduction in growth velocity was examined using the Promega Cytotox 96® Non-radioactive Cytotoxicity Assay. In order to quantify the number of cells that underwent lysis as a direct toxic effect of medium conditions, the amount of lactate dehydrogenase (LDH) released from cells co-incubated with calcium channel antagonists for 24 hours was measured. Measured LDH from cells incubated only in growth culture medium (control LDH) was then deducted from the reading of each test sample (test LDH). This was compared to LDH measured from a triplicate panel of cells to which a lysis buffer had been added, representing total cellular LDH (total LDH). Therefore, an estimate of the percentage cytotoxicity could be made as follows:

\[
\% \text{ cytotoxicity} = \frac{(\text{Test LDH} - \text{Control LDH}) \times 100\%}{\text{Total LDH}}
\]

Figures 4.20 and 4.21 illustrate results of cytotoxicity assays following treatment of human MC with mibefradil and TTL-1177 respectively at concentrations up to and including 15µM. Treatment with concentrations of mibefradil up to 12.5µM had no significant cytotoxic effects. However, there was a significant increase in LDH release upon treatment of MC with 15µM of mibefradil. Treatment with TTL-1177 at concentrations up to 15µM did not result in any significant increase in LDH release.
Figure 4.20: Assessment of cell toxicity following treatment of human MC with mibefradil. An estimate of drug-induced cytotoxicity was made following measurement of LDH release from cells treated with mibefradil at a range of concentrations for 24 hours. Error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. *p<0.05 compared to untreated cells.

Figure 4.21: Assessment of cell toxicity following treatment of human MC with TTL-1177. An estimate of drug-induced cytotoxicity was made following measurement of LDH release from cells treated with TTL-1177 at a range of concentrations for 24 hours. Error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments.
As shown in figure 4.22, treatment of MC with Ni$^{2+}$ at concentrations up to 400µM did not result in any increase in cytotoxicity as measured by LDH release. The apparent fall into negative values of cytotoxicity may be a result of interference with the absorbance readings by the green colour of nickel chloride in solution.

Figure 4.22: Assessment of cell toxicity following treatment of human MC with nickel chloride (Ni$^{2+}$). An estimate of drug-induced cytotoxicity was made following measurement of LDH release from cells treated with Ni$^{2+}$ at a range of concentrations for 24 hours. Error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments.
Figure 4.23 illustrates results for the L-type channel antagonist verapamil. No evidence of cytotoxicity was demonstrated after treatment with concentrations up to and including 150µM. However, treatment of MC with verapamil at a concentration of 200µM resulted in a significant increase in LDH release, representing cell damage or death in 32.2±7.5% of cells treated (p<0.0001).

Figure 4.23: Assessment of cell toxicity following treatment of human MC with verapamil. An estimate of drug-induced cytotoxicity was made following measurement of LDH release from cells treated with verapamil at a range of concentrations for 24 hours. Error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. *p<0.001
4.4 Effects of calcium channel inhibitors on downstream signalling mechanisms

An increasing body of work across a number of cell and animal models has reported an association between T-type calcium channels and cell proliferation. However, the identification of a mechanism linking a small inward flux of calcium ions into the cell via T-CaCN to cell proliferation remains absent. In an effort to identify a possible mechanism in mesangial cells, an examination of proteins known to be involved in MC proliferation was undertaken. Ca$^{2+}$ ions are integral to a plethora of cell signalling pathways. The experiments reported here studied expression of two mitogen-activated protein kinases (MAPKs) known as extracellular signal-related kinases (ERK1/2), in addition to p38 and Akt expression. These were proteins that had previously been easily identified in proliferating human MC using Western blotting in our laboratory.

Acting via Ras-GRF and Ras-GRP, an increase in intracellular Ca$^{2+}$ ions is one of number of stimuli leading to the activation of GTP-bound Ras and subsequent downstream signalling. GTP-bound Ras interacts with two regions on the serine/threonine kinase Raf known as the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) (Mott et al, 1996). Further downstream, activated Raf phosphorylates and activates mitogen-activated protein kinase kinases 1 and 2 (MAPKK, also known as MEK 1 and MEK 2). These are dual-specificity kinases, which in turn are capable of phosphorylating and activating ERK1/2. Following translocation to the cell nucleus, ERK1/2 is then responsible for the activation of promoters and transcription factors, including c-fos. Stimulation of these transcriptional regulators leads to expression of cell cycle proteins necessary for progression through the cell cycle, particularly G1-phase.
Figure 4.24 illustrates the results of Western blots of total cellular lysates of proliferating human MC for phosphorylated ERK1/2 (p44/42) following treatment with the T-CaCN inhibitors mibefradil (12.5µM) or TTL-1177 (15µM). Cells were prepared and cultured as previously described. Following 48 hours of serum-deprivation in 0.2% FCS, cells were incubated with standard growth medium +/- inhibitors for fixed time periods of up to 24 hours. Total cell lysates were isolated at intervals and protein concentration quantified. In both sets of treatment conditions, there was a clear increase in ERK1/2 phosphorylation 4 hours after stimulation with standard growth medium. However, T-CaCN inhibitors did not have any effect on the degree of ERK phosphorylation after 4 hours or more of incubation and its expression appeared identical in both treatment and control groups. Phospho-ERK1/2 expression then diminished back to pre-treatment levels within 8 hours.

<table>
<thead>
<tr>
<th>Mibefradil 12.5µM</th>
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<tr>
<td>TTL-1177 15µM</td>
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| Std growth medium | +   | +   | +   | +   | +   | +   | +   |
| Inhibitor added   | -   | +   | -   | +   | -   | +   | -   |
| Time of treatment (hrs) | 0   | 4   | 4   | 8   | 8   | 24  | 24  |

Figure 4.24: Phospho-ERK1/2 expression in human MC after treatment with T-CaCN inhibitors for 24 hours. A total of 10µg of total cellular protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.
No significant difference in p38 phosphorylation was seen after treatment with T-CaCN inhibitors in a similar experiment. As shown in figure 4.25, treatment with neither mibefradil nor TTL-1177 results in any significant difference in phospho-p38 expression over 24 hours.

Figure 4.25: Phospho-p38 expression in human MC after treatment with T-CaCN inhibitors for 24 hours. A total of 10µg of total cellular protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

Finally, an examination of the apoptosis signalling pathway was performed. It is possible that a delicate balance exists between proliferation and apoptosis that is regulated by T-CaCN and varying between cell type. Akt (also known as protein kinase B (PKB)) is a pro-survival signal that is capable of phosphorylating the pro-apoptotic protein Bcl-associated death promoter (BAD). Such phosphorylation leads to dissociation of BAD from the Bcl-2/Bcl-X complex with subsequent loss of the pro-apoptotic function of BAD (Datta et al, 1997). Furthermore, Akt is capable of activating Nuclear Factor kappa-B (NF-κB), resulting in the transcription of pro-survival genes.

Total cellular lysates of proliferating human MC were examined for the expression of phosphorylated Akt after treatment with T-CaCN inhibitors, hypothesising a predominant anti-apoptotic rather than a pro-proliferative role for T-CaCN. However, as illustrated by the sample blot in figure 4.26, there was no significant difference in phospho-Akt expression before or after treatment with either of the two T-CaCN inhibitors used. Expression of phospho-Akt appeared to remain constant throughout the experiments.
Figure 4.26: Phospho-Akt expression in human MC after treatment with T-CaCN for 24 hours. A total of 10µg of total cellular protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

In view of the lack of any apparent effect of co-incubation of proliferating MC with T-CaCN inhibitors on ERK phosphorylation when studying time-points of 4 hours or later, experiments studying this particular pathway were later repeated to examine earlier time points. Figure 4.27 illustrates the results of Western blots of total cellular lysates of proliferating human MC for phosphorylated ERK1/2 (p44/42) and total ERK1/2 following treatment with either TTL-1177 (15µM) or verapamil (100µM). Cells were prepared and cultured as previously described. Following 48 hours of serum-deprivation in 0.2% FCS, cells were incubated with standard growth medium +/- inhibitors for fixed time periods of up to 1 hour. Total cell lysates were isolated at intervals and protein concentration quantified.

The blots demonstrate a marked reduction in p-ERK as early as 15 minutes after treatment with TTL-1177, with an almost complete absence of detectable phosphorylated protein at 1 hour. Such an effect was not seen after treatment of MC with verapamil. Similar studies of p-Akt and total Akt were performed, which showed no significant differences between treated and untreated lysates, even at these earlier time points.
4.5 Discussion

The data presented in this chapter strongly suggest an important role for T-type calcium channels in the proliferation of human MC. Serum-stimulated human MC in primary culture had their growth velocity attenuated upon co-incubation with a range of T-CaCN antagonists, with concurrent evidence of a reduction in the rate of DNA replication. Meanwhile, the growth velocity of human MC was unaffected by inhibition of L-type calcium channels using verapamil.

The T- and L-type calcium channel antagonists used in this series of experiments were chosen in view of their published use in the study of these channels. There are, however, limitations to be considered. Mibefradil was introduced into the clinical field of
cardiovascular medicine as a ‘selective’ T-CaCN antagonist and was used in the treatment of hypertension and angina pectoris. Mibefradil (as Posicor®) was later withdrawn from the market due to drug interactions involving the cytochrome P450 system. It is now accepted that mibefradil has significant inhibitory effects on a number of other channels including HVA calcium channels, sodium, potassium and chloride ion channels. Whilst it is recognised that mibefradil preferentially inhibits LVA calcium channels over HVA channels by a factor of up to 100, questions are often raised regarding in vitro and in vivo results using this drug. However, despite such concerns, mibefradil has been the most widely studied T-CaCN antagonist over the past 10 years and therefore its use in our experiments was deemed essential.

Treatment of human MC with mibefradil resulted in an attenuation in cell growth velocity in a dose-dependent manner with an EC50 estimated at 10.3μM. DNA replication was also reduced upon treatment with mibefradil, indicating an inhibitory effect on cell cycle progression. As BrdU is only taken up into the cell nucleus once cells have progressed into S-phase, these results demonstrate a reduction in the proportion of cells in this part of the cell cycle. These results are consistent with those from similar experiments studying the proliferation of VSMC (Schmitt et al, 1995; Shattock et al, personal communication), blood mononuclear cells (Lijnen et al, 1999), PASMC (Rodman et al, 2005), and the U87-MG astrocytoma cell line (Panner et al, 2005).

