RENAL BRUSH BORDER MEMBRANE GLUCOSE TRANSPORT: REGULATORY MECHANISMS AND ADAPTATION TO DIABETIC HYPERGLYCAEMIA.

A thesis submitted

by

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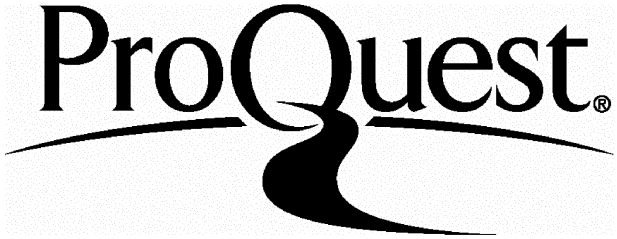

Abstract

There is now substantial evidence that implicates hyperglycaemia in the progression of diabetic nephropathy. Studies using mesangial cells have demonstrated that overexpression of the facilitative glucose transporter, GLUT1, is a key factor that predisposes this cell type to glucose-induced damage. Diabetes is also reported to evoke changes in proximal tubule function, yet the underlying mechanisms involved have not been studied in detail. The studies described in this thesis were designed to investigate the effect of streptozotocin-induced diabetes on proximal tubular glucose transport and to determine the cellular mechanisms involved in its regulation.

Streptozotocin-induced diabetes was found to increase facilitative glucose transport across the proximal tubule brush border membrane (BBM), a response that could be abolished by normalisation of the blood glucose levels. Changes in transport rate correlated with expression of GLUT2 at the proximal tubule BBM. Experiments investigating the regulation of renal glucose transport demonstrated that sodium-dependent and facilitative glucose transport, are regulated by different intracellular signaling events. The regulation of sodium-dependent glucose transport was found to occur via cAMP-induced insertion of SGLT1 protein into the proximal tubule BBM. In contrast, a pathway involving both protein kinase C and intracellular calcium was demonstrated to regulate facilitative glucose transport.

The data reported herein provided evidence that GLUT2 expression at the proximal tubule BBM provides a dominant low affinity/high capacity route for glucose reabsorption during hyperglycaemia, which may culminate in glucose-induced damage of this nephron segment. The adaptation of renal glucose transport to experimentally-induced diabetes and the mechanisms involved in the regulation of renal glucose transport display striking similarities to those reported for the small intestine.

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Abbreviation list

α,β-meATP = α,β-methylene adenosine 5'-triphosphate

β,γ-meATP = β,γ-methylene adenosine 5'-triphosphate

1,3 BPG = 1,3 bisphosphoglycerate

2-DOG = 2-deoxy-D-glucose

2MeSADP = 2-methylthioadenosine diphosphate

2MeSATP = 2-methylthio-adenosine 5'-triphosphate

3-0 MG = 3-0-methylglucose

8-Br-cAMP = 8-Bromo-adenosine 3',5'-cyclic monophosphate

AC = adenylate cyclase

ADP = adenosine diphosphate

AE1 = anion exchanger 1

AGE's = advanced glycation endproducts

AMP = adenosine monophosphate

AQP = aquaporin

AR = aldose reductase

ATP = adenosine triphosphate

ATPγS = adenosine 5'O-3-thiotriphosphate

AVP = arginine-vasopressin

AVPR2 = arginine-vasopressin receptor 2

BB rat = biobreed rat

BBM = brush border membrane

BLM = basolateral membrane

Bz-ATP = 2',3'-O-(4-Benzoyl-benzoyl)- adenosine 5'-triphosphate

cAMP = cyclic adenosine monophosphate

cARs = cAMP receptors

CCPA = 2-chloro-N^6- cyclopentyladenosine

CFTR = cystic fibrosis transmembrane conductance regulator

CGS-21680=2-[*p*-(carboxyethyl)phenylethylamino]-5'-*N*-ethyl-carboxamidoadenosine

col = colchicine

CPA = N^6-cyclopentyladenosine

Cy2 = cyanine-2

Cyt B = cytochalasin B

DAB = 3,3'-diaminobenzidine tetrahydrochloride

DAG = diacylglycerol

db/db mouse = model of type 2 diabetes

DCCT = Diabetes Control and Complications Trial

DOG = 1,2 dioctanoylglycerol

DPMA = *N*-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine

DPP IV = dipeptidyl peptidase-IV

DTT = Dithiolthreitol

ECM protein = extra cellular matrix protein

EGTA = glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid

ER = endoplasmic reticulum

ERK = extracellular signal-regulated kinase

G29 = glucagon 29 or pancreatic glucagon

G37 = glucagon 37 or oxyntomodulin

GFR = glomerular filtration rate

GIP = gastric inhibitory peptide

GK rats = Goto-Kakizaki rats

GLP-1 = glucagon-like peptide-1

GLP-2 = glucagon-like peptide 2

GLUT = facilitative glucose transporter

GLUT1CBP = GLUT1 C-terminal binding protein

GRPP = glicentin-related pancreatic polypeptide

GTP = guanosine thriphosphate

H_2O_2 = Hydrogen peroxide

HK = hexokinase

IBMX = 3-isobutyl-1-methylxanthine

IDDM = insulin-dependent diabetes mellitus

IP-1 = intervening peptide-1

IP-2 = intervening peptide-2

IP_3 = inositol 1,4,5-triphosphate

kDa = kilodaltons

KIU = Kallikrein Inactivator units

K_m = Michaelis constant, substrate concentration at half V_{max}

LLC-PK = a porcine renal epithelial cell line with proximal tubule phenotype

MDCK cells = Madin-Darbey Canine kidney cells with distal tubule phenotype

MEK = MAPK/ERK kinase

MPGF = major proglucagon fragment

mRNA = messenger ribonucleic acid

ms = milliseconds

NAD^+ = nicotinamide adenine dinucleotide

NADH = nicotinamide adenine dinucleotide, reduced form

NADP = nicotinamide adenine dinucleotide phosphate

NADPH = nicotinamide adenine dinucleotide phosphate, reduced form

NDI = Nephrogenic diabetes insipidus

NGS = normal goat serum

NIDDM = non-insulin-dependent diabetes mellitus

NO = nitric oxide

NOD mouse = non-obese diabetic mouse

O_2 = Superoxide

OH' = hydroxyl radicals

OK cells = opossum kidney cells

p38 MAP kinase = p38 mitogen activated protein kinase

PBGST = 0.1M PBS containing 1% NGS, 0.1% BSA and 0.1% Triton X-100

PBS = phosphate buffered saline (pH7.4)

PBS-T = phosphate buffered saline (pH7.4) containing 0.1% Tween 20

PCT = proximal convoluted tubule

PD = potential difference

PDK-1 = 3-phosphoinositide-dependent protein kinase-1

Phl = phloretin

PI 3-kinase = phosphatidylinositol 3-kinase

PIA = (R-(-)-N^6-phenylisopropyladenosine

PKA = protein kinase A

PKC = protein kinase C

PLA_2 = phospholipase A_2

PLC = phospholipase C

PLD = phospholipase D

PLP = periodate-lysine-paraformaldehyde

PMA = phorbol 12-myristate 13-acetate

PPP = pentose phosphate pathway

prob = probenecid

PST= proximal straight tubule

PTH = parathyroid hormone

PZ = phlorizin

RER = rough endoplasmic reticulum

ROS = reactive oxygen species

rpm = rotations per minute

RT-PCR = reverse transcription polymerase chain reaction

SDH = sorbitol dehydrogenase

SDS = sodium dodecyl sulphate

SEM = standard error of the mean

SGLT = sodium glucose linked transporter

SNARE = soluble N-ethylmaleimide sensitive factor attachment protein receptor

STZ = streptozotocin

TBM = tubular basement membrane

TGF = transforming growth factor-β

TGN = *trans*-Golgi network

T_m = transport maximum

TMH = transmembrane helices

TPA = 1,2-O-tetradecanolphorbol 13-acetate

UDP = uridine 5' diphosphate

UKPDS = UK Prospective Diabetes Study

UTP = uridine 5' triphosphate

UTPγS = uridine 5'-O-(3'thiotriphosphate

UTR = 3'-untranslated region

V_{max} = maximum transport capacity

Chapter 1.

Background

1.1. Introduction

The kidneys play an important role in plasma glucose homeostasis. Under normal blood glucose conditions filtered glucose is completely reabsorbed, it is only when the renal absorptive mechanism is saturated, as in diabetes mellitus with hyperglycaemia, that glucose appears in the urine. It has been hypothesised that in poorly controlled diabetes, the high concentrations of glucose filtered by the kidneys, induce changes in tubular glucose transport which may contribute to the pathophysiological aspects associated with diabetic nephropathy.

1.2. Mechanisms of epithelial glucose transport

The conventional mechanism of renal and intestinal glucose transport displays striking similarities. Glucose moves across the intestinal enterocyte and the renal proximal tubule cell via specific glucose transporters. A Na^+-dependent glucose transporter (SGLT) located at the brush border membrane (BBM) couples glucose transport to the inwardly directed Na^+ electrochemical gradient. This gradient is maintained by the basolaterally located Na^+/K^+-ATPase. GLUT5 is also expressed at the BBM, where its primary function is in fructose transport. The exit of glucose from the cell occurs via facilitative diffusion across the basolateral membrane and is mediated by the glucose transporter isoforms, GLUT1 and GLUT2 (reviewed in (Debnam & Unwin, 1996; Thorens, 1996)). Interestingly, this model of epithelial glucose transport has recently been challenged by the finding that GLUT2 contributes significantly to the uptake of sugars across the BBM during the assimilation of a meal (Kellett, 2001; Kellett & Helliwell, 2000).

In the kidney, both active and facilitative glucose transporters have distinct distribution profiles along the proximal tubule that relate to their specific kinetic characteristics (Dominguez *et al.*, 1992) (Fig. 1.1 and 1.2). This provides a proximal tubule environment in which the bulk of filtered glucose is reabsorbed in the early S1 segment, by the low affinity/high capacity

glucose transporters, SGLT2 (Lee *et al.*, 1994) and GLUT2 (Chin E *et al.*, 1993); whereas the high affinity/low capacity transporters, SGLT1 (Kanai *et al.*, 1994; Pajor AM *et al.*, 1992) and GLUT1 (Chin E *et al.*, 1993), scavenge the remaining glucose that is presented to the distal regions of the proximal tubule. Fructose reabsorption in the kidney has until recently been considered to occur in a sodium independent manner, via the facilitative glucose transport GLUT5, which is expressed at the BBM of the S3 segment of the proximal tubule (Sugawara-Yokoo *et al.*, 1999). However, studies by Horiba *et al.* have suggested the presence of a sodium-dependent fructose transporter, termed NaGLT1, which is expressed primarily at the BBM of the PCT (Horiba *et al.*, 2003a). This transporter shows only 22% homology to the known SGLT and GLUT glucose transporters (Horiba *et al.*, 2003b) and has a reported K_m for fructose of 4.5 mM, with a coupling ratio for sodium of 1:1 (Horiba *et al.*, 2003a). At present the exact function of this proposed transporter has yet to be characterised fully, but it is assumed that NaGLT1 mediates the active reabsorption of fructose, in a manor similar to SGLT2 (Horiba *et al.*, 2003a).

Figure 1.1. Schematic representation of sugar reabsorption in the proximal convoluted tubule (PCT).