Mibefradil did not induce any apoptosis after 24 hours treatment of human MC, adding weight to its proposed anti-proliferative effect. An alternative method of identifying apoptosis was not used in these experiments and it is acknowledged that the addition of another method (for example, Annexin-V staining) would have added to the robustness of the results, giving numerical data from flow cytometry in addition to observed apoptotic changes manually counted using the Hoechst staining method. As such, the apoptosis results presented in this chapter using mibefradil and other T-CaCN antagonists cannot prove without doubt the absence of apoptosis, although the Hoechst assay did demonstrate apoptotic cell nuclear fragmentation when used with the positive control staurosporin.
Interestingly, it has been proposed that an increase in T-CaCN activity may mediate cytokine-induced cell death in pancreatic beta-cells (Wang et al, 1999). Whilst this would appear to contradict published reports linking an increase in T-CaCN to an increase in cell proliferation, it is not unreasonable to suppose that T-CaCN may be involved in a balance of apoptosis and proliferation. One or other state might predominate in a given cellular or tissue environment, for example in the evolution of tumours. Mibefradil has been shown to be toxic to human MC at concentrations of 15µM and above, as evidenced by an increase in LDH release at 15µM mibefradil. At this range of doses, there was a progressive reduction in cell number throughout the growth velocity experiments, despite no increase in apoptotic figures using the Hoechst 33342 assay. This concentration is significantly higher than an estimated concentration in human circulation following treatment with mibefradil for angina pectoris. In the PRIDE trial, patients were treated with 100mg mibefradil daily (Lee et al, 2002). Allowing for assumptions of steady state plasma concentrations and equal distribution in the extracellular compartment of a 70kg patient, circulating concentrations of mibefradil might be expected to have been around 3-4µM. In summary, T-CaCN blockade with mibefradil would appear to have an anti-proliferative effect on human MC, supporting the hypothesis that these channels are important in cell proliferation. However, in view of its inhibitory effects on other ion channels, the results of mibefradil must be read with caution.

In view of the lack of a ‘specific’ T-CaCN antagonist, new inhibitors to these channels have recently been designed and synthesised (McCalmont et al, 2004; Haverstick et al, 2000). One of these compounds – known as TTL-1177 (formerly TH-1177) – was available for our experiments studying human MC. TTL-1177 had been shown to block voltage-gated and ‘capacitative’ calcium entry (Gray et al, 2004) and to inhibit the proliferation of a prostate cancer cell line in vitro. Additionally, TTL-1177 at an intraperitoneal (ip) dose of 10mg/kg/day extended by 38% the lifespan of SCID mice that had been inoculated with human prostate cancer cells (Haverstick et al, 2000). In the same in vivo study, the treatment of mice with very high doses of TTL-1177 (180mg/kg/day ip for 22 days) had no toxic effects. In our experiments, TTL-1177 attenuated human MC proliferation in a dose-dependant manner, with an EC₅₀ estimated
as 13.4 μM. As comparison, the EC\textsubscript{50} for inhibition of cell proliferation in two prostate cancer cell lines – PC-3 (hormone-resistant) and LNCaP (hormone-sensitive) – were 14μM and 4μM respectively (Haverstick et al, 2000). The results presented in this chapter demonstrate an inhibitory effect of TTL-1177 on DNA replication, with no apparent cytotoxic effects at concentrations up to 15μM. As shown in figure 4.4, cell number assays using concentrations of TTL-1177 at 25μM and 50μM demonstrated a rapid fall in cell number over three days. It is likely that this is a direct cytotoxic effect of the drug, although this would appear to contradict the \textit{in vivo} data published by Haverstick et al. Alternatively, this may be a result of an induction in apoptosis as at 24 hours there is a small non-significant increase in apoptotic figures after treatment.

As outlined in chapter 1, polyvalent cations including nickel (Ni\textsuperscript{2+}) facilitated the initial identification and description of the low voltage-activated calcium channel – now known as the T-CaCN. Ni\textsuperscript{2+} was chosen for study in these experiments due to its recognised preference for inhibiting the α\textsubscript{1}H T-CaCN subunit over other T-CaCN subunits. Incubation of proliferating human MC with Ni\textsuperscript{2+} (as nickel chloride solution) resulted in attenuation of expected cell growth in a dose-dependent manner. An EC\textsubscript{50} for Ni\textsuperscript{2+} could crudely be estimated at around 160μM. On reflection of the results, there would appear to be a threshold between 100-200μM at which apoptosis becomes predominant. Apoptosis appears to increase significantly (to over 20%) upon treatment of cells with Ni\textsuperscript{2+} concentrations of 200μM or more, although the proportion of apoptotic cells at higher doses did not appear to significantly increase. This is interesting as cell growth velocity fell by over 50% upon treatment with 200μM and continued to diminish and BrdU incorporation continued to fall as the Ni\textsuperscript{2+} concentration increased from 200μM to 400μM (figures 4.5 and 4.13). Cytotoxicity studies using the LDH assay showed no cytotoxic effect of Ni\textsuperscript{2+} treatment. However, in view of the problems experienced using this particular assay with nickel chloride in solution, supporting evidence would subsequently be needed by means of an alternative method of assessing LDH release, such as the use of an LDH immunoassay.

We may conclude that while Ni\textsuperscript{2+} inhibits MC proliferation without being directly cytotoxic, induction of apoptosis at higher concentrations is contributory to the
diminished growth velocity illustrated in figure 4.5. Further detailed analysis of apoptosis would be beneficial in establishing this hypothesis. Despite this uncertainty, these data support published work describing inhibition of endogenous and recombinant T-CaCN in a variety of cell types at concentrations of a similar order of magnitude (Yunker, 2003). Had electrophysiological studies of our MC been more robust, Ni$^{2+}$ would have played an important role as a selective T-CaCN antagonist in view of its particular potency against $\alpha_1$H T-CaCN.

Although Nickel ions are recognised as being able to block the current through Ca$\alpha$3.2 T-type channels with an IC$_{50}$ of around 10-20µM (Lee et al, 1999), these results have shown that concentrations of 100-150µM are needed to significantly block MC proliferation. This apparent inconsistency may be explained by data published by Gray and colleagues when comparing the degree of current inhibition of calcium channel blockers to their anti-proliferative action (Gray et al, 2004). The authors proposed a ‘threshold effect’ to explain their finding that >70% of the current needs to be blocked before any significant effect is seen on cell proliferation. This non-linear relationship between current inhibition and anti-proliferative effects is well recognised and was also demonstrated with Mibefradil and TTL-1177. It is possible that this threshold effect might act as a ‘binary switch’, allowing the initiation of all-or-nothing effects such as the calcium spikes necessary for G1/S transition to occur. Furthermore, it is important to note that the conditions in which the reported IC$_{50}$s for channel blockade are measured are not identical to the conditions used for the proliferation assays in this study. The presence of growth medium and serum may alter the kinetics of the reaction and the availability of drug, making direct comparison of effective concentrations difficult.

Verapamil is a phenylalkylamine structurally resembling some local anaesthetics, a group of compounds known to block T-CaCN. Verapamil continues to be used clinically, primarily due to its ability to potently block HVA L-type calcium channels in the cardiovascular system. Inhibition of T-CaCN by verapamil is recognised, but using concentrations some ten-fold higher than that required to block L-type channels. The data presented in this chapter illustrate this and would support the hypothesis that L-type channels are not directly involved in the regulation of MC proliferation, although this
hypothesis was not investigated in further detail. Verapamil was used in these experiments largely as a comparison to the effects of T-CaCN antagonists on MC proliferation. Concentrations of verapamil below 50µM had no effect on human MC growth velocity or DNA replication. 100µM verapamil inhibited the expected increase in cell number over 72 hours while concentrations of 200µM and above resulted in cell death, as evidenced by increased apoptosis scores and LDH release reflecting cytotoxicity. Once again, detailed electrophysiological studies would have been useful in establishing the potency of verapamil in human MC. The difficulties surrounding this technique have been discussed in chapter 3, but (as in non-transformed VSMC) it is likely that the L-type calcium channel is much more abundant in human MC than the T-CaCN.

A mechanism linking intracellular Ca\(^{2+}\) flux via T-CaCN and human MC proliferation was not identified from the data presented here. The signalling pathways chosen for study in these experiments were starting points in an admittedly ‘blind’ search for clues to a mechanism, as they are known to be pathways critical to MC proliferation and apoptosis. Cultured human MC expressed all proteins being studied, but T-CaCN inhibition did not affect the timing or the pattern of signalling protein expression in experiments looking for changes in signal protein expression after 4 hours. However, in later experiments looking at early changes in protein expression within the first hour of incubation with T-CaCN inhibitors, a significant and reproducible effect was seen. Such an effect was not seen with verapamil treatment and supports data from proliferation assays and BrdU incorporation studies. These data do not provide evidence of a causal direct link between T-CaCN channel activity and the Ras/ERK pathway, as there may be a number of other steps in between as yet unidentified and as the Ras/ERK pathway is common to the proliferative process of MC when stimulated by a number of factors. However, a recent report would appear to support the hypothesis that the Ras/ERK pathway is involved in T-CaCN signalling. Studies of a cell line of HEK-293 cells stably expressing Ca\(_{\alpha}3.1\) (\(\alpha_{1}G\)) and Kir2.1 (potassium channel) subunits demonstrated that Ca\(_{\alpha}3.1\) activation (using KCl-mediated depolarisation) triggered p21\(^{ras}\), with subsequent signal transduction being transferred via ERK (Choi et al, 2005). Further detailed work will need to be planned in order to establish the signalling pathways involved which may
fully explain our results, including repeating these experiments at similar time points and examining for other signalling proteins, including p38.

In summary, these data support the hypothesis that T-CaCN are intricately linked to the regulation of human MC proliferation. Pharmacological T-CaCN inhibitors attenuate cell growth velocity and DNA replication – an effect not seen upon treatment with L-type calcium channel antagonists. A potential signalling mechanism involving ERK phosphorylation was identified upon examination of selected signalling pathways after treatment of human MC with the T-CaCN inhibitor TTL-1177. Synchronisation of cell populations may help in this search and further study is needed to establish the sequence of signalling linking T-CaCN opening and the entire Ras/MAPK pathway.
5. Use of small-interfering RNA (siRNA) to target T-type calcium channels

5.1 RNA interference (RNAi)

5.1.1 Mechanism of action of siRNA molecules

The data presented in Chapter 4 implicate T-CaCN as important in the signalling mechanism involved in human MC proliferation. In order to investigate this hypothesis further, targeted reduction of $\alpha_1H$ T-CaCN isoform expression was undertaken using the technique of RNA interference (RNAi).

The RNAi pathway is a post-transcriptional gene-silencing mechanism, with siRNAs utilized as effector molecules (Dorsett and Tuschl, 2004). First discovered in the UK in plants (Ecker & Davis, 1986) and also in nematodes, siRNA duplexes 21 to 28 nucleotides in length guide the sequence-specific degradation of mRNAs and reduce the expression of targeted genes in mammalian cells (Elbashir et al, 2001). Naturally occurring siRNAs derive from the cytoplasmic processing of long dsRNAs by the enzyme DICER – a member of the RNase III family of nucleases (Bernstein et al, 2001). Long dsRNAs are cleaved by DICER into 21 to 28 nucleotide duplexes that are homologous to a sequence segment of the gene being suppressed. siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). Upon activation, RISC binds to complementary RNA molecules by base-pair interactions between the mRNA and the siRNA antisense strand. RISCs are then able to direct mRNA cleavage with consequent gene silencing (Dorsett and Tuschl, 2004).
Figure 5.1: RNA interference (RNAi). Mechanism of RNAi utilizing small interfering RNA (siRNA) molecules in order to effect gene-specific mRNA degradation. (Figure adapted from www.ambion.com).
5.1.2 Choice of RT-PCR cycle number

Total RNA from proliferating primary cultures of human MC was isolated and quantified. Repeated RT-PCR were then performed, varying the cycle number each time between 20 and 40 cycles. The relative abundance of $\alpha_{1}H$ T-CaCN cDNA on gel electrophoresis was then measured by luminescence (using Adobe Photoshop®) and plotted (figure 5.2). The linear phase of PCR for use in siRNA experiments was thus deemed to be at around 30 cycles.

Figure 5.2: Relative amounts of the $\alpha_{1}H$ T-CaCN isoform following amplification of human MC RNA by RT-PCR. RNA isolated from primary cultures of RNA was quantified and underwent RT-PCR with the cycle number being varied between 20-40 cycles. RT-PCR products were demonstrated by agarose gel electrophoresis, shown in upper panel. The luminescence of each product was then measured and plotted as shown in the lower graph.
5.2 Evidence illustrating *in vitro* T-type channel knockdown using siRNA and effects on cell proliferation

While antisense oligonucleotides (ASOs) have been widely used by investigators studying the functions of T-CaCN, including any potential role in proliferation, the literature describing an anti-proliferative effect following knockdown of T-CaCN using siRNA is limited.

ASO knockdown of the $\alpha_1$G T-CaCN isoform has been described in a retinoblastoma cell line. In these experiments, mibefradil had an inhibitory effect on cell growth velocity, while $\alpha_1$G knockdown resulted in a reduction in the inhibitory effect of mibefradil (Bertolesi et al, 2002). Furthermore, blocking of the expression of the $\alpha_1$G T-CaCN isoform subunit with ASOs in cell lines of glioma cells and neuroblastoma cells has also been shown to inhibit cell proliferation by up to 70% (Panner et al, 2005).

T-CaCN knockdown using siRNA transfection has only recently been reported, with a successful reduction in Ca,v,3.1 ($\alpha_1$G) mRNA resulting in a reduction in the rate of PASMC proliferation (Rodman et al, 2005). The Ca,v,3.1 ($\alpha_1$G) isoform was found to be the predominant T-CaCN expressed in PASMC in early *in vitro* passage and also in the media of human pulmonary arteries. The effect of knockdown of this isoform was examined using siRNA directed against Ca,v,3.1 channel mRNA transfected into PASMC in primary culture. The authors demonstrated a significant reduction in serum-stimulated cell proliferation and inhibition of cell cycle progression. However, there has been no published data reporting the *in vitro* effects of Ca,v,3.2 ($\alpha_1$H) T-CaCN isoform knockdown using siRNA.
5.3 Effects of siRNA transfection on $\alpha_1$H T-type channel isoform expression in Vero cells and human mesangial cells

5.3.1 Preliminary siRNA experiments

The efficacy of our first siRNA sequence (named siRNA-1) designed to knockdown the expression of the $\alpha_1$H T-CaCN isoform was initially tested on cultured Vero cells. Whilst it is accepted that the siRNA (and RT-PCR) primers were designed specifically against the human genome $\alpha_1$H sequence, such experiments were primarily to test our protocols and simultaneously to assess any potential effect the sequence may incidentally have. Although the siRNA-1 duplex being used had not been designed with a primate sequence as a template, the $\alpha_1$H T-CaCN isoform has been demonstrated in the primate thalamus by RT-PCR (Genbank No.: AY268439) (Alexander et al, 2006). Vero cells were subcultured 1:10 and seeded into 35mm dishes. 24 hours later the cells were 40-50% confluent and ready for transfection with the siRNA-1 duplexes. Growth medium was added 24 hours after transfection. 48 hours after transfection, RNA extraction was performed followed by RT-PCR as described in chapter 2. Cells were transfected with siRNA at final concentrations of between 200nM and 800nM for 48 hours. A negative control siRNA, not corresponding to any recognised human RNA sequence, was transfected at a similar range of concentrations to control groups of cells to create paired experiments of test and control conditions.
Figure 5.3 shows the relative abundance of $\alpha_1H$ T-CaCN RNA in cultured Vero cells after 30 cycles of RT-PCR. A concentration-dependent reduction in mRNA is seen as the transfected dose of siRNA-1 increases, with almost complete knockdown seen at concentrations over 400nM ($n=3$). In our Vero cell experiments we needed to use 400nM or 800nM to see any appreciable effects. The need for such a high concentration may have been due to a lack of specificity of the siRNA duplex or to problems with transfection. RNA expression was not affected by transfection with negative control siRNA.

Figure 5.3: RT-PCR results of $\alpha_1H$ mRNA knockdown using siRNA-1 in Vero cells. siRNA-1 was transfected at a range of concentrations into cultured Vero cells, with comparison made to negative control siRNA transfection. The upper band in each lane represents the amplification product of the housekeeping gene $\beta$-Actin whilst the lower bands represent the amplification products of the $\alpha_1H$ T-type channel isoform (product size 176bp) in each experimental condition. Ctrl = negative-control siRNA-transfected cells. Test = $\alpha_1H$ siRNA-transfected cells. Olig = oligofectamine only (no siRNA). ($n=3$).
Figure 5.4 illustrates results of a similar experiment performed on primary cultures of human MC. Analysis of $\alpha_1$H mRNA knockdown using siRNA-1 was repeated over four separate experiments. Representative images of agarose gel electrophoresis from two of these experiments are shown in figure 5.4. In experiment A, MC transfection with $\alpha_1$H siRNA-1 appears to be effective in knocking-down $\alpha_1$H expression. However, this result was not consistent throughout the four experiments performed, as illustrated in experiment B. In this example, MC transfection with $\alpha_1$H siRNA-1 would appear to have no effect on $\alpha_1$H expression, whilst expression of $\alpha_1$H was reduced upon transfection of the negative control siRNA.

![Image of agarose gel electrophoresis](image)

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**Figure 5.4: RT-PCR results of $\alpha_1$H mRNA knockdown using siRNA-1 in human MC.** The upper band in each lane represents the amplification product of the housekeeping gene $\beta$-Actin whilst the lower bands represent the amplification products of the $\alpha_1$H T-type channel isoform (product size 176bp) in each experimental condition. Results shown are examples of two distinct experiments using MC. (n=4).
5.3.2 Use of new siRNA duplexes

In view of the difficulties encountered with the initial siRNA duplex, new siRNA duplexes were designed for use in human MC. Two distinct siRNA sequences were designed by Qiagen® - labelled siRNA-2 and siRNA-3 – allowing us to utilise their expertise in designing duplexes that would have a high degree of activity and specificity. A scrambled negative control siRNA sequence (Negative Control siRNA #1, Ambion, UK) - which does not correspond to any part of the human genome sequence – was used as a negative control siRNA in all experiments. Transfection of each siRNA duplex at a concentration of 200nM resulted in a significant knockdown of $\alpha_1$H T-CaCN RNA compared to negative control siRNA transfection. After comparison of the effectiveness of each of the two duplexes using RT-PCR, siRNA-3 was deemed the better of the two duplexes in achieving adequate knockdown (results shown in figure 5.5). siRNA-3 was therefore used for all subsequent proliferation assays and protein lysate experiments.

![Figure 5.5: $\alpha_1$H RNA expression in human MC after transfection of 200nM $\alpha_1$H siRNA-3. Results shown after 30 cycles of RT-PCR. The upper band represents $\beta$-Actin products, illustrating equal RNA loading, and the lower band $\alpha_1$H products (product size 176bp). Compared to treatment of cells with negative control siRNA at 200nM, transfection of $\alpha_1$H siRNA resulted in a reduction in $\alpha_1$H RNA expression of 22±20% at day3, and 62±12% (as assessed by relative luminescence) at day 6. (n=3)
5.4 Effect of siRNA transfection on cell proliferation

5.4.1 Effect on cell number
Primary cultures of human MC were plated into 96-well plates at a density of 2500 cells per well. Cells were visualised 24 hours later and transfected in triplicate according to methods described in chapter 2, provided cell confluence was estimated to be 40-50%. Transfection efficiency is optimum in the absence of serum. Cell proliferation was stimulated using standard growth medium (RPMI-1640 / 10% FCS) added 24 hours after transfection. Cell number was assessed on the day of transfection then 48, 72 and 144 hours after transfection using Promega’s CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay as described in chapter 2. The medium of all wells was changed at day four in the first set of experiments and replaced with Optimem I plus standard growth medium (figures 5.6 & 5.7). A repeat transfection of siRNA was not performed in this group. In a further set of experiments the medium was left unchanged in all wells for the duration of the experiment (figures 5.8 & 5.9).

Medium changed at day four
Figure 5.6 illustrates growth velocity curves of human MC following siRNA transfection. Growth medium was initially added at day one after transfection, then all wells were washed and medium changed at day four. Negative control siRNA transfection has no effect on cell growth velocity when compared to vehicle-treated cells. Transfection of siRNA targeting the α_{1}H T-CaCN isoform resulted in a significant reduction in MC growth velocity over six days. Data at days 3 and 6 from the same series of experiments, demonstrating a significant reduction in cell number, is shown in figure 5.7.
Figure 5.6: Modulation of human MC proliferation following transfection of $\alpha_1$H siRNA-3. Cells were transfected 24 hours after plating, with standard growth medium added a further 24 hours later. 96 hours after transfection, all cells were washed with PBS and fresh standard growth medium added. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 5 repeated experiments. **p<0.001 when compared to cells in all other treatment groups.

Figure 5.7: Bar chart illustrating MC number 3 and days after $\alpha_1$H siRNA-3 transfection. Cells were transfected and treated as described in figure 5.6. Illustrated error bars are SEM. n=3 in each experiment, with results shown from 5 repeated experiments (left panel: day 3; right panel: day 6). **p<0.001 when compared to cells in all other treatment groups. (Dotted line represents cell number at time of transfection (Day 0)).
Medium unchanged throughout experiment

Figure 5.8 illustrates growth velocity curves of human MC following siRNA transfection. In this second set of experiments, growth medium was added at day one after transfection and remained unchanged throughout the experiment. Once again, transfection of a negative control siRNA had no effect on cell growth velocity when compared to vehicle-treated cells. Transfection of siRNA targeting the α₁H T-CaCN isoform again resulted in a significant reduction in MC growth velocity over six days. This reduction in total cell number at day six was much more significant than in the earlier experiments when medium was changed at day 4. Data at days 3 and 6 from the same series of experiments, demonstrating this significant reduction in cell number compared to negative control siRNA transfection, is shown in figure 5.9.
Figure 5.8: Modulation of human MC proliferation following transfection of α₁H siRNA-3 (no medium change). Cells were transfected 24 hours after plating, with standard growth medium added a further 24 hours later. This medium was not changed later in the experiment. Cell number was estimated using the MTS assay (y-axis). Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments. **p<0.001 when compared to cells in all other treatment groups.

Figure 5.9: Bar chart illustrating MC number 3 and 6 days after α₁H siRNA-3 transfection (no medium change). Cells were transfected and treated as described in figure 5.8. Illustrated error bars are SEM. n=3 in each experiment, with results shown from 3 repeated experiments (left panel: day 3; right panel: day 6). **p<0.001 when compared to cells in all other treatment groups. (Dotted line represents cell number at time of transfection (Day 0)).
### 5.4.2 Effect on DNA replication

The effect of siRNA transfection and subsequent knockdown of the $\alpha_1$H T-CaCN isoform on DNA replication was assessed. MC were plated onto 60mm dishes and underwent siRNA transfection. Growth medium was added 24 hours after transfection, similar to the proliferation assays. Approximately 32 hours after growth medium was added, BrdU was added to all plates. A further 16 hours later, cells were fixed and labelled with fluorescent antibody as previously described.