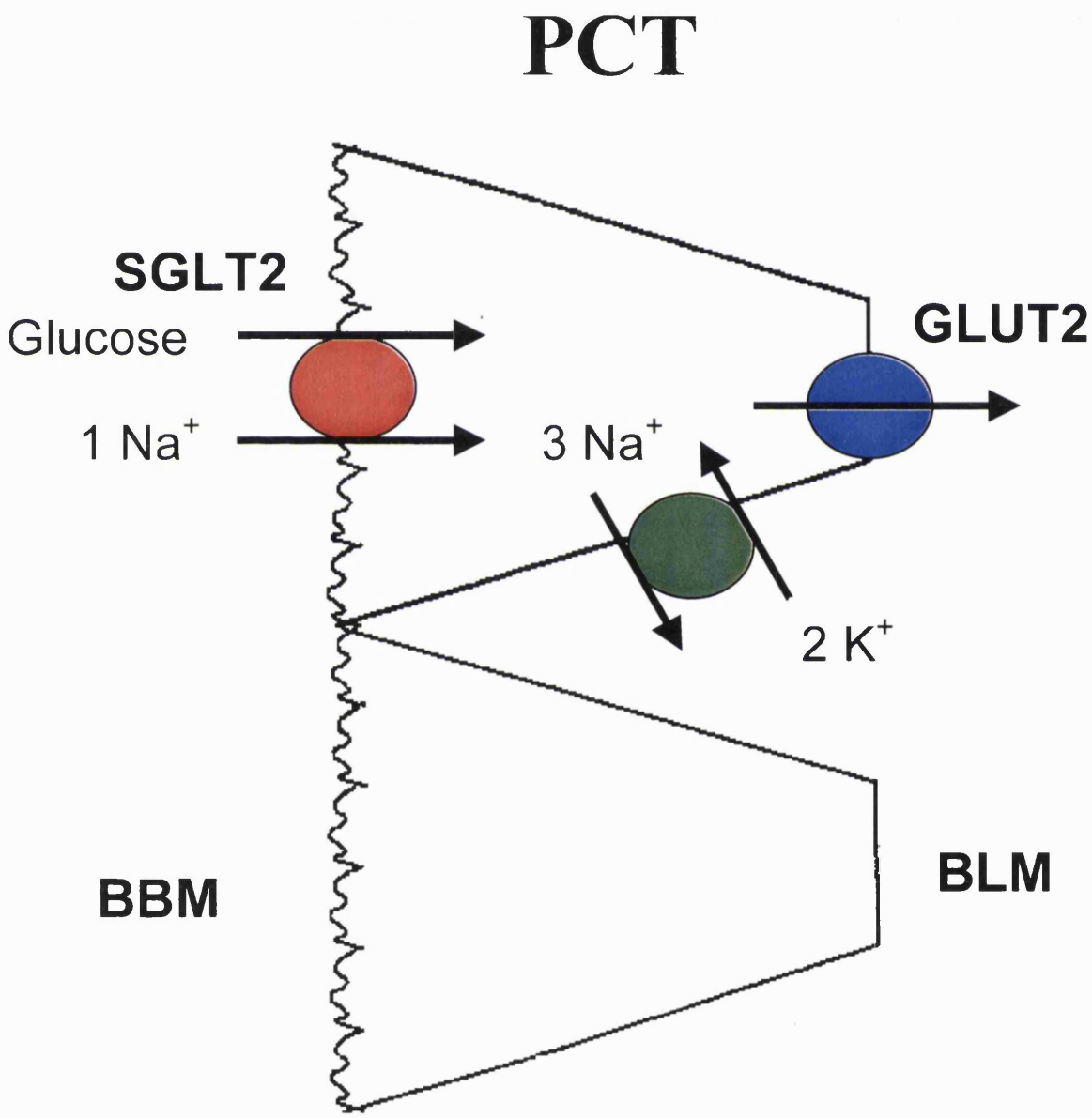
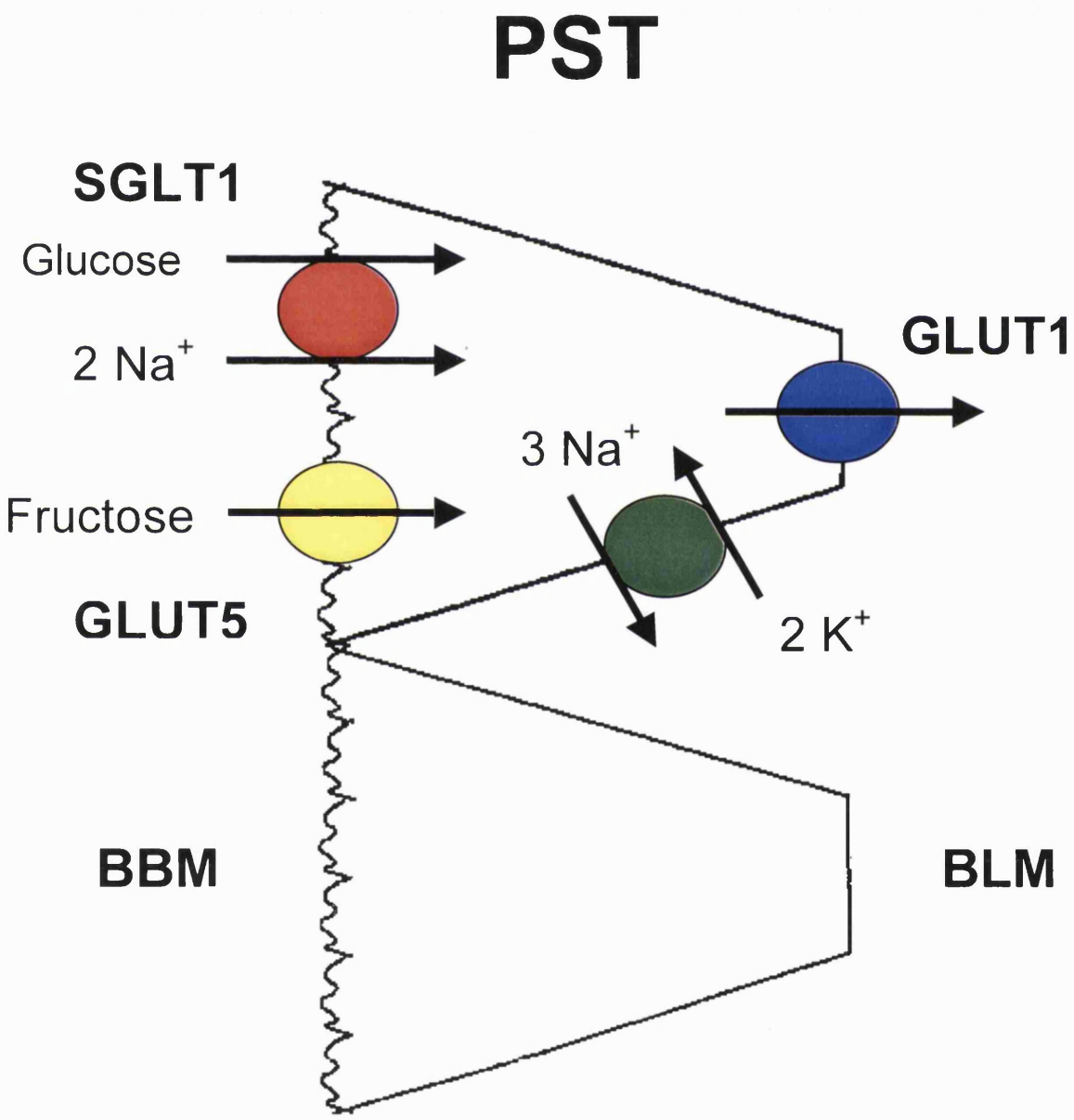


Figure 1.2. Schematic representation of sugar reabsorption in the proximal straight tubule (PST).



1.3. Sodium Glucose Linked Transporters (SGLTs)

1.3.1. Gene and protein properties

SGLT1 and SGLT2 are members of a large family of cotransporter proteins (gene name SLC5A) that are expressed in bacteria, yeast, invertebrates and vertebrates. SGLT1 was first cloned in 1987 (Hediger *et al.*, 1987) and following its successful expression in *Xenopus* oocytes, the transport characteristics of this protein have been extensively characterised (Table 1.1). Until the early 80's, SGLT1 was considered to be solely responsible for active glucose reabsorption in the kidney. Studies by Barfuss and Schafer in 1981, using *in vitro* perfusion of isolated rabbit proximal convoluted tubules and proximal straight tubules, demonstrated that the two distinct proximal tubular segments displayed different K_m values for active glucose transport, comparable with the now accepted K_m values for SGLT1 and SGLT2 (Table 1.1) (Barfuss & Schafer, 1981). Turner and Moran, using BBM vesicles isolated from the outer cortex and outer medulla, established differences in the sodium/glucose stoichiometry in the two regions (Turner & Moran, 1982). In addition to these findings, patients with autosomal recessive renal glycosuria display impaired renal but not intestinal glucose absorption (Elsas & Rosenberg, 1969). Indeed, a recent study has shown that this disease occurs as a consequence of a nonsense mutation in the SGLT2 gene, which upon translation forms a truncated, non-functional protein (van den Heuvel *et al.*, 2002). Following these observations, Hediger and colleagues successfully cloned the low affinity sodium-glucose transporter, SGLT2 (Wells *et al.*, 1992). Evidence suggests that another sodium-dependent glucose transporter, with functional characteristics, intermediate between SGLT1 and SGLT2 is also expressed in the kidney. This transporter was originally termed SAAT1, and was thought to be a neutral amino acid transporter with sequence similarities to SGLT1 (Kong *et al.*, 1993b). Following its expression in *Xenopus* oocytes, it was established that the transporter is in fact a low affinity sodium glucose cotransporter (Mackenzie *et al.*, 1994), and was therefore renamed SGLT3 (Diez-Sampedro *et al.*, 2000).

Human SGLT1 has been mapped to chromosome 22 q13.1 (Turk *et al.*, 1993). SGLT3 is also found on this chromosome but at position 12.1-12.3 (Dunham *et al.*, 1999), whilst, SGLT2 has been assigned to chromosome 16 p11.2 (Wells *et al.*, 1993). The SGLT genes have been shown to code for proteins of 659-672 residues, with a predicted mass of 73 kDa (Wright, 2001). The secondary structure of SGLT1 was originally thought to consist of 12 transmembrane helices (TMH) (Lee *et al.*, 1994), however, a more detailed secondary structure model reveals that the protein consists of 14 TMH, with both the NH_2 and COOH termini exposed to the extracellular space (Turk *et al.*, 1996). Amino acid sequence alignment has demonstrated that SGLT2 has 59% structural homology to SGLT1 (Wells *et al.*, 1992), whilst, SGLT3 shows 76% homology (Kong *et al.*, 1993b). Although the secondary structure of SGLT2 and SGLT3 have not been studied in detail they are predicated to be similar to that of SGLT1 (Wright, 2001).

SGLT1 protein is expressed at high levels in the small intestine, with lower expression occurring in the renal proximal tubule. Conversely, expression of SGLT2 occurs at high levels in the kidney, and to a much lesser degree in the intestine (Wells *et al.*, 1992). Localisation studies have demonstrated that SGLT1 is predominately expressed in the S3 segment of the proximal convoluted tubule (Lee *et al.*, 1994), with SGLT2 expression occurring in the S1 and S2 segment of the PCT (Kanai *et al.*, 1994; Silverman *et al.*, 1993). At present the exact localisation of SGLT3 in the kidney is unknown.

Expression of SGLT1 at the apical membrane is a widely accepted phenomenon, with the amino acid sequence conferring the membrane specificity (Kong *et al.*, 1993a). The exact region responsible for the targeting of the protein to this membrane remains controversial. Constructs containing deletions in the N-terminal domain of SGLT1 localised to the cytoplasm (Suzuki *et al.*, 2001), the authors of this study proposed that aspartic acid 28 was essential for the delivery of the protein to the apical membrane. In contrast, Lostao and colleagues demonstrated that substitution of arginine-427 within membrane domain 9, also abolished expression of the protein in the plasma membrane (Lostao *et al.*, 1995). In

addition to these findings, patients with glucose-galactose malabsorption display decreased membrane expression of SGLT1 in the small intestine. Missense mutations in these patients, at cystine 355 and leucine 147, eliminates sodium-dependent glucose transport by altering trafficking of the protein to the plasma membrane (Martin *et al.*, 1997).

1.3.2. Transport properties of SGLT proteins

SGLT1 can actively transport both glucose and galactose with a K_m of 0.5 mM (Wright, 2001), and a stoichiometry of 2:1, whereby 2 sodium molecules are transported for every 1 sugar molecule (Lee *et al.*, 1994). SGLT2 has a K_m for D-glucose of 2 mM and is generally considered to have a low affinity for galactose (Panayotova-Heiermann *et al.*, 1996), with a K_m value of greater than 20 mM. In contrast to SGLT1, the stoichiometry of SGLT2 is 1:1 (Kanai *et al.*, 1994). SGLT3 displays functional characteristics intermediate between SGLT1 and SGLT2; it is considered to be a low affinity transporter with a K_m for D-glucose of 6 mM, but with a sodium stoichiometry of 2:1 (Diez-Sampedro *et al.*, 2001). Phlorizin inhibits all three SGLT isoforms but with different affinities (Wright, 2001) (Table 1.1).

In addition to active sodium and glucose transport, SGLT proteins have also been shown to function as low conductance water channels. This hypothesis is derived from a number of observations obtained from experiments carried out on *Xenopus* oocytes expressing SGLT1. These include the finding that osmotic water permeability is proportional to the expression level of SGLT1, it is independent of the osmotic gradient and can be inhibited by phlorizin (Loo *et al.*, 2002). It has been demonstrated that the stoichiometry of human SGLT1 is 2 sodium, 1 glucose and 264 water molecules; with the water cotransport considered to be a consequence of the coupling of sodium and glucose through the SGLT protein (Loo *et al.*, 2002).

Table 1.1. Gene and functional properties of sodium-dependent glucose transporters

	SGLT1	SGLT2	SGLT3
Gene localisation	22q13.1	16p11.2	22p12.1-12.3
Homology to SGLT1		59%	76%
amino acid residues	664	672	659
K_m D-glucose (mM)	0.5	2	6
Na^+:glucose stoichiometry	2:1	1:1	2:1
K_m Na^+ (mM)	3	100	1.5
K_i for phlorizin (μM)	200	1	10
Sugar specificity	Glucose > Galactose	glucose	glucose

Kinetic studies by Wright and colleagues have determined the mechanism by which SGLT proteins cotransport sodium, glucose and water. It has been hypothesised that cotransport involves 6 distinct steps; firstly binding of sodium to the N-terminal domain of the protein causes a conformational change in the protein allowing glucose to bind to the C-terminal region, which is accompanied by entry of water into the sugar-binding pocket. The protein then undergoes a conformational change to expose the substrates to the intracellular space. Sodium then dissociates from the transporter as a consequence of the low intracellular sodium concentration, leading to relaxation of the protein conformation and subsequent extrusion of the glucose and water molecules. Finally the protein returns to its original state with the binding sites exposed to the extracellular medium (Wright, 2001).

1.3.3. Regulation of SGLT transporter proteins

It is well documented that activation of the protein kinase A (PKA) signalling pathway increases intracellular cAMP levels and promotes SGLT1-mediated glucose transport (Hirsch *et al.*, 1996; Sharp & Debnam, 1994b). Short-term modulation of SGLT1 transport occurs via insertion of additional SGLT1 protein into the plasma membrane (Hirsch *et al.*, 1996; Williams & Sharp, 2002) and through changes in the membrane electrochemical gradient (Sharp & Debnam, 1994b). Indeed, Wright *et al.* have estimated that in *Xenopus* oocytes intracellular vesicles contain 10-20 SGLT1 proteins and that the net basal rate of insertion of these vesicles into the plasma membrane is 10000 per second. This insertion rate has been shown to increase 100-fold following activation of PKA (Wright *et al.*, 1997). In this context, studies using Caco-2 cells have revealed that the major fraction of SGLT1 protein resides at the terminal web, which represents an intracellular reserve pool of protein ready for rapid insertion into the membrane (Kipp *et al.*, 2003).

Stabilisation of SGLT1 mRNA levels has been demonstrated to be involved in the long-term regulation of sodium-dependent glucose transport (Peng & Lever, 1995a). This occurs via cAMP-dependent binding of a 38 kDa nucleocytoplasmic protein, termed SG-URBP, to a uridine-rich sequence in the 3'-untranslated region of the SGLT1 mRNA, which protects the mRNA from decay (Lee *et al.*, 2000; Peng & Lever, 1995b). In addition to SG-URBP, an inhibitory transcription factor has also been identified, termed RS1 (Veyhl *et al.*, 1993). This 67 kDa protein has been localised to the intracellular surface of the plasma membrane. It has been proposed that the protein migrates to the nucleus where it acts as an inhibitory transcription factor for SGLT1 (Korn *et al.*, 2001). In contrast to the effect of cAMP on SGLT1, SGLT3 mRNA expression remains unchanged following treatment with cAMP (Clancey & Lever, 2000)

Activation of the protein kinase C pathway has also been demonstrated to affect SGLT-mediated glucose transport. Activation of this signalling

pathway by 1,2 dioctanoylglycerol (DOG) in oocytes, decreased the rate of sodium-dependent glucose transport and was accompanied by a reduction in the expression of SGLT1 protein in the plasma membrane (Hirsch *et al.*, 1996). Additionally, activation of protein kinase C (PKC) in cultured proximal tubule cells by 1,2-O-tetradecanolphorbol 13-acetate (TPA) (a synonym for PMA) has been shown to decrease mRNA levels of SGLT1 and SGLT3 (Clancey & Lever, 2000). Thus, it has been hypothesised that activation of the PKA and PKC signalling pathways regulates the expression of SGLT1 protein at the plasma membrane by exocytosis and/or endocytosis, respectively (Wright *et al.*, 1997).

Investigation into the regulation of SGLT1 by glucose has yielded conflicting results. Studies in the small intestine have shown that dietary glucose enhances SGLT-mediated glucose uptake both short-term (within 1 hour) and long-term (1-3 days) (Ferraris, 2001; Ferraris & Diamond, 1997). Short-term changes occur as a consequence of either insertion of additional cotransporter protein into the enterocyte BBM, or through changes in membrane potential difference (Sharp *et al.*, 1996). Long-term regulation occurs due to the functional expression of SGLT1 in a greater proportion of cells along the crypt-villus axis (Ferraris *et al.*, 1992). In contrast to the small intestine, long-term incubation of LLC-PK1 cells, a porcine proximal tubule kidney cell line, with high glucose concentrations results in a decrease in the mRNA (Ohta *et al.*, 1990) and protein expression (Handler & Moran, 1985) of SGLT1. Whether these differences reflect different regulatory mechanisms in distinct epithelial cell populations or that the changes in LLC-PK1 cells occurs as a consequence of the culturing of this cell type, has yet to be established.

1.3.4. Defects in SGLT proteins

Glucose galactose malabsorption (GGM) is a rare autosomal recessive disease first identified in 1962 (Lindquist B & Meeuwisse GW, 1962). To date, 200 people worldwide have been identified with the disorder (Wright, 1998). The disease is characterised by neonatal onset of severe, watery,

acidic diarrhoea, which is fatal within weeks unless lactose, glucose and galactose are removed from the diet. Interestingly, fructose absorption is unaffected in these patients (Wright *et al.*, 2002). Autoradiography of intestinal biopsies revealed that GGM patients display reduced galactose transport, which is accompanied by a 90% decrease in phlorizin binding sites (Schneider *et al.*, 1966). From these observations it was hypothesised that the disease resulted from a defect in SGLT-mediated glucose uptake. Studies from the laboratories of Wright and colleagues have confirmed this hypothesis, with mutations in the SGLT1 gene being responsible for either mistrafficking or misfolding of the protein, leading to reduced expression at the BBM (reviewed in (Wright, 1998)). Indeed, over 30 different mutations have been identified in GGM patients, demonstrating the susceptibility of the protein to minor modifications which lead to transport defects (Martin *et al.*, 1996). Mutations have also been identified that result in normal expression levels but the protein displays reduced affinity for glucose. This translocation defect arises from a missense mutation in residue Q457, which is localised close to the carboxyl terminus of the protein, an area known to be involved in sugar binding and translocation (Wright *et al.*, 2002).

Primary renal glucosuria is a rare disease that can be inherited in an autosomal recessive or autosomal dominant manner (Sankarasubbaiyan *et al.*, 2001). The incidence of the disease in the United States has been estimated at 0.16-6.3% of the population (Feld L.G., 2002). Patients with this disease present with normal blood glucose levels and normal oral glucose tolerance tests, but display isolated persistent glucosuria (Sankarasubbaiyan *et al.*, 2001). DNA sequence analysis has shown that the autosomal recessive form of the disease occurs due to a homozygous nonsense mutation in exon 11 of the SGLT2 gene, leading to the formation of a truncated SGLT2 protein. Such a mutation leads to the partial absence of TMH 10 and total absence of TMH 11-14, which abolishes the proteins ability to transport glucose (Elsas & Rosenberg, 1969; van den Heuvel *et al.*, 2002). This mutation in the SGLT2 isoform has therefore been proposed to be the cause of the persistent glucosuria seen in these patients.

1.4. Facilitative glucose transport

1.4.1. Gene and protein properties

The entry of glucose into most mammalian cells is catalysed by a family of sugar transporters with the gene symbol, SLC2A (solute carrier 2A) and protein symbol GLUT. These glucose transporters are part of a large superfamily of facilitative transporters, which includes organic anion and cation transporters and plant and bacteria hexose-proton symporters. Recently, additional members of this glucose transporter family have been identified based on sequence similarity, and a new GLUT numbering scheme has been proposed by Joost *et al.*, where the 13 known members of the GLUT family are divided into 3 subclasses. Class I is comprised of GLUT1 to GLUT4 on the basis of their specificity for glucose. Class II consists of the fructose specific transporter, GLUT5 and the related proteins GLUT7, GLUT9 and GLUT11, whilst class III is characterised by the lack of a glycosylation site in the first extracellular linker domain and includes GLUT6, GLUT8, GLUT10, GLUT12 and the recently cloned proton-myoinositol symporter, HMIT1 (Joost *et al.*, 2002; Joost & Thorens, 2001).

In renal tubules, a functional role for GLUT1, 2 and 5 has been established, therefore only the properties of these isoforms will be described in detail in this thesis (For reviews on the other isoforms see (Baldwin, 1993; Pessin & Bell, 1992). GLUT1 was first isolated and sequenced in 1985. Based on the predicted amino acid sequence, a model for the topology of the protein in the plasma membrane was proposed, consisting of 12 membrane spanning domains, with the NH_2 and COOH termini orientated intracellularly (Mueckler *et al.*, 1985). Human GLUT1 has been mapped to chromosome 1 p31.3-35 (Shows *et al.*, 1987), and codes for a protein of 492 residues with a calculated molecular weight of 54.2 kDa (Hruz & Mueckler, 2001). Using low stringency cross-hybridisation with the cDNA of GLUT1, Fukumoto *et al.* isolated and sequenced the gene for GLUT2. This gene was mapped to chromosome 3 q26.1-26.3 and codes for a 524 amino acid protein that has 55.5% homology to GLUT1 (Fukumoto *et al.*, 1988). Using a similar experimental approach the same group went on to clone the facilitative

fructose transporter, GLUT5 (Kayano *et al.*, 1990). As with GLUT1, GLUT5 was mapped to chromosome 1 in the region p22-p32, with the most likely location being p31, the gene was found to code for a protein of 501 amino acids, showing 41.7 and 40% homology to GLUT1 and GLUT2, respectively (Kayano *et al.*, 1990). In addition, GLUT2 and GLUT5 have a predicted membrane topology that display striking similarities to GLUT1 (Fukumoto *et al.*, 1988; Kayano *et al.*, 1990).

The three GLUT isoforms described above have also been isolated from other species. GLUT1 is highly conserved, showing 97-98% sequence homology between human, rat, rabbit, mouse and pig (Pessin & Bell, 1992). GLUT2 is less conserved, with 81% homology between human, rat and mouse, with the most divergent region being the extracellular domain that connects TMH1 and TMH2 (Pessin & Bell, 1992). Homology between species has also been established for GLUT5; rat GLUT5 shows 87.3% homology to the human transporter (Rand *et al.*, 1993) and rabbit GLUT5 has 72.3% and 67.1% homology to human and rat, respectively (Miyamoto *et al.*, 1994).

GLUT1 is the most ubiquitously distributed isoform; it is expressed in most tissues and cell types, although often at low levels in combination with other isoforms. Therefore, it is often considered to be responsible for basal cellular glucose uptake (Mueckler, 1994). GLUT2 is expressed at high levels in hepatocytes, pancreatic β-cells and the absorptive epithelia of the intestine and kidney (Fukumoto *et al.*, 1988; Thorens *et al.*, 1988). In hepatocytes and β-cells, GLUT2 is coupled to the expression of a high K_m glucokinase and this distinct expression pattern has been proposed to form a glucose sensing mechanism that responds to subtle changes in blood glucose levels (Unger, 1991). In the kidney and intestine, expression of GLUT2 is conventionally thought to occur exclusively at the BLM, where it is involved in the cellular efflux of glucose and fructose (Dominguez *et al.*, 1992). However, Kellett and colleagues have convincingly demonstrated that GLUT2 is also expressed at the enterocyte BBM (Affleck *et al.*, 2003; Kellett,

2001; Kellett & Helliwell, 2000). High levels of GLUT5 expression occur in the intestine (Castello *et al.*, 1995) and the S3 segment of the proximal tubule (Sugawara-Yokoo *et al.*, 1999). Expression at these sites occurs at the BBM and is consistent with a role in the handling of dietary fructose.

Thus, it has been established that different GLUT isoforms are expressed on either the apical or basolateral membrane of absorptive epithelia. However, the mechanisms involved in the selective targeting of the proteins to their specific membranes have not been fully clarified. Czech *et al.* have proposed that the cytoplasmic COOH-terminal sequence is involved in the insertion of GLUT1 into the plasma membrane (Czech *et al.*, 1993). However, Asano *et al.* demonstrated that the region consisting of amino acids 300-327 (located around TMH7) is essential for membrane targeting of GLUT1 (Asano *et al.*, 1992). Indeed, a further study by this group examining the apical and basolateral expression of GLUT1 and GLUT5 chimeras, revealed that the large intracellular loop between TMH6 and TMH7 is responsible for conferring membrane specificity (Inukai *et al.*, 1997). Differences in the results of these studies may be a consequence of the cell type used for the expression of the chimeras or the density at which they were expressed. In addition, it has been suggested that the GLUT transporters harbour one or more motifs that direct the protein to its specific cellular location (Joost & Thorens, 2001).

1.4.2. Transport properties of GLUT proteins

Many of the properties of the facilitative glucose transporters have been established using the *Xenopus* oocyte expression system. Variation in predicated K_m values and substrate specificity has been reported, which appears to be a consequence of species variation and experimental protocol (Table 1.2). K_m values for GLUT1 are generally quoted in the region of 5-10 mM. In contrast, values for GLUT2 reflect a low affinity transporter with a K_m of 15-25 mM (Bell *et al.*, 1993; Debnam & Unwin, 1996; Helliwell *et al.*, 2003). In both cases these values represent measurements made at zero trans-transport using 2-deoxy-D-glucose. The K_m value for GLUT5 mediated

Table 1.2. Published K_m values for glucose transport by GLUT1 and GLUT2; and fructose transport by GLUT5, obtained using the *Xenopus* oocyte expression system.

	GLUT1	GLUT2	GLUT5
Equilibrium exchange using 3-0 MG	17 mM (human) (a) 21.3 mM (human) (b) 20.1 mM (rat) (c) 26.2 mM (rat) (d)	42 mM (human) (a)	
Zero trans-transport using 2-DOG	6.9 mM (rat) (e)	17 mM (human) (e) 11 mM (human) (f) 13 mM (human (g)	6 mM (human) (h) 11.6 mM (rat) (I) 11 mM (rabbit) (j)

3-0 MG, 3-0-methylglucose; 2-DOG, 2-deoxy-D-glucose.

(a) (Gould *et al.*, 1991)
(b) (Keller *et al.*, 1989),
(c) (Gould & Lienhard, 1989),
(d) (Nishimura *et al.*, 1993),
(e) (Burant & Bell, 1992),
(f) (Colville *et al.*, 1993),
(g) (Pessin & Bell, 1992),
(h) (Burant *et al.*, 1992),
(i) (Inukai *et al.*, 1995),
(j) (Miyamoto *et al.*, 1994)

fructose uptake is generally quoted as being 6 mM, this value arising from the first study into the properties of human GLUT5 expressed in *Xenopus* oocytes (Burant *et al.*, 1992).