Figure 5.10 illustrates results of an experiment assessing BrdU incorporation into primary cultures of human MC following siRNA transfection. Transfection of MC with a negative control siRNA had no effect on BrdU incorporation at 48 hours when compared to vehicle-treated controls. However, transfection of an siRNA duplex targeting the $\alpha_1$H T-CaCN isoform resulted in a clear and significant reduction in DNA replication.

![Figure 5.10: BrdU incorporation in human MC after transfection with 200nM $\alpha_1$H siRNA-3.](image)

The total percentage of BrdU-positive cells is shown 48 hours after stimulation of transfected cells with 10% FCS (in standard growth medium). Illustrated error bars are SEM. n=3. *p<0.05 when compared to cells transfected with negative control siRNA.
5.5 Effect of $\alpha_1$H T-type channel isoform knockdown using siRNA on downstream signalling mechanisms

A further attempt was made to try to identify a potential mechanism linking stimulation and $\text{Ca}^{2+}$ entry via the T-CaCN to cell cycle activity and cell proliferation. Similar to experiments described in chapter four, an examination of the expression of proteins known to be involved in a cascade of signals regulating MC proliferation was performed following transfection with $\alpha_1$H siRNA or a negative control siRNA duplex. Primary cultures of human MC were prepared and transfected as described in chapter 2. Growth medium was added 24 hours after transfection with total cell lysates then isolated at intervals and protein concentration quantified.

Figure 5.11 illustrates expression of phosphorylated ERK1/2 following transfection with either $\alpha_1$H siRNA or a negative control siRNA duplex. Similar to results described in chapter 4, there is an initial increase in expression over the first six hours that diminishes to pre-treatment levels within 24 hours. However, no difference was demonstrated between treatment groups throughout the experiment.

Figure 5.12 illustrates the expression of the pro-survival signalling protein Akt following similar siRNA transfection. Throughout the 24 hours of the experiment, no significant difference was demonstrated between treatment groups.

As a result of the subsequent demonstration of changes in p-ERK expression within 15 minutes of incubation of proliferating human MC with TTL-1177, in later work it would be interesting to repeat these siRNA experiments looking for similar early changes in protein expression. siRNA knockdown-induce changes in the cell signalling machinery my subsequently lead to identification of the link between calcium entry via T-CaCN and the proliferative signalling cascade.
**Figure 5.11:** Expression of phospho-ERK1/2 after transfection with α₁H siRNA-3, followed by stimulation with growth medium for 0-24 hours. A total of 5µg of protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

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**Figure 5.12:** Expression of phospho-Akt and total Akt after transfection with α₁H siRNA-3, followed by stimulation with growth medium for 0-24 hours. A total of 5µg of protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

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5.6 Discussion

The results presented in this chapter further support the hypothesis that T-CaCN are critical to the process of human mesangial cell proliferation. Following a description of the technique in mammalian cells in 2001 (Elbashir et al, 2001), RNA-interference has revolutionised the investigation of the functions of individual genes and proteins. The key attraction of this technique is the high degree of specificity of gene knockdown it affords. In the series of experiments described here, knockdown of the $\alpha_1$H T-CaCN isoform has a profound effect on cell growth rate and DNA replication. A clear mechanism explaining how T-CaCN have their effect on cell proliferation remains unclear, although given the results of subsequent repeated experiments at earlier time points shown in chapter 4, a more detailed analysis of signalling proteins immediately upon siRNA knockdown would now be warranted in further work.

The first siRNA sequence targeting $\alpha_1$H mRNA described in our preliminary experiments was designed using the following set of widely held ‘rules’ governing duplex design:

- The target region from a given cDNA sequence begins 50-100 nucleotides (nt) downstream of the start codon
- Use a 23nt sequence motif consisting of AA(N$_{19}$), where A=adenine and N=any nucleotide
- The target sequence is approximately 50% G-C rich
- Exclude sequences sharing homology with other genes

Due to the expense and relatively limited window of usefulness associated with primary cultures of human MC, the efficacy of siRNA duplexes was examined in the first instance using cultures of the Vero cell line. While it must be accepted that the siRNA duplexes and RT-PCR primers were designed against the human $\alpha_1$H T-CaCN cDNA sequence, the use of this primate cell line allowed us to carry out preliminary experiments without wasting resources during refinement of the technique. Our preliminary siRNA experiments showed variable results in terms of mRNA knockdown in both Vero cells and human MC. Additionally, both sets of experiments seemed to show that
concentrations of siRNA in the range of 400-800nM needed to be transfected in order to achieve significant knockdown. At concentrations this high, non-specific off-target or toxic effects of siRNA transfection cannot be excluded.

Further siRNA duplexes were subsequently designed by Qiagen® using their established commercial programme. The degree of mRNA knockdown achieved using duplex siRNA-3 was significantly more robust and reproducible. Knockdown of α1H T-CaCN isoform mRNA has been demonstrated at three and six days after siRNA duplex transfection by semi-quantitative RT-PCR as quantitative real-time PCR (qPCR) techniques were unavailable at the time of study. Clearly qPCR would be a better tool in assessing degree of knockdown and would produce more accurate data than could be produced by gel electrophoresis of RT-PCR products. However, evidence did exist in these results of inhibition of expression of the α1H T-CaCN isoform when compared to transfection with a negative control siRNA, supported by proliferation assay and BrdU endpoint data, while accepting that the exact degree of mRNA knockdown could not be quantified from these results.

An inhibitory effect on MC proliferation and BrdU incorporation following siRNA transfection was subsequently shown, supporting the evidence for successful mRNA knockdown compared to negative control transfection. Cells transfected with α1H T-CaCN isoform siRNA and stimulated with growth medium that remained unchanged throughout the six days of the experiment illustrated an almost complete inhibition of the expected increase in cell number. However, any continued inhibitory effect on cell proliferation appeared to be lost when cell culture medium was replaced with fresh growth medium after four days. It must be accepted that the observed fall in growth velocity following α1H T-CaCN isoform siRNA transfection may be due to apoptosis – this cannot be ruled out from the data presented. However, the illustrated BrdU incorporation experiments demonstrate evidence of a reduction in S-phase progression. Although intracellular inclusion of the siRNA duplexes should have been entirely completed within the first 24 hours after transfection, changing the growth medium still seemed to affect cell growth rate. It is possible that a ‘fresh’ stimulus of growth factors results in alternative pathways to the as yet unidentified T-CaCN signalling pathway.
being activated. This would clearly need further investigation. Furthermore, the use of a plasmid in transfection of the siRNA duplex could potentially extend the effect of the siRNA and may alter this observation markedly.

Other limitations must also be considered when examining our siRNA results. Firstly, the degree of successful siRNA transfection was not assessed in our experiments, with transfection efficiency effectively being assumed to be equal in all experiments. In order to increase the robustness of these results, an assessment of the efficiency of the delivery mechanism could be performed. This could perhaps be done using fluorescent-labelled siRNA targeting a common housekeeping gene such as GAPDH. Secondly, the degree of T-CaCN current knockdown and reduction in T-CaCN protein expression following siRNA transfection was not assessed.

As described in chapter 3, the accurate identification and measurement of a T-CaCN current in primary cultures of human MC is technically difficult. As a result, it was not possible to determine the effect of siRNA transfection on T-CaCN current density in our experiments and this was not attempted. As an alternative strategy, the effectiveness of the siRNA duplex could perhaps be assessed in alternative cells in which a T-CaCN is easier to measure, such as HEK-293 cells over-expressing the $\alpha_1$H T-CaCN isoform.

Furthermore, the lack of a sensitive and specific antibody capable of detecting a small number of copies of the $\alpha_1$H T-CaCN channel protein creates a similar problem. We were not able to assess the degree of knockdown in protein expression in our cells. However, despite these limitations, the demonstration of both $\alpha_1$H mRNA knockdown and a marked attenuation in MC growth rate following siRNA transfection would support our hypothesis that the $\alpha_1$H T-CaCN isoform is critical to MC proliferation.

The work of Rodman et al reports results of pulmonary artery smooth muscle cell (PASMC) studies from which comparisons with our results may be drawn (Rodman et al, 2005). PASMC were shown to express a transient inward calcium current, which peaked upon membrane depolarisation to -40mV and was eliminated by mibebradil but not nifedipine (an L-type calcium channel inhibitor). This LVA T-type current was small,
with a maximum current of 5±2pA being recorded. Although a T-type current was recorded from only a small number of MC in our studies, the size of the inward calcium current is comparable to that recorded by Rodman in PASMC. The presence of $\text{Ca}_v3.1$ ($\alpha_1G$) and $\text{Ca}_v3.2$ ($\alpha_1H$) T-CaCN isoforms in the human pulmonary vasculature was demonstrated by immunostaining of cultured cells. Polyclonal antibodies against $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ were prepared for the authors – the use of which had not previously been described. Following $\text{Ca}_v3.1$ ($\alpha_1G$) knockdown with siRNA, the authors reported a correlation between the degree of reduction in $\text{Ca}_v3.1$ protein expression and inhibition of PASMC proliferation. With this in mind, it would be interesting to re-explore our results utilising an antibody such as those used in Rodman’s experiments.

In summary, the use of siRNA as a tool in knocking down T-CaCN in human MC illustrates further supportive evidence for a role of T-CaCN in the signalling and/or regulation of MC proliferation.
6. The role of T-type calcium channels in cell cycle regulation of human mesangial cells

6.1 The cell cycle and MC proliferation

As outlined in chapter one, the mammalian cell cycle is a highly regulated sequence of events governed by the expression of specific proteins and protein kinases at precise stages in the cycle. At particular points in this sequence the relative expression of cyclin-dependant kinases (CDKs) or their inhibitors (CDKIs) determines the degree to which cell cycle progression may occur.

MC injury may precipitate proliferation, hypertrophy or apoptosis – each event occurring via subtle differences in cell cycle protein expression. Models of mesangial proliferative glomerulonephritis allow detailed examination of cell-cycle protein expression at specific points. The expression of cyclins A and E were examined in proliferating MC in our experiments. Cyclin A and E expression has been shown to increase upon proliferation of MC in the Thy1.1 rat model of mesangioproliferative GN. The expression of cyclin D1, CDK4 and CDK2 is also augmented, with this sequence occurring on re-entry to the cell cycle and throughout G1-phase (Shankland et al, 1996; Marshall & Shankland, 2006). Evidence in PASMC (Rodman et al, 2005) and unpublished data in VSMC (Shatock et al, personal communication) would indicate an important role for T-CaCN in regulating progression through the G1/S-phase transition. In these experiments, T-CaCN inhibitors led to an increase in the proportion of cells held in G1. In support of this, our results reported in chapters four and five demonstrate a reduction in the proportion of cells undergoing DNA replication upon treatment with T-CaCN inhibitors or after α1H T-CaCN isoform knockdown using siRNA. This may be as a result of a reduction in the proportion of cells progressing from G1-phase through into S-phase.