Facilitative glucose transporters have been shown to transport a variety of substrates (Table 1.3). In 1991, Gould *et al.* demonstrated that in oocytes expressing GLUT2, glucose transport could be inhibited by fructose (Gould *et al.*, 1991). This novel finding was confirmed to occur *in vivo* with a predicted K_m of 16.4 mM (Cheeseman, 1993). Colville *et al.* using human GLUT2 expressed in oocytes, went on to demonstrate that GLUT2 could also transport galactose, with the affinity of GLUT2 for these substrates differing considerably (K_m for glucose, 11.2 mM, fructose, 66.7 mM and galactose, 85.5 mM) (Colville *et al.*, 1993). GLUT1 has also been shown to transport galactose, with a K_m of 17.1 mM, but this transporter shows no affinity for fructose (Burant & Bell, 1992). GLUT5 is now widely considered to be involved in the uptake of fructose with the protein showing no affinity for any other substrate (Helliwell *et al.*, 2000a). Early studies using the *Xenopus* oocyte expression system demonstrated that GLUT5 is capable of transporting glucose (Miyamoto *et al.*, 1994; Rand *et al.*, 1993). However, further functional characterisation of the transporter using rabbit BBM vesicles revealed that this isoform does not transport glucose *in vivo* (Miyamoto *et al.*, 1994).

Phloretin and cytochalasin B inhibit facilitative hexose transport by GLUT1 and GLUT2, with GLUT5 being insensitive to both compounds (Miyamoto *et al.*, 1994). Independent researchers have established the K_i of cytochalasin B for GLUT1 and GLUT2. K_i values for GLUT1 are reported to be 0.1 μM (Burant & Bell, 1992; Zoccoli *et al.*, 1978) with values for GLUT2 ranging from 6.9 to 7.5 μM, reflecting the lower affinity of this isoform for this compound (Burant & Bell, 1992; Colville *et al.*, 1993).

Table 1.3. Gene and functional properties of facilitative glucose transporters.

	GLUT1	GLUT2	GLUT5
Gene localisation	1p31.5-35	3q26.1-26.3	1p31
Homology to GLUT1	—	55.5%	41.7%
amino acid residues	492	524	501
Molecular weight (kDa)	54	58	49-60
K_m D-glucose (mM)	5-10	15-25	6 (for fru)
Inhibitors	Phl & Cyt B	Phl & Cyt B	insensitive
Sugar specificity	glu > gal	glu > gal > fru	fru

Phl, phloretin; Cyt B, cytochalasin B; glu, glucose; gal, galactose; fru, fructose.

The mechanisms involved in the transport of glucose across the plasma membrane by the facilitative glucose transporters have not been fully established. A simple alternating conformational model has been proposed, whereby the GLUT transporter protein forms a channel that spans the membrane and contains two distinct glucose binding sites which can be presented to either the cytoplasm or to the external medium. Substrate binding induces a conformational change in the protein's structure, which results in reorientation of the binding site and subsequent release of the glucose molecule (Baldwin, 1993). Mutational studies have provided evidence that TMH7 and 11 may be in close proximity and constitute part of the channel through which the substrate is transported (Bell *et al.*, 1993). Inconsistency in some of the available kinetic data has prompted some researchers to reject this model in favour of more complex hypotheses. One such suggestion is the simultaneous-site model, in which GLUT1 proteins have been proposed to function as dimers, with each protein's binding site

presented to the opposite side of the membrane (Carruthers, 1990; Naftalin & Rist, 1994). A more recent proposal is the β-barrel model, whereby the generally accepted topology of the GLUT proteins has been challenged by the hypothesis that the GLUT proteins consist of β-sheets that are arranged in the membrane to form a β-barrel (Fischbarg & Vera, 1995). At present the extensive kinetic data compiled over the past 20 years fails to completely support any of the proposed models.

1.4.3. Regulation of facilitative glucose transport

The activity and expression level of GLUT1 is altered by a variety of different agents and conditions. GLUT1 is positively regulated by cAMP (Hiraki *et al.*, 1989), phorbol esters (Hiraki *et al.*, 1988), serum-derived growth factors (Hiraki *et al.*, 1988) and transforming growth factor β (TGF-β) (Inoki *et al.*, 1999; Kitagawa *et al.*, 1991). Conditions of cellular stress, such as hypoxia (Loike *et al.*, 1992), hyperosmolarity (Barros *et al.*, 2001; Hwang & Ismail-Beigi, 2001), calcium stress (Dominguez *et al.*, 1996) and mechanical stress (Gnudi *et al.*, 2003) have also been shown to increase GLUT1 expression. In some cells types, including proximal tubules (Dominguez *et al.*, 1994), glucose negatively regulates GLUT1 protein and mRNA expression (Tordjman *et al.*, 1990; Walker *et al.*, 1988; Walker *et al.*, 1989; Wertheimer *et al.*, 1991). However, in cultured mesangial cells, GLUT1 protein expression increases in relation to glucose concentration and has been proposed to be involved in the glucose induced cellular damage characteristic of diabetes mellitus (Heilig *et al.*, 1997). The intracellular signalling events involved in the adaptation of GLUT1 to these various stimuli have not been studied in detail, however, Barros *et al.* have proposed that p38 mitogen-activated (MAP) kinase, a stress sensitive member of the MAP kinase superfamily is involved in the regulation of GLUT1 (Barros *et al.*, 1997).

Studies into the regulation of GLUT2 and GLUT5 have concentrated mainly on the effect of dietary manipulation. It has been reported that the substrates

for GLUT2 and GLUT5 can positively regulate the expression and activity of these transporter proteins. GLUT2 is regulated by both glucose and fructose, whilst GLUT5 is regulated by fructose only (Burant & Saxena, 1994; Mesonero *et al.*, 1995). This data is consistent with the proposal that GLUT2 can transport both fructose and glucose (Burant & Bell, 1992; Colville *et al.*, 1993) whereas, *in vivo* GLUT5 only transports fructose (Burant *et al.*, 1992; Inukai *et al.*, 1995).

The regulation of GLUT2 by ingested sugars was originally thought to occur exclusively at the BLM, however, recent reports have shown that increased luminal glucose or fructose concentrations also promotes GLUT2 expression at the enterocyte BBM (Kellett & Helliwell, 2000) (Gouyon *et al.*, 2003a). Targeting of GLUT2 to the BBM can be mimicked by activation of the PKC signalling pathway, by phorbol 12-myristate (PMA) and blocked by the PKC inhibitor, chelerythrine, and has been shown to correlate with activation of PKC-βII (Helliwell *et al.*, 2000b). This PMA induced activation of PKC-βII occurs via phosphatidylinositol 3-kinase (PI 3-kinase)-dependent activation of 3-phosphoinositide-dependent protein kinase-1 (PDK-1), which in turn stimulates the phosphorylation of PKC-βII at Thr-500, leading to the autophosphorylation of Thr-641 and Ser-660. Phosphorylation of PKC-βII at these three sites locks the enzyme into a catalytically active conformation, which stimulates the insertion of GLUT2 into the BBM (Helliwell *et al.*, 2003).

From these novel findings Kellett and colleagues have proposed a model for the regulation of sugar absorption. The expression level and intrinsic activity of GLUT2 at the BBM is low at the early stages of digestion, therefore transport initially occurs via SGLT1. This sodium-dependent transport activates PKC-βII and causes contraction of the peri-junctional actomyosin ring, which in turn stimulates the insertion of GLUT2 containing vesicles into the BBM, together with the activation of transporter protein already present in the membrane (Kellett, 2001). Insertion of GLUT2 at the BBM results in increased transepithelial delivery of sugars to the circulation leading to raised plasma insulin levels. Increased plasma insulin subsequently stimulates the

activity of PI 3-kinase leading to enhanced formation of catalytically active PKC-βII. As absorption proceeds, luminal sugar levels progressively fall resulting in reduced transepithelial sugar absorption. This is paralleled by reduced plasma glucose and insulin levels and a decrease in membrane associated PKC-βII. Overall these affects culminate in loss of GLUT2 from the BBM and down-regulation of sugar absorption (Helliwell *et al.*, 2003).

Other major signalling pathways have also been implicated in the regulation of GLUT2 and GLUT5 at the BBM. Activation of the extracellular signal-regulated kinase (ERK) / MAP kinase pathway has little effect on the overall rate of fructose transport, yet alterations in the intrinsic activity of GLUT2 and GLUT5, coupled with a reduction in the expression level of GLUT5 have been demonstrated. In contrast, activation of the p38 MAP kinase pathway significantly increases the rate of fructose transport as a result of a doubling of GLUT2 expression and intrinsic activity, with little effect on GLUT5 (Helliwell *et al.*, 2000a). *In vivo* these pathways would potentially work in synergy, indeed Helliwell *et al.* have demonstrated that the ERK MAP kinase pathway restrains the activity of the p38 MAP kinase pathway (Helliwell *et al.*, 2000a). This may be of relevance in conditions of hyperglycaemic stress, such as type 1 diabetes. In this condition the stress-activated pathway, p38 MAP kinase would be activated, in addition insulin levels would be low and therefore activation of the ERK pathway would be minimal. Thus, the ERK pathway would provide little restraint of the p38 pathway, resulting in significant activation of GLUT2 at the BBM. In conclusion, regulation of GLUT2 and GLUT5 at the enterocyte BBM occurs via a number of major signalling pathways, however the impact of these pathways on BLM expression of GLUT2 have yet to be established.

In addition to a effect of dietary sugars, increased expression of GLUT5 has also been reported during starvation (Castello *et al.*, 1995). Early studies into the regulation of GLUT5 proposed a role of enhanced cellular cAMP levels (Brot-Laroche, 1996; Mesonero *et al.*, 1995). Indeed, Mahraoui *et al.* demonstrated that increased cellular cAMP levels, resulting from treatment of

Caco-2 cells with forskolin, induced stabilisation of GLUT5 mRNA levels via the interaction of a unidentified cytoplasmic protein with a *cis*-acting element in the UTR of the mRNA (Mahraoui *et al.*, 1994). A recent study has confirmed that the half-life of GLUT5 mRNA is correlated with intracellular cAMP levels and that binding of different RNA/protein complexes, induced by various conditions, act as stabilising or destabilising factors regulating the translation of the GLUT5 mRNA (Gouyon *et al.*, 2003b). In contrast to the effect of cAMP on GLUT5 regulation, glucose induced accumulation of GLUT2 mRNA in hepatocytes is reduced by cAMP, possibly via the presence of a repressor element within the GLUT2 promoter sequence (Rencurel *et al.*, 1997). In keeping with this finding, elevated intracellular cAMP levels also decreases the expression of GLUT2 at the BLM of Caco-2 cells (Brot-Laroche, 1996) and isolated rat enterocytes (Williams & Sharp, 2002). Vascular infusion of gastric inhibitory peptide (GIP) and glucagon-like peptide 2 (GLP-2) have been shown to promote trafficking of GLUT2 protein to the enterocyte BLM, with glucagon-like peptide 1 (GLP-1) having no affect (Cheeseman & O'Neill, 1998). In addition, vascular infusion of GLP-2 has also been reported to enhance fructose uptake at the BBM via insertion of GLUT2 protein (Au *et al.*, 2002).

1.4.4. Defects in GLUT proteins

In 1991, De Vivo *et al.* first described a condition where infants displayed a reduction in erythrocyte GLUT1 protein expression (De Vivo *et al.*, 1991). Klepper *et al.* went on to report this condition, termed glucose transporter protein syndrome, in a further 15 patients, with the symptoms including infantile seizures, impaired development and hypoglycorrhachia (reduced levels of glucose in the cerebrospinal fluid) (Klepper *et al.*, 1999). Glucose transport studies in erythrocytes isolated from these patients, revealed a 50% reduction in the rate of glucose transport compared with parents or normal controls (Klepper *et al.*, 1999), which in some cases was accompanied by a reduction in GLUT1 immunoreactivity (De Vivo *et al.*, 1991). DNA sequencing of these patients has identified a missense mutation

in the GLUT1 gene, therefore confirming the molecular basis of the disease (Klepper *et al.*, 1999).

Fanconi-Bickel syndrome (FBS) is a rare autosomal recessive condition, with 82 cases of the disease being documented in the literature (Santer *et al.*, 1998). Patients present during infancy with a failure to thrive, rickets, hepatorenal glycogen accumulation leading to enlargement of the liver and kidney, postprandial hyperglycaemia, fasting hypoglycaemia and hypergalactosaemia. Renal proximal tubular dysfunction is also apparent, presenting as glycosuria, hyperaminoaciduria, hyperphosphaturia, hypercalciuria, renal tubular acidosis and polyuria (Santer *et al.*, 1998). Following advances in the characterisation of the facilitative glucose transporters it was proposed that mutations in GLUT2 protein were responsible for the pathophysiology of the disease. This hypothesis was confirmed by the identification of 3 mutations in the GLUT2 gene of patients with FBS (Santer *et al.*, 1997), which would lead to the translation of truncated GLUT2 protein with reduced functional properties. Indeed, more than 30 different mutations in the GLUT2 gene of patients with FBS have been identified with no single mutation showing prevalence (Santer *et al.*, 2002; Yoo *et al.*, 2002).

1.5. Diabetes

1.5.1. Clinical implications

Diabetes mellitus is a chronic disease characterised by hyperglycaemia which leads to complications that include diseases of the eye (retinopathy), nerves (neuropathy) and kidney (nephropathy).

There are two common forms of diabetes mellitus. Type 1 diabetes, which accounts for 5-10% of cases (Olefsky, 2001), usually occurs in children or young adults and is also termed insulin-dependent diabetes mellitus (IDDM) or juvenile–onset diabetes. The disease is caused by the auto-immune destruction of the insulin producing β-cells of the pancreas and ultimately

leads to failure of the pancreas to secrete insulin (Eisenberth GS *et al.*, 1994).