This chapter reports the results of experiments aimed at studying the effects of T-CaCN inhibition on cell cycle progression in human MC, and attempts at the synchronisation of populations of MC in order to examine cyclin expression.
6.2 Effects of T-type calcium channel antagonists on cell cycle phase

Flow cytometric analysis was used in order to determine the cell cycle profile of proliferating human MC treated with T-CaCN inhibitors as used experimentally in chapter 4. The determination of cell cycle distribution using flow cytometry is based on measurement of the DNA content of cells. Cells in G1-phase should contain the normal diploid amount of DNA. In G2-phase cells have double the normal content of DNA, while cells undergoing DNA synthesis in S-phase have an intermediate amount. Cells are co-incubated with BrdU for the last 16 hours of inhibitor treatment that incorporates only into cells in S-phase. Cells are then labelled with a FITC-conjugated anti-BrdU antibody. DNA is then stained by propidium iodide (PI) before flow cytometric analysis.

PI-stained cells are gated (shown in figure 6.1B) for analysis. When analysed in a dot plot with FITC-antibody staining on the y-axis (i.e. BrdU incorporation) as shown in figure 6.1C, an accurate determination of cell cycle distribution can be gained by gating each area. The percentage of cells in each of G1-, S- and G2-phases can then be expressed as a proportion of the whole gated population.

Figure 6.1 illustrates results of flow cytometric analysis of proliferating human MC cultured without T-CaCN inhibitors, but in the presence of nocodazole as described in the methodology in section 2.7. Panel A shows the total population of cells analysed. The gated area in panel B illustrates PI-stained cells, which are then analysed in further detail in panel C, with gated areas R2, R3 and R4 representing populations of cells in G1-, G2- and S-phases respectively.

Changes in cell cycle distribution are seen upon treatment with mibefradil or TTL-1177 – relatively selective inhibitors of T-CaCN. Representative examples of dot plot analyses after treatment with these drugs are illustrated in figures 6.2 and 6.3 respectively.
Figure 6.1: Dot plot analysis of serum-stimulated human mesangial cells to determine cell cycle phase using flow cytometry. Cells were plated into 60mm dishes and serum-starved in 0.2% FCS for 48 hours prior to treatment with 10% FCS for 48 hours. BrdU was added to the culture medium for the final 24 hours and nocodazole was added for the final 16 hours of culture. Cells were then prepared for flow cytometric analysis and labelled as described in chapter 2. Panel A above represents the total population of cells analysed. Panel B indicates the PI-stained cells upon which further analysis is illustrated in Panel C. In Panel C, the FL3-A channel measures PI staining (i.e. DNA content) while the FL1-H channel measures FITC-labelling (i.e. BrdU incorporation). Therefore, gated area R2 represents cells in G1-phase, area R3 represents cells in G2-phase and area R4 cells in S-phase. The number of cells in each gate was calculated using CellQuest software.
Figure 6.2: Cell cycle phase analysis of MC following treatment with 15µM mibebradil for 48 hours. Dot plot flow cytometry analysis of serum-stimulated human MC. Cells were treated, labelled and analysed as described earlier. The labelled gated areas R2, R3 and R4 were used to calculate the proportion of cells in G1-, G2- and S-phases respectively, as described earlier.

Figure 6.3: Cell cycle phase analysis of MC following treatment with 15µM TTL-1177 for 48 hours. Dot plot flow cytometry analysis of serum-stimulated human MC. Cells were treated, labelled and analysed as described earlier. The labelled gated areas R2, R3 and R4 were used to calculate the proportion of cells in G1-, G2- and S-phases respectively, as described earlier.
Figure 6.4 illustrates the cell cycle phase distribution of serum-stimulated MC treated with mibefradil at concentrations of 10µM and 15µM for 48 hours compared to vehicle-treated controls. There was a 48% reduction in the proportion of cells in S-phase following treatment with 10µM mibefradil compared to vehicle treated controls, with the percentage of S-phase cells recorded falling from 16.7±1.1% to 8.7±0.5%. Following treatment with 15µM mibefradil the proportion in S-phase fell by 65% (16.7±1.1% to 5.9±1.0%). Alongside the fall in cell number recorded in S-phase, a corresponding rise in the proportion of cells held in G1-phase was observed, with the proportion of cells in G2/M–phase showing no significant alteration following treatment.

Figure 6.4: Percentage of human MC in each cell cycle phase following treatment with mibefradil. The proportion of cells in each cell cycle phase is shown after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either none or increasing concentrations of mibefradil. MC were collected, labelled and analysed as described earlier. The proportion of cells in each phase was determined from dot plot analyses as described in Figure 6.1. Illustrated error bars are SEM. n=3. *p<0.01 when compared to the number of untreated control cells in G0/G1 phase. **p<0.01 when compared to the number of untreated control cells in S-phase.
Figure 6.5 illustrates results of flow cytometric analysis of serum-stimulated human MC following treatment with TTL-1177 at a final concentration of 15µM for 48 hours compared to vehicle-treated controls. Similar to the results following mibefradil treatment, co-incubation with 15µM TTL-1177 led to a 50% fall in the proportion of recorded cells in S-phase. The percentage of cells in S-phase fell from 16.7±1.1% in untreated cells to 8.4±1.1% after 48 hours treatment with 15µM TTL-1177. Once again, there was a corresponding rise in the percentage of cells in G1-phase, with no significant change in the number of cells in G2/M-phase.

Figure 6.5: Percentage of human MC in each cell cycle phase following treatment with TTL-1177. The proportion of cells in each cell cycle phase is shown after 48 hours of stimulation with 10% FCS (in standard growth medium) and simultaneous treatment with either no drug or TTL-1177 at a final concentration of 15µM. MC were collected, labelled and analysed as described earlier. The proportion of cells in each phase was determined from dot plot analyses as described in Figure 6.1. Illustrated error bars are SEM. n=3. *p<0.05 when compared to the number of untreated cells in G0/G1 phase. **p<0.05 when compared to the number of untreated control cells in S-phase.
6.3 Expression of cell cycle proteins in proliferating human MC

6.3.1 Cell cycle protein expression following treatment of MC with T-CaCN inhibitors

The area of particular interest with regard to T-CaCN is around the G1/S border of the cell cycle. Results illustrated in section 6.2 would support the hypothesis that T-CaCN are important in the complex signalling cascade occurring around at the end of G1-phase. At this point, one would expect the level of cyclin E to be at its maximal with expression subsequently falling sharply once in S-phase, with the level of cyclin A being low and expected to increase through S-phase. Therefore, one might expect T-CaCN blockade to reduce any fall in cyclin E expression and attenuate the expected rise in cyclin A. In order to investigate this hypothesis, cultured human MC were plated into 60mm dishes and grown to approximately 50-60% confluence before being serum starved in 0.2% FCS for 48 hours. Cells were then washed and treated with 0.2% FCS, 10% FCS or T-CaCN at varying concentrations for a further 48 hours. At this point, cells were lysed for Western blot analysis as described in chapter 2. Protein lysates underwent gel electrophoresis before transfer onto a nitrocellulose membrane. Monoclonal antibodies against cyclin A and cyclin E were used for immunodetection.

Illustrated in figure 6.6 are examples of Western blots for cyclin A and cyclin E of total cellular lysates of human MC following treatment with T-CaCN inhibitors. There would appear to be no significant difference in expression of these cyclins before and after treatment with mibebradil, TTL-1177 or nickel chloride (Ni^{2+}). Despite repeated experiments and modifications of treatment time (from 24 to 72 hours), lysate volume and antibody concentrations, there continued to be no significant differences.
Figure 6.6: Expression of cyclin A and cyclin E in human MC after treatment with T-CaCN inhibitors for 48 hours. A total of 10µg of total cellular protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

Figure 6.7: Expression of cyclin A and cyclin E in human MC after treatment with the T-CaCN inhibitor Ni²⁺ for 48 hours. A total of 10µg of total cellular protein lysate was loaded into each lane for electrophoresis. The experiment was repeated three times and the images above are representative of three Western blots.

Upon reflection of these results, the presented findings were not entirely surprising. Although cells had been serum-starved in 0.2% FCS for 48 hours, it is unlikely that all cells were synchronised into the same phase of the cell cycle. Therefore, one would expect the expression of cyclin A, E and other proteins to vary between cells in each treatment group. Attempts were then made to synchronise populations of MC for further investigation.
6.3.2 Synchronisation of populations of human MC

In order to examine cell cycle protein expression after treatment with T-CaCN inhibitors, populations of cultured human MC needed to be synchronised into the same phase of the cell cycle at a given time. Two methods of synchronisation of MC were attempted – serum-deprivation and a thymidine double-block technique.

In order to investigate the effects of serum-deprivation on cyclin expression, human MC were plated into 100mm dishes and allowed to grow to approximately 50% confluence. At this point, cells were washed in PBS and fresh medium added – either containing 10% FCS (standard culture medium) or 0.2% FCS (serum-deprivation). After 72 hours, cells were washed with PBS once more and medium containing 10% FCS was added to all plates, with protein lysates isolated immediately, at 7 hours and at 24 hours. Figure 6.8 illustrates cyclin A and cyclin E expression following serum-deprivation with 0.2% FCS for 72 hours. No difference between treatment groups was demonstrated over the 24 hours period of the experiment, with cyclin expression appearing to remain constant throughout.

![Cyclin A and Cyclin E expression](image)

| Serum starved? | + | + | + | - | - | - |
| Time since 10%FCS (hrs) | 0 | 7 | 24 | 0 | 7 | 24 |

**Figure 6.8: Expression of cyclin A and cyclin E in human MC after serum-deprivation.** Following serum-deprivation with 0.2% FCS for a period of 72 hours, cells were ‘released’ upon addition of medium containing 10% FCS and lysates collected at intervals. A total of 10µg of protein lysate was loaded into each lane for electrophoresis. Primary antibody concentration was 1:200. Secondary antibody concentration was 1:1000. The experiment was repeated three times and the images above are representative of three Western blots.
An alternative method of cell synchronisation has been described in vascular smooth muscle cell lines using the technique of double thymidine block. The experimental protocol is described in chapter 2. The thymidine double block technique exposes large populations of cells to a high concentration of thymidine for two periods of 12 hours, at an interval of 12 hours. The aim is to achieve a population of cells in which the majority are collected at the G₁/S border, ready for further study with drugs that are postulated to affect G₁/S progression, such as T-CaCN inhibitors (Harper, 2005).

The results of this technique in populations of primary cultures of human MC were disappointing. Had cells been synchronised, one might expect an increase in cyclin A levels as cells progress through G₁ and S-phases following release from G₁/S block, and a simultaneous fall from high to low concentrations of cyclin E. Neither finding was demonstrated in our experiments, as illustrated in figure 6.9 below.

![Cyclin A and Cyclin E expression](image)

**Figure 6.9: Expression of cyclin A and cyclin E in human MC following double thymidine block.** Following thymidine treatment, cells were ‘released’ into the cell cycle upon addition of medium containing 10% FCS and lysates collected at intervals. A total of 10µg of protein lysate was loaded into each lane for electrophoresis. Primary antibody concentration was 1:200. Secondary antibody concentration was 1:1000. The experiment was repeated three times and the images above are representative of three Western blots.
6.4 Discussion

The flow cytometry results presented in this chapter support the hypothesis that T-CaCN inhibition delays progression into S-phase of the cell cycle. However, detailed examination of cell cycle proteins was difficult due to problems achieving cell synchronicity.

FACS analysis of primary cultures of human MC was undertaken to specifically look at effects of T-CaCN inhibition at the G1/S border. In order to facilitate investigation of events at this stage of the cell cycle, nocodazole was used in an effort to hold cells at G2/M once through S-phase. This would then make any increase in the proportion of cells in G1-phase easier to identify. In the experiments reported here, nocodazole had a partial effect in reducing the number of cells in G1-phase and increasing cells held at G2/M. As a result, it was possible to demonstrate the effect of inhibition of G1/S progression following treatment with T-CaCN inhibitors. High concentrations of nocodazole are cytotoxic and therefore any effects at these doses would be difficult to interpret. The results shown in chapter 2 demonstrated a maximal degree of G2/M block with 80nM nocodazole, with no further increase with higher doses. 80nM was therefore used in all experiments. However, this meant that much smaller alterations in the proportion of cells in each cell cycle phase following treatment with T-CaCN were demonstrated. Mibebradil and TTL-1177 have been shown in chapter 4 to cause a fall in DNA replication as estimated by BrdU incorporation. The technique used in our FACS experiments essentially confirmed this result, using additional staining with PI in order to estimate nuclear DNA content.

The reduction in S-phase progression with an increase in the proportion of cells in G1 is supported by data in pulmonary artery smooth muscle cells (Rodman et al, 2005). Treatment of PASMC with mibebradil resulted in a similar increase in G1 cell number and a fall in the proportion in S-phase. These results were reported despite nocodazole not being used in Rodman’s experiments.
A limitation to these reported experiments in MC relates to the small number of T-CaCN inhibitors available for use. These experiments focused on mibebradil and TTL-1177 only, with numerous attempts at running the experiments needed in order to generate reproducible results. In view of the limited nature of the relatively precious resource of human MC primary cultures, in addition to the time taken to analyse cells and results, further experiments studying the effects of Ni$^{2+}$ or verapamil were not completed. It is accepted that this does diminish the strength of these results somewhat, as any results of verapamil treatment showing no alteration to cell cycle phase after treatment would have supported our hypothesis.

Synchronisation of primary cultures of human MC was challenging and was not possible in our experiments. The purpose of attempting this line of investigation was as a means of supporting the FACS results using total cellular lysates from MC after treatment with T-CaCN. As a result, based on knowledge of cyclin expression at specific points in the cell cycle, any effect of T-CaCN inhibition on cell cycle progression could be demonstrated.

Serum-deprivation had been used in many of the experimental techniques described in this thesis in an attempt to establish ‘growth arrest’ of primary cultures of human MC. Serum-deprivation of MC for a period of 72 hours was therefore performed prior to assessment of cell cycle protein expression (primarily cyclin A and cyclin E) upon serum-stimulation. There was no clear pattern of progression of cyclin expression over the 24 hours after exposure to standard growth medium (10% FCS) and therefore the effect of T-CaCN inhibition on cell cycle progression and cyclin expression could not be assessed. This experiment was attempted three times before changing to an alternative method of cell synchronisation – the thymidine double-block technique.

The thymidine double-block technique has been described as a tool for the synchronisation of populations of VSMC (Harper, 2005). Cells are plated in standard growth medium following trypsinisation and subculture. At 40-50% confluence, culture medium was replaced with standard growth medium containing thymidine in excess at a concentration of 2mM. The subsequent 12-hour incubation was intended to block cells in
After washing and replenishing cells with standard growth medium for another 12 hours, cells were free to progress through the cell cycle. A further 12-hour exposure to excess thymidine (2mM) in standard growth medium aimed to collect all cells at the G1/S border. Cells were then washed with PBS and ‘released’ into standard growth medium, with the aim of having a synchronised population of cells moving through the cell cycle. Once again, based on studies of expression of cyclin A and cyclin E, no significant difference was seen between populations of cells treated with thymidine in this way and those which were not. A number of factors could be responsible for this apparent failure. These would include problems with the protocol, such as too much or too little time exposed to thymidine and the concentration of thymidine used, unfamiliarity with the technique or possible cytotoxic, pro-apoptotic or other unidentified signalling effects of thymidine. Each of these should be investigated in detail in order to establish the efficacy of this technique in primary cultures of human MC and establish reproducible synchronised populations of MC – with hindsight this would be a separate project beyond the scope of this thesis.

A number of methods for cell cycle synchronisation of mammalian cells have been reported, many of which allow synchronisation of populations of cells at the G1/S border. A number of pharmacological agents have been used to arrest the cell cycle at specific phases. These include mimosine and lovastatin causing G1 arrest, hydroxyurea leading to S-phase arrest, and nocodazole causing G2/M arrest. Mimosine, an iron/zinc chelator, induces DNA double-strand breaks in treated cells leading to G1 arrest (Mosca et al, 1992), although it may also cause apoptosis. Lovastatin, a mevalonate synthesis inhibitor, has been shown to inhibit serum-stimulated rat MC proliferation in vitro, following a 48-hour period of serum-deprivation (O’Donnell et al, 1993). However, lovastatin is also recognised as having pro-apoptotic effects in a variety of cell lines, including human malignant glioma cells (Jones et al, 1994) and prostate stromal cells (Padayatty et al, 1997). This limits its usefulness as a reliable tool in assessing cell cycle progression. Drugs such as these, and thymidine used in experiments described in this chapter, are therefore likely to result in a variable degree of toxicity and alterations in normal cell metabolism – perhaps more so in primary cultures. A number of authors
have described causal associations between the use of synchronisation agents and side effects including cell death (Kung et al, 1990; Pardee & Keyomarsi, 1992).

Serum deprivation is one of a number of drug-independent methods of synchronising cells from asynchronous populations. Others include cell contact inhibition and centrifugal elutriation. Serum deprivation can be used as a technique for achieving cell synchrony, as cell cycle proteins (particularly D-type cyclins) are up-regulated in the presence of growth factors in serum, facilitating early G\(_1\) progression. Therefore, the absence of these mitogens through cell incubation in medium containing very low concentrations of serum or none at all should result in cells exiting the cell cycle into quiescence (G\(_0\)-phase). This technique was utilised in a number of experiments reported in this thesis, including in the investigation of the effects of T-CaCN inhibition following serum-stimulation (and the assumed entry of cells into G\(_1\)). However, a criticism of these experiments would point to a lack of definitive proof of quiescence prior to starting experiments. Furthermore, the rate at which different cell types – primary cultures or transformed cell lines – become quiescent, enter into G\(_1\)-phase or progress through G\(_1\) to S-phase may vary between cell type and even within cultures of the same cell population (Davis et al, 2001). On reflection, cell quiescence could have been demonstrated using flow cytometry and progression through the cell cycle following release from quiescence could have been more closely followed (perhaps at 3, 6, 12, and 24 hours) using BrdU incorporation. Finally, the study of an alternative cell cycle protein – cyclin D\(_1\) – may have yielded more information about the usefulness of this technique as a tool for synchronisation. Upon serum stimulation of human MC after a 48-hour period of serum deprivation in 0.4% FCS, cyclin D\(_1\) has been shown to fall from high levels in quiescence. Levels then rise throughout G\(_1\) towards S-phase (Zeng et al, 2002).

In summary, the data presented in this chapter again support the hypothesis that T-CaCN are critically involved in the regulation of human MC cell cycle progression. Inhibition of T-CaCN with mibefradil or TTL-1177 resulted in accumulation of cells in G\(_1\)-phase and a fall in the S-phase population. Synchronisation of a population of MC was not achieved during these experiments – further study would be needed to establish a reliable, non-toxic method to achieve this in MC.
7. Discussion

7.1 Summary of findings

The expression of T-type calcium channels in human MC and their potential role in MC proliferation is described for the first time in this thesis. The data presented demonstrate an inward Ca\(^{2+}\) current that would appear consistent with that carried by a T-CaCN and expression of the $\alpha_1$H T-CaCN isoform at the mRNA level. Pharmacological inhibition of T-CaCN is shown to attenuate serum-stimulated proliferation of primary cultures of human MC, to limit S-phase progression as assessed by BrdU incorporation and to increase the proportion of cells held in G\(_1\)-phase of the cell cycle. Similarly, transfection of MC with siRNA designed to knockdown expression of the $\alpha_1$H T-CaCN isoform also attenuates cell growth velocity upon serum stimulation and reduces the proportion of cells entering S-phase (Mulgrew et al, 2009). The identification of a mechanism linking Ca\(^{2+}\) entry via T-CaCN to regulation of proliferation remains unclear, both in MC and other cells. However, results presented in this thesis would suggest that the MAP-kinase signalling pathway may be involved as ERK phosphorylation was significantly diminished within one hour of treatment with TTL-1177. This finding would warrant further detailed study.

A number of limitations should be considered when reviewing these results as a whole. With regard to isoform expression in human MC, only the $\alpha_1$H isoform (and not $\alpha_1$G or $\alpha_1$I) was found to be expressed at the mRNA level in proliferating cells. While positive controls for the $\alpha_1$G and $\alpha_1$H isoforms were available to support these results, a reliable positive control for $\alpha_1$I only became available after completion of experiments reported in this thesis. However, while $\alpha_1$G and $\alpha_1$H isoforms have been widely described in a number of tissues, $\alpha_1$I is almost exclusively found in the central nervous system. Expression of the $\alpha_1$H isoform at the protein level was not demonstrable, despite using two commercially available polyclonal antibodies. This may have been due to a lack of specificity of the antibodies or to the likely relatively low number of channels expressed at any one time. Other antibodies have been used and success described by other authors.
(Panner et al, 2005; Rodman et al, 2005; Wolfe et al, 2003), but these antibodies were not commercially available.

The current measured in MC was small and current recording technically difficult. The current characteristics demonstrated upon progressive depolarising steps at low voltages between -90mV and -20mV are consistent with an inward Ca\textsuperscript{2+} current carried via T-CaCN. Inhibition of this current following incubation of MC with T-CaCN inhibitors was not demonstrated. Had such an experiment been attempted, the absence of a measurable current may or may not have been due to pharmacological T-CaCN inhibition, as many untreated cells had no recordable current. It could be argued that this was not because the current did not exist but a consequence of technical problems or perhaps variations in T-CaCN current expression during the cell cycle. Similar problems recording T-CaCN currents have been reported in VSMC preparations. A masking effect of the more robust and more abundant L-CaCN may add to this difficulty (Cribbs, 2006).

The use of Fura-2 to further examine calcium entry was considered and briefly attempted. Depolarisation of cultured MC with potassium chloride (KCl) was attempted, with effects of fluorescence before and after treatment of cells with TTL-1177. Variable concentrations of KCl were required (up to 10mM) to generate minor changes in fluorescence representing calcium entry, with no consistency seen in results before or after treatment with the T-CaCN inhibitor. As a result, it was felt that significantly more time would be taken up optimising this technique before adding greatly to the results. Further experiments were therefore stopped.