The second form of diabetes mellitus is type 2, also known as non-insulin-dependent diabetes mellitus (NIDDM) or mature-onset diabetes. Type 2 diabetes accounts for 90-95% of all patients with diabetes and is increasing in prevalence (Olefsky, 2001). This disease generally occurs in people over the age of 40 and although there is evidence for a genetic link, environmental risk factors such as obesity, a sedentary lifestyle and ageing also play a role (Olefsky, 2001). In this type of diabetes the pancreatic β-cells remain intact and continue to secrete insulin, however, there is a reduction in the expression of the insulin receptor in the insulin-sensitive cells of the body, resulting in a reduced response to the hormone even if it is in abundance, a situation known as insulin resistance (Alberti *et al.*, 1992).

The common symptoms associated with both type 1 and 2 diabetes are hyperglycaemia, polyuria, persistent thirst, unusual weight loss, lethargy, blurred vision, tingling or numbness in hands and feet, cuts and bruises that are slow to heal and frequent, recurring infections (Watkins PJ, 1983).

Treatment of type 1 diabetes involves the administration of insulin by injection, however recently, more patient-friendly methods of administration are being researched and are undergoing clinical trials, including inhaled, buccal and oral application (Owens *et al.*, 2003). Diet and exercise are also important factors that aid glycaemic control. The principles of the diet of a type 1 diabetic patient is that sugar should be avoided except for the treatment of hypoglycaemia, and carbohydrate intake is steady from day to day. Meals should be taken at regular times, with snacks taken between meals to avoid hypoglycaemic episodes (Watkins PJ, 1983).

Treatment of type 2 diabetes, in the first instance, involves changes in dietary habits, such that the amount and frequency of carbohydrate intake is carefully regulated and sugar is completely eliminated from the diet. Since a

high proportion of type 2 diabetic patients are obese, weight loss through exercise and reduced calorific intake can lessen insulin resistance (Alberti *et al.*, 1992). Oral hypoglycaemic drugs are given only when dietary treatment has failed (Watkins PJ, 1983).

There are two types of oral hypoglycaemic drugs, with different modes of action. Sulphonamides act by stimulating the release of insulin from the pancreatic β-cells (Alberti *et al.*, 1992). They are generally the preferred drug for treatment of type 2 diabetes as they are the most potent, with few side effects (Watkins PJ, 1983). In contrast biguanides act by reducing hepatic glucose production (Watkins PJ, 1983), they are occasionally used as a first line of treatment in grossly obese patients, chiefly because they can help with weight reduction. Unlike the sulphonamides, this class of hypoglycaemic drugs can have unpleasant side effects such as nausea, diarrhoea and vomiting (Watkins PJ, 1983).

Patients with diabetes have a life expectancy of approximately 15 years less than those without the disease (Olefsky, 2001), and due to its complications, causes an increased rate of morbidity. For example, diabetic nephropathy accounts for 40% of all new cases of end stage renal disease (Ritz & Orth, 1999) and diabetic retinopathy is the leading cause of blindness in adults aged between 20 and 74 (Ferris, III *et al.*, 1999). Heart disease and strokes occur 2-4 times more frequently in diabetic patients than in those who are healthy (Olefsky, 2001).

The Diabetes Control and Complications Trial (The Diabetes Control and Complications Trial Research Group, 1993) demonstrated that strict control of blood glucose levels prevents and retards development of diabetic retinopathy, nephropathy and neuropathy. The DCCT findings showed that intensive control of blood glucose levels reduced the risk of retinopathy by 76%, nephropathy by 50% and neuropathy by 60% (National kidney and urologic diseases Information Clearinghouse, 2001). Hypertension is also a major factor in the progression of diabetic complications. The UK

Prospective Diabetes Study (UKPDS) (UK Prospective Diabetes Study Group, 1998) and the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (Opie, 1998) have demonstrated that stringent control of blood pressure, below 130/85 mm Hg, is beneficial in slowing the development and progression of diabetic nephropathy and retinopathy. Intensive blood pressure control was also found to reduce the risk of coronary heart disease in diabetic patients.

1.5.2. Pathogenesis of diabetic nephropathy

Diabetes is the most common cause of end stage renal failure (National kidney and urologic diseases Information Clearinghouse, 2001). Patients with type 1 diabetes are at a higher risk of developing renal complications than those with type 2 diabetes. Statistics show that 40% of type 1 diabetic patients develop severe nephropathy and kidney failure by the age of 50, whilst 10-20% of type 2 patients develop renal complications (National kidney and urologic diseases Information Clearinghouse, 2001).

Five stages leading to diabetes induced renal failure have been described (National kidney and urologic diseases Information Clearinghouse, 2001).

Stage I: Renal blood flow increases (hyperfiltration) and the kidneys become enlarged (hypertrophic). Patients can remain in this stage indefinitely or advance to stage 2 after a number of years.
Stage II: Glomerular damage starts to become apparent, with albumin being detected in the urine (microalbumunuria). Patients may remain at this stage for many years especially if blood pressure and blood glucose levels remain well controlled.
Stage III: The kidneys progressively lose the ability to filter waste products. Again patients can remain at this stage for many years.
Stage IV: This stage is referred to as 'advanced clinical nephropathy'. The glomerular filtration rate decreases dramatically and large amounts of protein appear in the urine. High blood pressure almost always occurs.

Stage V: The final stage of kidney failure. Patients require dialysis or transplantation.

For patients with type 1 diabetes the average length of time from onset of kidney disease to stage IV is 17 years, whilst the average length of time to progress to renal failure is 23 years (National kidney and urologic diseases Information Clearinghouse, 2001). Progression of kidney failure may occur more rapidly (5-10 years) in patients with untreated high blood pressure (National kidney and urologic diseases Information Clearinghouse, 2001).

1.5.2.1. Diabetic glomerulosclerosis and tubulointerstitial fibrosis

Glomerulosclerosis is defined by fibrotic scarring of the glomerulus leading to proteinuria. Scaring occurs as a consequence of accumulation of fibronectin, laminin and collagen, which leads to mesangial matrix expansion and thickening of the glomerular basement membrane (Wolf & Thaiss, 1995). Tubulointerstitial fibrosis is characterised by accumulation of extracellular matrix (ECM) proteins, such as fibronectin and collagen, within the tubular interstitium leading to tubulointerstitial scaring (Norman & Fine, 1999).

There is evidence from both type 1 (Osterby & Gundersen, 1975) and type 2 diabetic patients (Rodby, 1997) and from the streptozotocin (STZ)-induced, type 1 animal model of diabetes (Seyer-Hansen, 1976), that glomerular and tubuloepithelial hypertrophy and basement membrane thickening are among the earliest pathological alterations in diabetes. Since the tubulointerstitium comprises the bulk of the kidney, tubular hypertrophy is quantitatively responsible for most of this renal enlargement (Nath, 1992; Wolf, 1995).

The various cell types along the nephron can behave differently during diabetes depending on their genetic makeup, local concentrations of growth factors and the presence and expression levels of the receptors for these growth factors (Schwieger & Fine, 1990). For example, *in vivo* and cell culture experiments, where cells are cultured under conditions of high glucose, show that in the glomerulus there is early, self limited, mesangial

cell proliferation, followed by development of hypertrophy. Whereas proximal tubule cells undergo hypertrophy, as defined by increased cell size and by stimulation of protein synthesis and collagen production, without proliferation (Wolf & Ziyadeh, 1999). Hypertrophy of the glomerulus and proximal tubule cells precedes, and may contribute, to the renal abnormalities that occur at the later stages of diabetes, such as glomerulosclerosis, tubular atrophy and interstitial fibrosis (stage II-V) (Ziyadeh, 1993). This occurs partly because the network of cytokines and growth factors that induce cellular hypertrophy can also stimulate extracellular matrix synthesis and deposition (El Nahas AM, 1992; Wolf & Ziyadeh, 1997).

Cell culture systems have enabled researchers to investigate the possible mechanisms by which high extracellular glucose results in cellular damage. *In vivo* studies, using experimental animal models of both type 1 and type 2 diabetes, have confirmed most of the *in vitro* findings. Specifically, exposure of mesangial cells to high glucose has been shown to promote thickening of the basement membrane and production of extracellular matrix components (Steffes *et al.*, 1989). This occurs by transcriptional activation of type IV collagen and fibronectin genes which leads to increased synthesis of the two proteins (Ayo *et al.*, 1990; Fumo *et al.*, 1994; Nahman, Jr. *et al.*, 1992). There is also evidence that exposure to high glucose concentrations reduces the degradation of these matrix components (McLennan *et al.*, 1994). In proximal tubule cells, hypertrophy is accompanied by an increase in the thickness of the tubular basement membrane (TBM). Unlike the glomerulus, it appears that exposure of human proximal tubule cells to elevated glucose has no effect on collagen or fibronectin gene expression but results in an alteration in their degradative pathways (Phillips *et al.*, 1999b).

1.5.2.2. Mechanisms of hyperglycaemia-induced glomerular and tubular cell damage.

It is now well established that hyperglycaemia is a key factor involved in the pathogenesis of diabetic nephropathy. Four major hypotheses as to how cellular damage occurs have been proposed.

1) Formation of reactive oxygen species
2) Increased activation of aldose reductase (polyol pathway)
3) Activation of protein kinase C (PKC) isoforms
4) Increased formation of advanced glycation end products (AGE's).

1.5.2.2.1. Role of reactive oxygen species (ROS) in the pathogenesis of diabetic nephropathy.

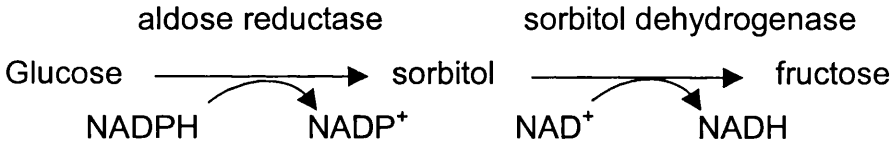
There have been a number of studies that support a role of ROS in the pathogenesis of diabetic nephropathy. Glomerular and tubule cells have been shown to generate ROS with the potential to cause oxidative stress (Baud *et al.*, 1992; Gwinner *et al.*, 1998). Additionally, glucose induced synthesis of TGF-β and subsequent ECM protein production can be suppressed by antioxidant administration, thereby preventing glomerular and tubular hypertrophy. Additionally, albuminuria and hyperfiltration, resulting as a consequence of diabetes, can also be normalised by antioxidant treatment (Ha & Lee, 2000).

Free radicals can be produced by a number of factors. Hydrogen peroxide (H_2O_2) production occurs during oxidation of enediols formed from Amadori products (Nagaraj *et al.*, 1994). Superoxide (O_2) formation can result from mitochondrial oxidation of NADH to NAD^+ (Freeman & Crapo, 1982) or as a consequence of the formation of prostaglandin (Kukreja *et al.*, 1986). Both H_2O_2 and hydroxyl radicals (OH') may then be produced from the accumulated O_2. In many cell types, ROS are also generated as secondary messengers during signal transduction by agonists implicated in diabetic complications such as angiotensin II (Wolf, 2000), platelet derived growth factor (Wolf & Ziyadeh, 1999) and advanced glycation end products (Ha & Lee, 2000). Oxidative stress occurs not only as a consequence of ROS production but also as a result of impaired scavenging of free radicals (Dunlop M, 2000).

It appears that under conditions of hyperglycaemia, there is an interaction between PKC and ROS. PKC activation can enhance ROS production and ROS have also been shown to increase PKC activity, through increased production of diacylglycerol (DAG) (Nishikawa *et al.*, 2000). Inhibition of PKC has been shown to block fibronectin accumulation in mesangial cells resulting from both H_2O_2 and high glucose environments (Ha & Lee, 2000).

1.5.2.2.2. Involvement of the polyol pathway in the pathogenesis of diabetic nephropathy.

Metabolism of glucose by the polyol pathway consists of two reactions, using the enzymes aldose reductase and sorbitol dehydrogenase.



Firstly, glucose is reduced to sorbitol using NADPH as the hydrogen donor and results in a decrease in the NADPH/$NADP^+$ ratio. Secondly, sorbitol is oxidised to fructose using NAD^+ as the hydrogen acceptor, which leads to an increase in the NADH/NAD^+ ratio. The enzyme aldose reductase has a low affinity for glucose; therefore, at normal glucose concentrations this pathway only represents a small proportion of glucose metabolism. However, the involvement of this pathway during hyperglycaemia increases dramatically. In fact, the formation of sorbitol from glucose, by the polyol pathway, in tissues of diabetic patients was recognised 30 years ago (Gabbay, 1973).

An early study in normal rats showed the highest levels of aldose reductase immunoreactivity is found in the medulla, with relatively little being observed in the cortex (Ludvigson & Sorenson, 1980). In the cells of the medulla it has been proposed that sorbitol may function as part of the organic osmolyte defence against extracellular solute fluctuations. However, it appears from cell culture studies, using cultured mesangial and proximal tubular cells that during conditions of hyperglycaemia accumulation of sorbitol also occurs in

the cortex. Experiments using human proximal tubule cells exposed to 25 mM D-glucose have demonstrated increased glucose metabolism by the polyol pathway resulting in accumulation of intracellular sorbitol and production of fibronectin. This fibronectin generation was shown to be dependent on the polyol pathway as addition of sorbinil, an aldose reductase inhibitor, led to a reduction in the fibronectin concentration (Phillips *et al.*, 1999b). Additionally, studies using the STZ-induced animal model of diabetes have shown that administration of sorbinil, at the time of induction of diabetes, normalises the expected increase in glomerular filtration rate (GFR) and urinary albumin excretion (Larkins & Dunlop, 1992).

Four mechanisms have been proposed by which increased glucose metabolism by the polyol pathway may contribute to the complications of diabetic nephropathy (Dunlop M, 2000; Larkins & Dunlop, 1992).

a) Osmotic changes due to the accumulation of sorbitol.

The intracellular accumulation of sorbitol, or indeed other polyols, could induce cellular hypertonicity, resulting in an influx of fluid and changes in membrane permeability and cellular function. In mesangial cells, accumulated sorbitol has been proposed to alter cellular myoinositol levels and reduce Na^+/K^+-ATPase activity, both of which would have potentially detrimental effects in diabetes (Dunlop M, 2000). However, the hypothesis of polyol accumulation *per se* being the sole cause of cellular damage seems unlikely, as in human proximal tubule cells, although galactose metabolism leads to the production of its polyol, galactiol, it does not lead to the accumulation of fibronectin (Phillips *et al.*, 1999b).

b) PKC activation as a result of increased polyol pathway activity.

When sorbitol is metabolised to fructose the ratio of $NADH/NAD^+$ is increased. One consequence of an alteration to this ratio is an increase in *de novo* DAG generation and subsequent activation of PKC (DeRubertis & Craven, 1994).

The consequences of increased PKC activity are discussed in section 1.5.2.2.3.

c) Free radical production as a result of increased polyol pathway activity

An increase in the cytosolic NADH/NAD^+ ratio as a consequence of the conversion of sorbitol to fructose can lead to an increase in free radical production (Williamson *et al.*, 1993). In addition, increased NADH levels can promote growth factor release and nitric oxide (NO) production. It has been proposed that the increase in NO may be responsible for glomerular hyperfiltration and hyperfusion seen in early diabetic nephropathy (Wolf & Thaiss, 1995).

d) Increased fructose formation due to polyol pathway activation

Fructose, the second product of the polyol pathway has been shown to be increased several fold in tissues with an activated polyol pathway. Its accumulation has been demonstrated to contribute to non-enzymatic fructosylation of proteins and results in the production of 3-deoxyglucosone, a precursor to advanced glycation end products (Niwa, 1999). Therefore accumulation of fructose may provide a link between the polyol pathway and non-enzymatic glycation.

These proposed mechanisms are not mutually exclusive; it is possible that interactions occur between them, which leads to hyperglycaemia-induced renal damage as a result of increased polyol pathway activity.

1.5.2.2.3. Involvement of PKC activation in diabetic nephropathy.

PKC comprises a family of serine threonine kinases that can be activated reversibly by the neutral lipid diacylglycerol (DAG). There are at least 12 isoforms of PKC, which can be divided into 3 subfamilies (Table 1.4). Under normal cellular conditions activation of PKC plays important roles in

transducing biological events including cell growth, differentiation, apoptosis and cellular responses to environmental stress.

Table 1.4. Classification of PKC isoforms and their regulatory mechanisms.

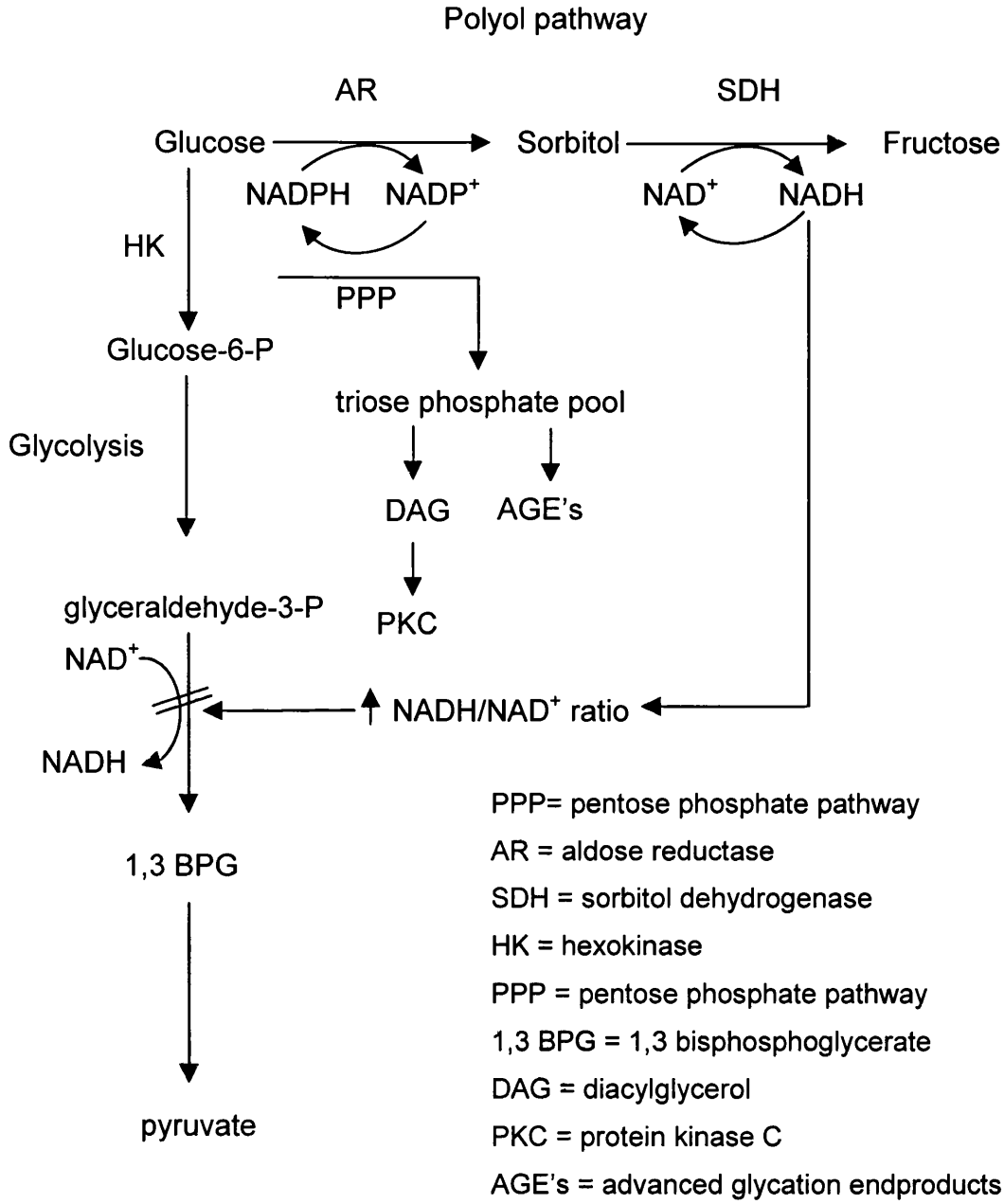
PKC Subfamily	Isoforms	Regulatory mechanisms
Classical	α, βI, βII, γ	DAG, Ca^{2+}, Phosphatidylserine
Novel	δ, ε, η, θ	DAG, Phosphatidylserine
Atypical	ι, λ, ζ	Phosphatidylserine, not fully classified

The direct synthesis of DAG from glucose has been demonstrated in a variety of tissues including pancreatic islet cells (Dunlop & Larkins, 1985), retina (Lee *et al.*, 1989) and glomeruli isolated from diabetic rats (Craven & DeRubertis, 1989). This direct synthesis of DAG occurs by conversion of triose phosphate intermediates produced when glucose-6-phosphate is metabolised by glycolysis or the pentose phosphate pathway (PPP) (Dunlop M, 2000). Increased throughput of glucose to the PPP occurs by two mechanisms. Under normal cellular conditions NADPH inhibits the PPP, however when exposed to a hyperglycaemic environment there is an increase in the activity of aldose reductase which results in a reduction in the cellular NADPH levels. This provides a constant throughput of glucose to produce PPP intermediates. Further flux through this pathway occurs due to a decrease in the NAD^+ levels, resulting from conversion of sorbitol to fructose, which inhibits the NAD^+ requiring enzyme glyceraldehye-3-phosphate, preventing 1,3 bisphosphoglycerate (1,3 BPG) production by glycolysis (Fig. 1.3).

DAG is the regulatory stimulator of PKC and its elevation is the presumed mechanism for the increased PKC activity observed in several tissues obtained from diabetic animals or cells exposed *in vitro* to high glucose concentrations (Larkins & Dunlop, 1992). In elucidating the cellular mechanisms involved in PKC activation, Haneda *et al.* have demonstrated *in*

vivo and *in vitro* that the DAG-PKC-ERK (extracellular regulatory protein kinase) pathway is involved in the cellular damage that occurs during hyperglycaemic conditions (Haneda *et al.*, 2001). ERK is a member of the mitogen activated protein (MAP) kinase family. Incubation of mesangial cells under conditions of high glucose in the presence of PD98059, a specific inhibitor of MAPK/ERK kinase (MEK), which is an upstream kinase activator of ERK, has been shown to inhibit the glucose induced production of TGF-βI and the ECM proteins, collagen (type I) and fibronectin (Isono *et al.*, 2000). Further involvement of this pathway has been demonstrated using animal models of diabetes. Thiazolidinedione compounds, such as troglitazone and pioglitazone, administered to STZ-diabetic animals prevented the activation of PKC by reducing DAG content as a result of DAG kinase activation (Haneda *et al.*, 2001). This reduction in the cellular content of DAG prevented the activation of PKC and ERK. Furthermore, these compounds lead to prevention of glomerular hyperfiltration and normalised the expression of TGF-β mRNA and protein and that of the ECM proteins (Isshiki *et al.*, 2000). Administration of LY333531, a PKC-β inhibitor, to db/db mice (model of type 2 diabetes) resulted in a decrease in PKC activation and, using histological methods, was shown to reduce fractional mesangial volume (Koya *et al.*, 2000). Therefore, activation of DAG-PKC-ERK pathway both *in vivo* and *in vitro* results in functional abnormalities and histological changes characteristic of diabetic nephropathy.

Figure.1.3. Diacylglycerol production as a consequence of glucose metabolism



Metabolism of glucose by the polyol pathway alters the ratio of $NADP^+$/NADPH and NADH/NAD^+. Increased utilisation of NADPH drives the PPP, whereas increased formation of NADH can inhibit the formation of 1,3 BPG resulting in an increase in the triose phosphate pool. (Adapted from (Dunlop M, 2000)

The PKC isoforms α, βI, βII, δ, ε and ζ have been shown to be expressed in mesangial cells. Glomeruli isolated from STZ-diabetic animals display an increase in membrane-associated PKC-δ and ε, whilst the levels of PKC-β1 are decreased. In cortical tubules isolated from these animals, total PKC-δ and ε also increased (Ha *et al.*, 2001). This finding suggests that diabetes-induced alterations in the various PKC isoforms affect the function of cortical tubules as well as the more established effects on the glomerulus.

PKC activation has been shown to occur via a number of pathways. Activation can occur as a consequence of the polyol pathway, whereby inhibition of aldose reductase *in vitro* has been demonstrated to reduce cellular DAG content and inhibit the translocation of PKC-δ and ε (Kapor-Drezgic *et al.*, 1999). However, this does not affect PKC-α and β isoforms, suggesting that these are activated by a mechanism independent of the polyol pathway (Whiteside & Dlugosz, 2002). There is increasing evidence that ROS can directly and indirectly activate PKC during conditions of hyperglycaemia (Ha & Kim, 1999). ROS may directly activate PKC by the induction of redox changes in the sulfhydryl groups on the cysteine rich regions of the PKC isozyme (Gopalakrishna & Jaken, 2000). Ha and co-workers have demonstrated that the antioxidant, taurine, can inhibit the alterations in PKC-δ and ε in cortical tubules and glomeruli isolated from STZ-diabetic animals (Ha *et al.*, 2001). Redox changes may also render individual isoforms more responsive to DAG activation during signal transduction (Gopalakrishna & Jaken, 2000). Indirect activation of PKC-α, δ and ε has been shown to occur by activation of vasoactive peptides, specifically angiotensin II and endothelin 1 (Whiteside & Dlugosz, 2002). Proteins rich in AGE's have also been shown to selectively activate mesangial cell PKC-βII through a mechanism involving oxidative stress without the involvement of DAG (Scivittaro *et al.*, 2000). In this context glycated albumin stimulates PKC-β which leads to an increase in collagen IV protein expression (Cohen *et al.*, 1999).

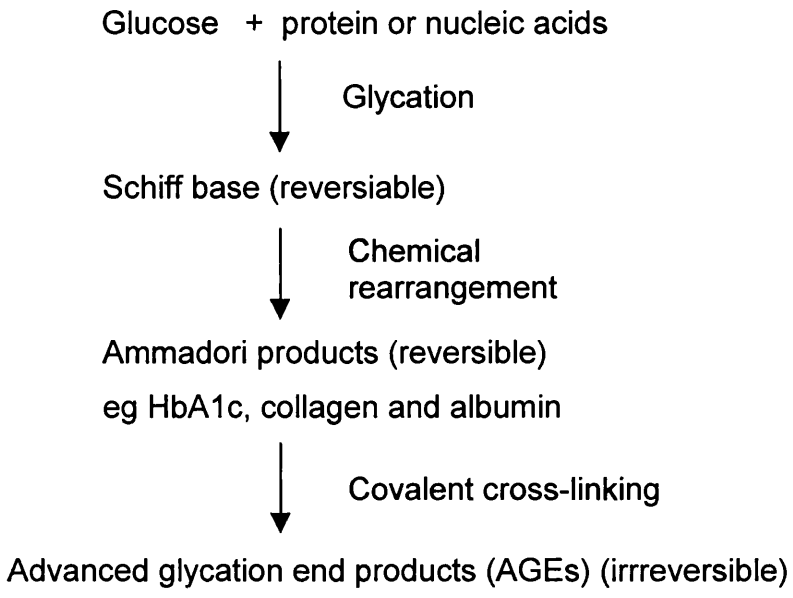
In summary, activation of PKC has been shown to result in increased production of cytokines, such as TGF-β, and extracellular matrix proteins, such as collagen and fibronectin (Murphy *et al.*, 1998). PKC activation has also been shown to increase prostaglandin synthesis by glomeruli (Xia *et al.*, 1995). Overall, these changes may play a role in renal basement thickening, hyperfiltration and increased permeability seen during diabetes.