Assessment of adequacy of siRNA duplex transfection into cultured MC has been discussed in chapter 5, with suggestions as to how this might be performed. However, the data presented clearly demonstrates α\textsubscript{1}H mRNA knockdown, a reduction in growth velocity and a reduction in progression into S-phase following α\textsubscript{1}H siRNA transfection when compared to transfection of a negative control siRNA.
Synchronisation of populations of MC was problematic and not achieved in these experiments. Further work would be needed to establish a reproducible, reliable and non-toxic method of synchronising an asynchronous population of MC in order to study T-CaCN expression and cell cycle progression in more detail.

7.2 T-type calcium channel isoform expression and function

Results of experiments described in this thesis would support the hypothesis that T-CaCN play an important role in the regulation of human MC proliferation. These findings add weight to an increasing body of evidence supporting a role for T-CaCN in the proliferation of a variety of cell types. Expression of T-CaCN isoforms is known to vary between cell types (table 3.1). The functions of T-CaCN in central nervous system signalling unrelated to cell proliferation have been extensively reviewed (Perez-Reyes, 2003) and are well recognised, but they are not discussed in further detail here.

In view of the similarities between VSMC and MC, it was a little surprising to find expression of only the \( \alpha_{1}H \) isoform in MC, particularly as the \( \alpha_{1}G \) isoform is often more abundant and physiologically active in VSMC. In MC, inhibition of the \( \alpha_{1}H \) isoform by RNAi resulted in significant attenuation of growth velocity and cell cycle progression. However, it is possible that in addition to a reduction in \( \alpha_{1}H \) expression and function, there may have been alterations in the expression of other T-CaCN isoforms, perhaps resulting in a small upregulation of \( \alpha_{1}G \) expression. This was not examined in these experiments and it would be interesting to revisit this in future work. The effect of \( \alpha_{1}H \) knockdown on the expression and function of other ion channels (\( Na^{+} \), \( K^{+} \) or L-type \( Ca^{2+} \) channels) should also be examined, as it is likely that interaction between different ion channels is critical to the underlying mechanism linking T-CaCN to cell proliferation.

Evidence supporting a role for T-CaCN in cell proliferation has been described in rat VSMC (Akaike et al, 1989), human PASMC (Rodman et al, 2005), prostate cancer cells (Haverstick et al, 2000) and glioma and neuroblastoma cells (Panner et al, 2005). Each
series of experiments describes attenuation of proliferation upon inhibition or knockdown of T-CaCN. Furthermore, over-expression of the α1H isoform into glioma cells or neuroblastoma cell lines increased cell proliferation by a factor of two (Panner et al, 2005), although the effect of over-expression of T-CaCN into HEK-293 cells has generated conflicting results and remains controversial. Both an increase in cell proliferative rate (around a 40% increase) (Wang et al, 2002) and no effect whatsoever (Chemin et al, 2000) have been reported. Proposed mechanisms may involve calmodulin, PKC, transcription factors (e.g. c-fos), cell cycle proteins or other mitogenic signalling pathways. Rho-associated kinase (ROCK) has recently been reported as demonstrating a regulatory role over T-CaCN. ROCK activation by lysophosphatidic acid (LPA) reduced native T-type currents in Y79 retinoblastoma cells and upregulated native CaV3.2 (α1H) current in DRG neurons (Iftinca et al, 2007).

This thesis has primarily addressed the role of T-CaCN in human MC proliferation. Other potential functions of T-CaCN in MC have not been investigated. One important function proposed for T-CaCN in VSMC is that of regulation of contractility and maintenance of vascular tone. The role, if it exists, of T-CaCN in MC contractility and associated regulation of glomerular blood flow has not been examined. With their critical role in regulating glomerular haemodynamics, the effect on MC contractility in vitro (perhaps after treatment of cells with AII) should be examined using a T-CaCN inhibitor. In VSMC, T-CaCN have been implicated in arterial vasoconstriction with the development of mibefradil a direct consequence of this observation. Mibefradil was produced and marketed (as Posicor®) to be a novel antihypertensive as it reduced peripheral vascular resistance by selectively targeting VSMC T-CaCN. Furthermore, T-CaCN are thought to have an important role in maintaining post-glomerular vascular tone. While both T- and L-type Ca2+ channels are expressed in afferent glomerular arterioles, efferent arterioles express only T-CaCN (Hansen et al, 2001). Any potential benefit of T-CaCN inhibition using mibefradil over L-CaCN inhibition in renoprotection by reducing intraglomerular pressure and glomerulosclerosis has not been demonstrated (Griffin et al, 2001). As mibefradil is now recognised as having effects on other ion channels in addition to the T-CaCN, a more highly-specific T-CaCN inhibitor should be
used. Following demonstration of in vitro inhibition of cell cycle progression from G\textsubscript{1} into S-phase by benidipine, an L- and T-type calcium channel blocker (Ono et al, 2002), recent clinical studies have shown promising results compared to amlodipine when assessing blood pressure control and proteinuria (Ohishi et al, 2007). If in vivo anti-proliferative effects of benidipine were to be demonstrated, then this compound may be worth further examination in experimental renal disease.

### 7.3 Pharmacological targeting of T-type calcium channels

T-CaCN have been targeted pharmacologically in order to study their many varied functions ever since the channel was first described over 30 years ago. The tools with which to selectively inhibit T-CaCN currents were relatively limited until the early 1990’s. Notwithstanding the introduction of mibefradil in 1994, very few other drugs have been available for this purpose. However, recently new selective drugs have been reported, with others in development, including TTL-1177 described in this thesis.

Mibefradil, Ni\textsuperscript{2+} and TTL-1177 have been used extensively in experiments in this thesis and have been described and discussed in detail in chapters 1 and 4. Although classically an L-type Ca\textsuperscript{2+} channel inhibitor, verapamil at higher doses is reported to inhibit T-CaCN currents. A number of other L-CaCN inhibitors have also been reported to show variable inhibitory effects on T-CaCN, but with such a lack of selectivity for T-CaCN their usefulness in the study of T-CaCN functions is limited (Heady et al, 2001). Selectivity in pharmacological agents for T-CaCN, and ultimately for specific T-CaCN isoforms, remains a goal in drug development.

A number of novel molecules have recently been in development. T-CaCN inhibitors based on a 3,4-dihydroquinazoline backbone have been reported to be selective blockers of transfected human Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels (Lee et al, 2004; Rhim et al, 2005). The activity of these compounds in untransfected, intact biological systems has yet to be reported. TTL-1177 is one of a number of novel compounds developed to target T-CaCN-dependent human cancer cell lines (McCalmont et al, 2004). The strategy
employed in developing these drugs relied upon the ability of ionic nickel (Ni\(^{2+}\)) to inhibit Ca\(^{2+}\) entry through T-CaCN (Lee et al, 1999), particularly the \(\alpha_1\)H (Ca\(_{v3.2}\)) isoform. McCalmont and colleagues undertook a series of searches of the National Library of Medicine database using the initial search criteria ‘calcium’ and ‘nickel’, yielding a number of citations describing compounds inhibiting Ca\(^{2+}\) entry. Any compounds that were difficult to synthesise or complex were excluded from further evaluation, as were compounds that were more potent at inhibiting proliferation than Ca\(^{2+}\) entry. The remaining compounds were used to create a structure-activity relationship yielding a library of new chemical entities. These are currently in development for potential use in the treatment of several human cancers (Gray & Macdonald, 2006).

The reported use of TTL-1177 \textit{in vivo} in the treatment of a prostate cancer cell line in mice provides encouragement that systemic administration of a T-CaCN inhibitor can have specific effects on localised aberrant cell proliferation (Haverstick et al, 2000). The goal would of course be to have a selective and specific treatment, targeting the cells or organ of interest and minimising systemic side effects. Although TTL-1177 and mibebradil have been shown to have anti-proliferative effects on prostate cancer and VSMC respectively \textit{in vivo} after systemic administration, novel methods of local drug delivery would be preferable to avoid future drug withdrawal as a result of drug interactions and side effects – the fate which befell Posicor\textsuperscript{®}.

### 7.4 T-type calcium channel knockdown using RNA interference

Initial experiments targeted T-CaCN using pharmacological agents that are known to have variable degrees of selectivity for the T-CaCN, but may have non-specific effects on many ion channels, including other Ca\(^{2+}\), Na\(^{+}\) and Cl\(^{-}\) channels. The technique of RNA interference (RNAi) was utilized to achieve specific knockdown of the \(\alpha_1\)H (Ca\(_{v3.2}\)) T-CaCN isoform while minimising effects on other channel mechanisms. As discussed in chapter 5, the degree of transfection efficiency was not evaluated in the experiments reported in this thesis, nor the degree of current inhibition following siRNA transfection.
Despite these limitations, α₁H mRNA knockdown was achieved and MC proliferation attenuated using RNAi. The possibility of non-specific effects of the transfection protocol was excluded by using a negative control siRNA sequence. Transfection of this negative control sequence had no significant effect on proliferative rate, the rate being similar to non-transfected cells.

These results support other published evidence describing the effect of T-CaCN knockdown with siRNA on the proliferative rate of human cells. In addition to the work on MC described here, the work of Rodman and colleagues studying human PASMC has been discussed in chapter 5. In both cell models, knockdown of the α₁H isoform resulted in an attenuation in cell proliferative rate and a reduction in the proportion of cells progressing into S-phase (Rodman et al, 2005). To date there have been no in vivo studies using siRNA in a model of MC proliferation. The challenge in translating this in vitro work with siRNA into an in vivo model primarily relates to achieving specificity for the target cells and minimising side effects. T-CaCN isoforms have important roles in normal human physiology. In an effort to achieve target specificity a number of strategies have been examined. Non-viral approaches include systemic injection of unmodified or chemically modified siRNA, physical methods of localised release such as electropulsation, encapsulation in liposomes or cationic lipids, or chemical coupling of siRNAs to carrier molecules (Aigner, 2007). Another alternative would be the use of magnetic assisted transfection (Bertram, 2006). It is likely that advances in the development of nanotechnology will improve drug delivery to specific signalling molecules in specific tissues or cell types. The slight variations in the cellular environment of tumours has leant itself to the possibility of being targeted by nanotechnology.

A concern regarding systemic knockdown of T-CaCN comes from an α₁H knockout mouse model (α₁H⁻/⁻) (Chen et al, 2003). α₁H knockout mice were smaller than littermate controls and showed abnormalities in coronary artery relaxation and increased cardiac fibrosis. The authors made no reference to renal development or function. It would be interesting to examine the response of α₁H knockout mice to Thy1.1 exposure and experimental glomerulonephritis, looking at MC proliferative rate and T-CaCN
expression in proliferating MC – does $\alpha_1G$ become prominent, or do signalling mechanisms alter in any way?

### 7.5 Clinical use of T-type calcium channel inhibition

A number of agents are known to have activity in blocking T-CaCN, either selectively or in addition to effects on other ion channels. A number of pharmacological agents including anti-psychotics and anaesthetics are known to have inhibitory effects on T-CaCN. The only drug to enter clinical practice and used therapeutically to target T-CaCN specifically has been mibefradil under the name of Posicor® (Hoffman-LaRoche). With its novel target and attractive physiological and pharmacokinetic profile, the use of Posicor® for the treatment of angina pectoris, hypertension and congestive cardiac failure was initiated in the late 1990s. Experiments in the early development of mibefradil demonstrated binding to the [3H]-desmethoxyverapamil receptor in cardiac membranes. It had similar potency to verapamil but with no negative inotropic effect (Fang and Osterreider, 1991). Randomised clinical trials demonstrated beneficial effects of mibefradil in the management of chronic stable angina pectoris (Braun et al, 1996) and essential hypertension (Oparil et al, 1997). Animal studies illustrated a reduction in ischaemia-related deaths in a rat model of chronic heart failure upon treatment with mibefradil that was similar to the ACE-inhibitor cilazapril – a finding not seen with any other calcium channel antagonist (Mulder et al, 1997).

Subsequently, the MACH-1 trial, a multicentre, randomised, double-blind study across North America and Europe of almost 2600 patients with chronic heart failure (NYHA class II to IV), was initiated. This showed no overall benefit of mibefradil when used as an adjunct to optimum treatment with diuretics and ACE-inhibitors. In fact, an increase in mortality was observed in the mibefradil group in patients also taking the anti-arrhythmic drug amiodarone, other anti-arrhythmics and drugs associated with ‘torsade de pointes’ conduction abnormalities. It was hypothesised that the negative trend of this study was due to toxic plasma concentrations of anti-arrhythmics as a result of inhibition
of hepatic cytochrome p-450 enzymes by mibefradil (Levine et al, 2000). Posicor® was voluntarily withdrawn from the market in 1998 – only six months after its introduction.

Other drugs in clinical use that inhibit T-CaCN to a minor degree in addition to their primary molecular target include the neuroleptic pimozide, and trazodone, primarily prescribed for the treatment of depression and insomnia. More promising may be benidipine, now in clinical use and recognised as a T-CaCN and L-CaCN antagonist, as described earlier.

The likelihood is that if and when specific T-CaCN inhibitors are used in clinical practice they will be used as an antiproliferative agent clinically in cancer therapeutics in the first instance. Aberrant cell proliferation seen in solid organ tumours, such as prostatic, breast or central nervous system tumours, have been targeted in vitro and in animal models. Mariot et al demonstrated a voltage-dependent T-type calcium current, mediated via α₁H T-CaCN, playing an important role in the differentiation and proliferation of human prostate cancer cells, with these channels shown to promote calcium entry at resting membrane potentials (Mariot et al, 2002). Haverstick and colleagues, using both in vitro and in vivo models, described the inhibition of prostate cancer proliferation using the compound TTL-1177, synthesized to specifically block Ca^{2+} entry via T-CaCN. In vitro studies of LNCaP and PC-3 prostate cancer cells demonstrated a concentration-dependent inhibition of proliferation when treated with TTL-1177, an effect shown to be cytostatic rather than cytotoxic as evidenced by a resumption of normal proliferative growth velocity upon drug washout. In an in vivo model of prostate cancer, SCID mice inoculated by intraperitoneal injection with PC-3 cells had a 34% increase in lifespan when treated with daily doses of TTL-1177 compared to control animals (Haverstick et al, 2000). Further work has shown a significant growth advantage to human embryonic kidney-293 (HEK-293) cells when transfected with α₁H T-CaCN and that this effect is inhibited by TTL-1177. Although the precise mechanism of action leading to opening of voltage-operated channels (VOC) in electrically non-excitable cells remains unclear, the authors proposed a role for T-CaCN in mediating ‘capacitative’ calcium entry (Gray et al, 2004).
Additional evidence for T-CaCN in malignant cell growth comes from experiments studying the phaeochromocytoma-derived PC12 cell line in which Del Toro et al demonstrated that the $\alpha_1$H T-CaCN gene is up-regulated, via a HIF-dependent mechanism, in a time- and dose-dependant manner upon exposure to a hypoxic environment (Del Toro et al, 2003). This type of HIF mediated action is recognised as being a hallmark of malignant solid tumour growth. A pharmaceutical research company in Virginia, USA is focussing research on targeting cell cycle progression in mammalian tumours, developing novel agents to inhibit T-CaCN which may go on to clinical use, one of which is TTL-1177 (www.tautherapeutics.com).

Therapies to target non-malignant cell proliferation, such as MC proliferation, are probably a further step in the use of T-CaCN targeting beyond cancer therapeutics. It must also be remembered that MC proliferation is not always pathogenic and has an important regenerative role. Studies of experimental models of glomerulonephritis have illustrated a pattern of cellular changes which would suggest that excess ECM deposition and progressive glomerular scarring does not always follow MC proliferation. In the initial stages following MC injury at least, proliferation of MC seems to be an essential part of successful glomerular repair. For example, post-streptococcal glomerulonephritis, certain forms of lupus nephritis and anti-Thy1.1 nephritis are characterized by mesangial expansion and hyperplasia, which frequently resolves completely once the inflammatory insult has subsided. Much of the evidence highlighting the importance of MC proliferation comes from the anti-Thy1.1 nephritis model of mesangio-proliferative disease in rats. Injection of an antibody to the MC antigen Thy1.1 causes acute complement-dependent mesangiolysis within the first 24 hours, followed by platelet and macrophage infiltration, intense MC proliferation and transient matrix accumulation. Despite the severity of the glomerular injury, with this ‘single-hit’ model there is complete recovery of the normal glomerular architecture within six to eight weeks (Jefferson & Johnson, 1999). However, repeated injections of anti-Thy1.1 antibody leads to early proliferation of MC followed by accumulation of ECM and progressive glomerulosclerosis (Harendza et al, 1999).
Strategies to inhibit MC proliferation using the anti-Thy1.1 model have been associated with a reduction in ECM accumulation (Jefferson & Johnson, 1999), suggesting that MC proliferation is also crucial in the pathogenesis of glomerulosclerosis and in this sense is pathogenic. Similar findings have been reported in other experimental models of mesangioproliferative disease. Mice transgenic for the SV40 antigen developed MC proliferation followed by progressive mesangial sclerosis (MacKay et al, 1990). Similarly, mice transgenic for bovine growth hormone developed glomerular cell proliferation followed by mesangial matrix accumulation (Pesce et al, 1991). Whilst it is clear that a prolonged insult to the glomerulus leads to MC proliferation and progressive glomerular scarring, the development of therapies will need to inhibit non-reparative proliferation whilst promoting resolution of the normal glomerular architecture.

The availability of the T-/L-type calcium channel blockers efonidipine in Japan has led to the publication of interesting work, in both animal models and clinical studies. Sugano et al studied spontaneously hypertensive rats that had undergone subtotal nephrectomy. Treatment with the R-enantiomer of efonidipine had no effects on systemic blood pressure but significantly reduced proteinuria compared to vehicle-treated rats. α₁G expression was increased in renal tissue of rats subjected to subtotal nephrectomy, while treatment with R(-)-efonidipine blunted the increase in tubulo-interstitial fibrosis, α-smooth muscle actin expression and TGF-β-induced Rho-kinase activity, suggesting the renoprotective effects are not simply due to haemodynamic changes (Sugano et al, 2008). Furthermore, in a rat model of interstitial fibrosis due to chronic unilateral ureteric obstruction (UOU), efonidipine improved tubulo-interstitial fibrosis more effectively than nifedipine (an L-type calcium channel blocker) independently of systemic blood pressure. The authors concluded that this effect was mediated via suppression of fibrogenic signalling (Matsuda et al, 2011).

In clinical studies, efonidipine has recently been studied in diabetic and hypertensive patient groups. A comparison of efonidipine and amlodipine in type 2 diabetic patients with hypertension and nephropathy (who were already taking an ARB) suggested that the additional blockade of T-CaCN with efonidipine blunted the observed decrease in estimated glomerular filtration rate seen in the amlodipine-treated arm of this small study,
while reducing plasma aldosterone levels and arterial stiffness over a 12 month follow-up period (Sasaki et al, 2009). Efonidipine was also shown to be effective in controlling both heart rate and blood pressure in patients with mild-to-moderate hypertension (Oh et al, 2010).

In summary, after initial success with T-CaCN inhibition in clinical use in the treatment of hypertension, mibefradil was withdrawn due to drug interactions and is now used solely in experimental studies. The development of novel, increasingly selective inhibition of the T-CaCN continues at pace, with new therapies for cancer treatment driving much of the research, and with novel T-/L-type calcium channel inhibitors available in the Far East generating interesting results which may lead to its use as a renoprotective agent either alone or alongside other established therapies such as ACEi or ARB. Therapies to treat proliferative renal diseases may then develop or transfer specialities after this, in much the same way as cyclophosphamide is now used for a variety of roles.

7.6 Future strategies

This research has provided significant evidence for the first time that supports the likely important role of T-CaCN in the signalling and regulation of human MC proliferation. It adds weight to an increasing body of evidence identifying T-CaCN as a key regulator of cell cycle progression and cell growth in a number of non-excitable cells and in malignant cell growth. It is hoped that this may lead to more specific, targeted therapies for proliferative renal diseases.

The identification of an entirely novel target such as this brings with it a series of new challenges in drug development and delivery. Furthermore, care must be taken to ensure that important physiological roles of T-CaCN essential to normal cell and organ function are affected minimally, if at all. However, early in vivo work in a mouse model receiving systemic treatment for prostate cancer with a T-CaCN inhibitor seemed to have no adverse effects and survival was prolonged. The exact mechanism allowing this to take
place remains unclear. Perhaps the turnover of many cells expressing T-CaCN, such as MC or VSMC, is very low. As a result, the effect on T-CaCN function following treatment with T-CaCN inhibitors is imperceptible. Meanwhile, T-CaCN may be increased in rapid uncontrolled cell proliferation, such as in tumours, resulting in the anti-proliferative effect of T-CaCN inhibitors in this situation. It would be interesting to study renal biopsy specimens, perhaps using in-situ hybridisation techniques, examining MC expression of T-CaCN in proliferative and non-proliferative renal diseases.

The underlying mechanism linking flux of Ca$^{2+}$ ions into the cell via T-CaCN and cell cycle regulation and proliferation remains unclear. It may be the case that T-CaCN are somehow linked to a ‘scaffold’ protein or other signalling promoter which then initiates a signalling cascade promoting cell proliferation. One proposed regulatory protein has been calmodulin (CaM), although further work would be needed to study the effects of T-CaCN inhibition on CaM expression and function, along with the associated expression of CaM-dependent kinases. The identification of a mechanism was not possible in this work and would therefore be a starting point for a future project, as would studying the expression of T-CaCN isoforms when MC are put under experimental physiological stress, for example using hydrostatic pressure techniques.

In addition to increasing our knowledge of T-CaCN signalling mechanisms in human MC with further in vitro work, the transfer of the work described in this thesis into an animal model should prove very interesting. This work has already started using a rat model of proliferative glomerulonephritis. Results from these experiments, thus far published in abstract form, demonstrate that TTL-1177 in acute Thy1 nephritis successfully reduces glomerular hypercellularity and indices of glomerular sclerosis at 10 and 14 days (Cove-Smith et al, 2009 (abstract)). Furthermore, TTL-1177 inhibits rat MC proliferation in vitro in a similar way to results of human MC described in this thesis (Cove-Smith et al, 2008 (abstract)). Building on this research may be the next step in the development of a novel therapeutic strategy for proliferative renal disease.
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