PKC-β seems to be the major pathogenic PKC isoform and an inhibitor specific for this isoform (LY333531) is presently being tested in clinical trials (Whiteside & Dlugosz, 2002). Other isoform specific PKC inhibitors may also prove useful in the treatment and prevention of diabetic nephropathy.

1.5.2.2.4. Formation of Advanced Glycation End Products (AGE's) as a consequence of hyperglycaemia.

Glucose has been shown to form glycation products with extra and intracellular proteins to produce Schiff bases. This occurs by a non-enzymatic process whereby the N-terminal amino acids, preferentially the lysine and valine residues are glycosylated. These Schiff bases can then spontaneously transform to form more stable Amadori products. These early glycation products can undergo an irreversible, complex series of biochemical events, to form advanced glycation end products (AGE's) (Fig. 1.4.), collectively these reactions are termed the Maillard reaction (Alberti *et al.*, 1992). Ageing in non-diabetic patients is associated with progressive accumulation of AGE's but, due to the hyperglycaemia associated with diabetes, the process of AGE accumulation is greatly accelerated in this condition (Larkins & Dunlop, 1992).

Figure 1.4 Formation of AGEs via the Maillard reaction.



The intracellular production of AGE precursors can damage target cells via three mechanisms. Firstly, intracellular protein glycation can alter protein function; secondly, extra cellular matrix (ECM) modified by AGE precursors has abnormal functional properties; and thirdly, the precursors can bind to AGE receptors on adjacent macrophages, resulting in ROS production and subsequent release of cytokines and hormones (Cochrane & Robinson, 1995). Mesangial cells have also been shown to express AGE receptors. Stimulation of these receptors has been shown to result in the release of different cytokines and the subsequent production of extracellular matrix. Furthermore, injection of AGE's into normal mice has been shown to increase mesangial cell area and stimulate TGF-β mRNA synthesis and extracellular type IV collagen production (Yang *et al.*, 1994). Further evidence for the involvement of AGE's in diabetic nephropathy comes from experiments using aminoguanidine, an inhibitor of AGE formation, which has been shown to prevent basement membrane thickening in diabetic rats (Brownlee *et al.*, 1988). In fact, this phenomenon can be mimicked using aldose reductase inhibitors, therefore providing a link between the polyol

pathway and non-enzymatic glycation. Non-enzymatic glycation increases the rate of free radical production by nearly 50 fold (Mullarkey *et al.*, 1990), demonstrating how these pathways can be inter-linked in their potential to cause cellular damage. It has also been hypothesised that AGE's can develop in DNA strands which would result in long-lasting phenotypic changes of the cells (Wolf & Thaiss, 1995).

1.5.2.2.5. Pathway interactions

At present it is unknown exactly how the mechanisms described above interact to cause diabetic nephropathy. Nishikawa *et al.* proposed that a potential relationship might exist between the polyol pathway-induced redox changes and the three other mechanisms. Increased oxidative stress as a result of activation of the polyol pathway leads to an increase in the triose phosphate pool, which in turn results in increased DAG production and subsequent PKC activation, together with an increase in AGE precursors. An inconsistency in this hypothesis is the fact that some major target cells, such as endothelial cells, display relatively low aldose reductase activity and that aldose reductase inhibitors fail to reduce the hyperglycaemia induced increase in DAG concentration (Nishikawa *et al.*, 2000). This group also proposed that the link between these 4 mechanisms lie in the over production of reactive oxygen species, whereby, ROS activates aldose reductase, inducing DAG accumulation and subsequent PKC activation, together with an increase in AGE production (Nishikawa *et al.*, 2000).

Recent evidence suggests that the underlying factor in the progression of diabetic nephropathy may be increased production of the cytokine TGF-β. As described above, the activation of PKC and the formation of ROS and AGE's all lead to the accumulation of TGF-β. There is also extensive evidence from both *in vivo* and *in vitro* studies and studies on human diabetic patients that TGF-β is involved in the development of renal hypertrophy (Wolf & Ziyadeh, 1999). In fact, proximal tubule cells isolated from TGF-β

knockout mice failed to undergo hypertrophy and collagen synthesis when cultured under conditions of high glucose (Wolf & Ziyadeh, 1999).

Further evidence for the involvement of this cytokine in diabetic renal growth and ECM accumulation comes from the demonstration that short-term treatment (9 days) of STZ diabetic mice with anti-TGF-β antibody reduced glomerular hypertrophy and therefore prevented the early manifestations of diabetic renal damage (Sharma *et al.*, 1996). Chronic administration (8 weeks) of anti-TGF-β antibody to db/db mice demonstrated that matrix expansion was prevented as a result of the decreased expression of type IV collagen and fibronectin. Plasma levels of TGF-β were returned to that of the control animals and perhaps most interesting was the normalisation of GFR (Ziyadeh *et al.*, 2000).

Further evidence for the involvement TGF-β in renal hypertrophy comes from *in vitro* and *in vivo* application of antisense TGF-β oligodeoxynucleotides. *In vitro* incubation of proximal tubule cells with TGF-β antisense reduced high glucose stimulated TGF-β1 secretion and protein synthesis. *In vivo* administration of the antisense to STZ-diabetic mice by osmotic minipumps reduced renal TGF-β1 protein content, levels of type IV collagen and fibronectin and lead to a reduction in renal hypertrophy (Han *et al.*, 2000).

Another interesting consequence of increased production of TGF-β, is its ability to stimulate an increase in GLUT1 mRNA and protein expression, leading to an increase in glucose uptake by mesangial cells (Inoki *et al.*, 1999). Therefore, increased levels of endogenous TGF-β, produced by high glucose activation of PKC, AGE's and ROS may in turn stimulate glucose uptake and exacerbate the metabolic alterations associated with diabetic nephropathy. It could therefore be hypothesised that the TGF-β system is involved in the initial and progressive stages of diabetic renal disease in both type 1 and type 2 diabetes. The fact that diabetic nephropathy was almost completely abolished by anti-TGF-β antibody treatment without reducing blood glucose levels suggests that the impact of the other effects of

hyperglycaemia must be relatively small compared with the effects of TGF-β (Reeves & Andreoli, 2000).

Overall there have been substantial advances in the understanding of the mechanisms leading to diabetic nephropathy. Numerous research groups are working towards establishing the exact detailed sequence of events in the pathogenesis of the disease. This is of great importance as many of the mechanisms described can be modified by dietary or pharmacological means and are therefore potential targets for therapeutic intervention.

1.6. Effects of streptozotocin-induced diabetes on intestinal glucose transport

The effects of streptozotocin (STZ)-induced diabetes on intestinal glucose transport have been extensively studied. Since the process of glucose transfer across the enterocyte and proximal tubule cell displays striking similarities, insights into the diabetes-induced changes in intestinal glucose transport may be of relevance to the understanding of the effects of the disease on renal glucose transport. Changes in potential difference (PD), the driving force for sodium-dependent glucose transport, have been reported as early as 2 hours after administration of streptozotocin (Sharp & Debnam, 1994a). This hyperpolarization of the PD was attributed to decreased sodium conductance of the BBM. Chronic treatment of animals with STZ also results in enhanced PD as a consequence of reduced sodium permeability (Debnam & Ebrahim, 1989). This is accompanied by a higher proportion of functionally mature enterocytes on the villus (Debnam *et al.*, 1990; Fedorak *et al.*, 1989) and an increase in villus length (Debnam *et al.*, 1995). Interestingly, SGLT1 protein is not detected in the ileum of normal animals, however chronic STZ treatment results in the induction of SGLT1 transporter mRNA and protein and increases the villus length in this region (Castello *et al.*, 1995; Debnam *et al.*, 1995). In addition to changes in sodium-dependent glucose transport, protein levels of the facilitative fructose

transporter, GLUT5 are also increased at the BBM during diabetes (Corpe *et al.*, 1996).

Acute and chronic diabetes has also been associated with increased basolateral efflux of glucose (Debnam *et al.*, 1990; Sharp *et al.*, 1997) and increased mRNA levels of GLUT1 and GLUT2 (Burant *et al.*, 1994). These early studies assumed that the facilitative transporters were expressed exclusively at the BBM, however later studies provided evidence for expression of GLUT1 (Boyer *et al.*, 1996) and GLUT2 (Chowrimootoo G *et al.*, 1993; Corpe *et al.*, 1996) at the enterocyte BBM. Taken together these enterocyte adaptations during diabetes provide a situation that allows enhanced glucose absorption.

1.7. Effect of high glucose concentrations on mesangial cells

Mesangial cells exposed *in vivo* and *in vitro* to high extracellular glucose levels would be expected to exhibit increased intracellular glucose concentration. GLUT1 is the major facilitative glucose transporter in mesangial cells and evidence suggests that transport of glucose into the cells by this transporter regulates the activity of the downstream pathways that culminate in cellular damage. Heilig *et al.* have demonstrated that increased glucose uptake rather than intracellular glucose concentration *per se* is the major determinant in cellular damage. They showed that mesangial cells overexpressing the human GLUT1 gene and grown in 8 mM glucose displayed all the characteristics of normal mesangial cells grown at 35 mM glucose. This includes an increase in GLUT1 protein expression and glucose uptake, increased cellular sorbitol content and increased collagen, fibronectin and laminin synthesis (Heilig *et al.*, 1995b). A later study by this group showed that, under the same growth conditions, these cells overexpressing GLUT1 displayed elevated levels of native GLUT1, aldose reductase and PKC protein (Henry *et al.*, 1999). This work was later extended by the finding that mesangial cells expressing antisense GLUT1 were protected from glucose induced cellular damage (Heilig *et al.*, 2001). From these data it was hypothesised that a positive feedback mechanism

exists which is pathological during hyperglycaemia and predisposed the mesangial cells to glucose induced damage.

1.8 Effect of streptozotocin-induced diabetes on renal tubular glucose transport

The effect of STZ-induced diabetes on sodium-dependent renal glucose transport has yielded conflicting results. Reports have shown both increased (Blank *et al.*, 1985) and decreased (Harris *et al.*, 1986; Yasuda *et al.*, 1990) SGLT-mediated glucose transport. Quantification of SGLT1 mRNA and protein levels have also proved inconclusive, with levels reported to decrease (Yasuda *et al.*, 1990), increase (Vestri S *et al.*, 2001) and remain unchanged (Dominguez *et al.*, 1994; Harris *et al.*, 1986). These discrepancies may be due to the dose of STZ used in the studies and the severity of the experimentally induced diabetes (Blank *et al.*, 1985). In the kidney, it has been hypothesised that expression of the sodium-dependent glucose transport, SGLT2, at the BBM of the PCT, quantitatively reabsorbs the bulk of the glucose filtered by the glomerulus (Kanai *et al.*, 1994). It would therefore be expected that this transporter would be an important factor in the handling of the increased filtered load of glucose, presented to the PCT during diabetes. Levels of SGLT2 mRNA have been reported to increase during experimentally induced diabetes (Vestri S *et al.*, 2001), but functional changes and alterations in protein expression have yet to be reported. STZ-induced diabetes has been shown to increase both protein (Asada *et al.*, 1997) and mRNA (Chin *et al.*, 1997) expression levels of the BBM fructose transporter, GLUT5. Reports consistently demonstrate that levels of GLUT2 mRNA (Chin *et al.*, 1997; Vestri S *et al.*, 2001) and protein (Asada *et al.*, 1997; Dominguez *et al.*, 1992; Dominguez *et al.*, 1994) undergo the greatest degree of adaptation during experimental diabetes, with increases in this transporter at the BLM being linked to the maintenance of increased glucose efflux in the presence of hyperglycaemia (Dominguez *et al.*, 1994; Kamran M *et al.*, 1997). Interestingly, levels of tubular GLUT1 protein and its mRNA have been shown to decrease in diabetes (Chin *et al.*, 1997; Dominguez *et al.*, 1994; Vestri S *et al.*, 2001). This adaptation in

tubular epithelial cells is in contrast to the effects of diabetes on mesangial cell expression of GLUT1, where increased expression of GLUT1 has been strongly correlated to hyperglycaemia induced mesangial cell damage (Heilig *et al.*, 1997; Heilig *et al.*, 2001; Mogyorosi & Ziyadeh, 1999).

1.9. Aims of thesis

It is evident from the literature that there is a direct correlation between hyperglycaemia and the progression of diabetic nephropathy. Experiments focusing on mesangial cells have linked overexpression of the facilitative glucose transporter, GLUT1, to the hyperglycaemia-induced damage that occurs in this cell type. However the cellular processes leading to glucose-induced proximal tubule damage have not been studied in detail. It is of interest that expression of facilitative glucose transporters at the enterocyte BBM has been reported during diabetes (Chowrimootoo G *et al.*, 1993; Corpe *et al.*, 1996). The experiments described in chapters 2 and 3 were designed to establish the effect of STZ-induced diabetes on proximal tubule glucose transport. Experiments in the subsequent chapters aimed to elucidate the signalling events that regulate glucose reabsorption by the proximal tubule, and to investigate possible hormonal factors involved in the response to STZ-induced diabetes. Therefore, the overall aim of the experiments reported in this thesis was to advance the understanding of the events leading to diabetes-induced proximal tubule dysfunction.

Chapter 2.

The effect of streptozotocin-induced diabetes on renal glucose transport.

2.1. Introduction

As stated in Chapter 1 the overall effects of streptozotocin (STZ)-induced diabetes on renal SGLT-mediated glucose transport remains controversial. Classically, it is assumed that GLUT-mediated glucose transport occurs at the basolateral membrane (BLM) (Dominguez *et al.*, 1992; Dominguez *et al.*, 1994). However, recent studies have proposed a role for these facilitative transporters at the brush border membrane (BBM) of the intestinal enterocyte, with increased BBM levels of GLUT1 (Boyer *et al.*, 1996), GLUT2 (Corpe *et al.*, 1996) and GLUT5 protein (Corpe *et al.*, 1996) reported in enterocytes isolated from STZ-treated animals. Recent reports have also demonstrated raised intestinal BBM levels of GLUT2 in response to high luminal concentrations of glucose (Kellett & Helliwell, 2000) and vascular infusion of glucagon-like peptide 2 (Au *et al.*, 2002). A role for GLUT transporters in renal BBM glucose transport has yet to be established.

The importance of understanding how diabetes affects renal glucose handling is evident from the observation that renal glucose uptake plays a key role in reducing plasma glucose during hyperglycaemia (Cersosimo *et al.*, 1997). In addition, since plasma glucose concentration can influence glucose handling and utilization by the kidney (Biava *et al.*, 1966; Khandelwal *et al.*, 1979), changes in glucose transport in diabetes may lead to tubule cell injury and associated renal interstitial changes (Larkins & Dunlop, 1992; Wolf & Thaiss, 1995). In this context, it is also known that hyperglycaemia increases GLUT1 expression in mesangial cells, which has been linked to increased transforming growth factor-β (TGF-β) production and this interation may contribute to the development of diabetic nephropathy (Heilig *et al.*, 1995b; Heilig *et al.*, 1997).

The aim of this first series of experiments was to determine the effect of acute and chronic STZ-induced diabetes on renal glucose uptake. Changes in sodium-dependent and facilitative glucose transport were assessed using BBM vesicles prepared from non-diabetic, diabetic and overnight fasted

diabetic animals to establish the role of hyperglycaemia in the regulation of renal glucose transport.

2.2. Materials and Methods

2.2.1. Induction of diabetes

Diabetes was induced in 230-260g male Sprague-Dawley rats by administration of a single, tail vein injection of streptozotocin (55 mg/kg). Streptozotocin was dissolved in freshly prepared 0.05 M citrate buffer (pH 4.5) and administered under light isoflurane anesthesia. Animals were glycosuric 24 hours after streptozotocin treatment (Diastix, Bayer PLC, Berkshire, UK). Animals were divided into two groups to represent acute (2-4 weeks) and chronic (8-10 weeks) diabetes, with weight matched animals being used as controls. Animals were allowed *ad libitum* access to food (diet RM1, SDS Ltd, Witham, Essex, UK) and water up to the time of experimentation, with the exception of those subjected to an overnight fast, where the food source was removed 16 hours prior to sacrifice. Animals were terminally anaesthetised with intraperitoneal pentobarbitone sodium (Sagatal, Rhone-Merieux, Harlow, UK, 90 mg/kg). Blood was taken via cardiac puncture for analysis of blood glucose levels and the kidneys removed and placed in ice-cold 154 mM NaCl. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

2.2.2. Analysis of plasma glucose levels

Blood obtained by cardiac puncture was placed into heparinised tubes containing 7500 Kallikrein Inactivator units (KIU) of the broad range protease inhibitor, Aprotonin, and centrifuged for 10 minutes at 6000 x g. The resulting plasma was snap frozen in liquid nitrogen and stored at –70°C until use. Plasma glucose levels were measured using a glucose oxidase method (Sigma, Dorset, UK. Assay kit number 510), according to the manufacturers instructions.

The principle of this assay relies on the conversion of glucose to gluconic acid and hydrogen peroxide (H_2O_2) by the enzyme glucose oxidase. Followed by the oxidation of o-dianisidine, which results in a colour change and is measured at 450nm, using a Beckman Du 650 spectrophotometer (Beckman-Coulter Bioresearch, Buckinghamshire, UK).

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine (colourless)} \xrightarrow{\text{Peroxidase}} \text{oxidised o-Dianisidine (brown)}$$

2.2.3. Brush border membrane (BBM) vesicle preparation

Excised kidneys were transferred into ice-cold 154 mM NaCl, individually they were placed onto a cold glass surface and the surrounding fat and capsule removed; kidney weights were then recorded. Each kidney was sliced into 2 mm sections and the cortex dissected away. Cortical fragments from both kidneys of 3 animals from each group was pooled, weighed and then used for the preparation of BBM vesicles.

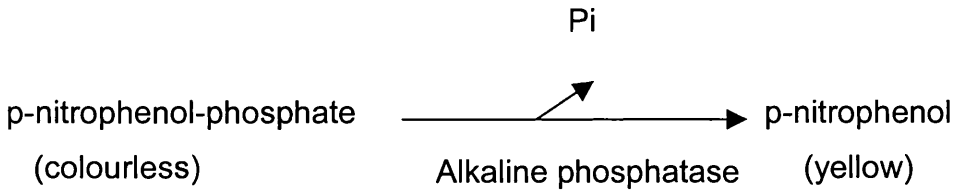
The method used to prepare renal BBM vesicles has been described previously (Biber *et al.*, 1981), all steps were carried out at 4°C. Cortical slices were added to 30 ml of buffer containing 300 mM mannitol, 12 mM Tris-HCl and 5 mM ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) (pH 7.4) and homogenised for 2 minutes (Ultra Turrax homogeniser, Janke & Kunkel, FRG) at half speed. 42 ml of ice cold distilled water was added, followed by $MgCl_2$ to a concentration of 12 mM, and then stirred on ice for 15 minutes. The solution was then centrifuged at 2000 x g for 15 minutes and the supernatant re-centrifuged at 21000 x g for 30 minutes. The resulting pellet was re-suspended in 20 ml buffer containing 150 mM mannitol, 6 mM Tris-HCl and 2.5 mM EGTA (pH 7.4), using ten cycles of a hand operated glass-Teflon homogeniser. $MgCl_2$ was again

added to a concentration of 12 mM and, after stirring on ice for 15 minutes, low and high-speed centrifugations, as described above, were repeated. The pellet was then re-suspended in 20 ml buffer containing 300 mM mannitol, 12 mM Tris-HCl and 2.5 mM EGTA (pH 7.4) and centrifuged at 21000 x g for 30 minutes. The purified BBM pellet was finally re-suspended in the same buffer to a protein concentration of 3-6 mg/ml using 5-6 passes through a syringe fitted with a 21-gauge needle. The concentration of protein in the initial homogenate and BBM preparation was determined using the Bradford method (Bradford, 1976). The principle of this assay relies on the binding of the dye, Coomassie Brilliant Blue G-250, to proteins. Binding results in a shift in the absorption maximum of the dye, from 465 to 595 nm, therefore the absorption at 595 nm is relative to the protein concentration.

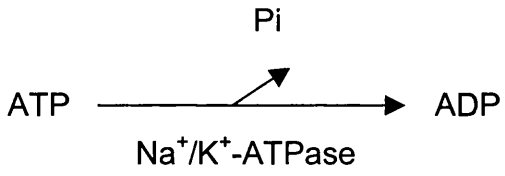
2.2.3.1. Validation of BBM Vesicle purity

An important aspect of using BBM vesicles is being able to establish that the vesicles are composed mainly of BBM and that contamination with BLM or the membranes of cellular organelles is minimal. Two enzyme assays were employed, which measure the activity of marker enzymes specific to either the BBM or BLM. The activity of the BBM marker enzyme, alkaline phosphatase, was measured in the initial homogenate and BBM preparation using the method of Forstner *et al.*, in order to derive an enrichment value for this marker enzyme (Forstner *et al.*, 1968). The level of contamination with BLM was determined by establishing an enrichment value for the ouabain-sensitive Na^+/K^+-ATPase, using the method of Proverbio and Del Castillo (Proverbio & Del Castillo, 1981).

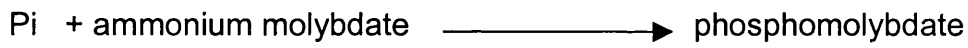
The principle of determining the activity of the BBM marker enzyme alkaline phosphatase relies on the cleavage of phosphate (Pi) from the substrate, p-nitrophenol-phosphate, under alkaline conditions. This produces p-nitrophenol, which appears yellow and can be detected at 450 nm.

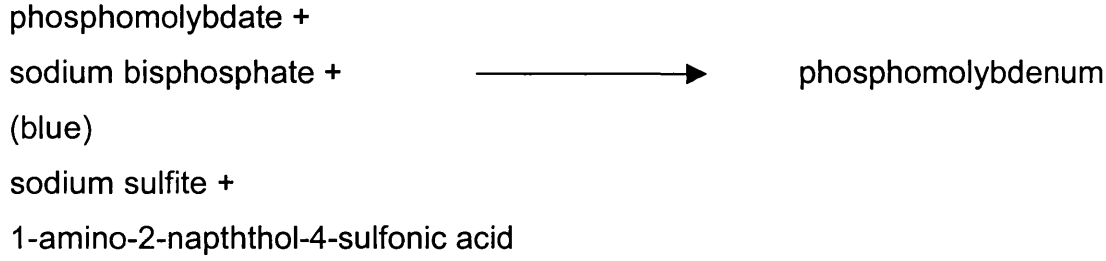


The principle of determining the activity of the BLM Na^+/K^+-ATPase is more complex. Firstly, the sample is treated with 20% trichloroacetic acid to remove protein and lipid phosphorous. ATP is then added to the resulting suspension and incubated at 37°C for 30 minutes, where it is cleaved by the enzyme to produce ADP and phosphate.



The resulting phosphate is then measured using the Fiske and Subbarrow method (Sigma, Dorset, UK, assay kit number 670), where under acid conditions, ammonium molybdate reacts with the phosphate to produce phosphomolybdate. Addition of a mixture of sodium bisphosphate, sodium sulfite and 1-amino-2-napththol-4-sulfonic acid, reduces the phosphomolybdate to form a phosphomolybdenum blue complex, which is measured at 660 nm.





The isoform of the Na^+K^+ATPase that is located at the BLM is sensitive to ouabain (Proverbio & Del Castillo, 1981), therefore the activity of the enzyme is measured in the presence and absence of 1 mM ouabain and the calculated difference taken to represent the activity of the BLM isoform.

2.2.4. Glucose uptake studies

The conditions required for studying glucose uptake using BBM vesicles have been optimised, initially for intestinal BBM glucose uptake studies, and regularly used in Dr Debnam's laboratory for a number of years (Sharp & Debnam, 1994b). Uptake studies were carried out on freshly prepared BBM vesicles, equilibrated to 20°C for 10 minutes. The transport process was initiated by mixing equal volumes (20 μl) of vesicle suspension and uptake buffer (pH 7.4) consisting of 200 mM sodium thiocyanate (NaSCN) and 12 mM Tris/HCl, containing [^{3}H]-D glucose such that the final concentration of glucose was 30-960 μM. This range of glucose concentration allows the V_{max} and K_m of SGLT-mediated glucose transport to be derived. Uptake was terminated after 4 seconds by the addition of 3 ml of 154 mM NaCl containing 0.5 mM phlorizin, a competitive inhibitor of SGLT-mediated transport, followed by vacuum filtration through 0.45 μm nitrocellulose filters (Sartorius, Germany). Three further washes of the vesicles trapped on the filter were performed. In order to assess GLUT-mediated transport, a higher glucose concentration (20 mM) was used, this value being in keeping with the low affinity of GLUT transporters for glucose (Dominguez *et al.*, 1994). These experiments were carried out in the presence of 1 mM phlorizin in order to abolish SGLT-mediated transport.

2.2.4.1. Validation of BBM vesicle transport capacity

Another important aspect of using BBM vesicles for transport studies is being able to demonstrate that they display the expected transport properties. An overshoot graph was prepared from uptake data obtained using 100 μM D-glucose in the presence or absence of 1 mM phlorizin, at time points

between 1 second and 20 minutes. Vesicle trapped space represents the volume the vesicles can accumulate at the point of equilibrium and was calculated from uptake data obtained at 20 minutes using 100 μM D-glucose. To establish the contribution of passive diffusion to the values obtained for SGLT and GLUT-mediated glucose transport, uptake of 100 μM or 20 mM L-glucose, the isoform that is not actively transported by the glucose transporters (Kinne *et al.*, 1975), was determined. In addition, the contribution of GLUT-mediated glucose uptake at the higher glucose concentration was established using 20 μM cytochalasin B and 0.5 mM phloretin, as these compounds have been shown to be specific inhibitors of GLUT-mediated transport (Colville *et al.*, 1993; Kellett & Helliwell, 2000).

2.2.5. Calculations

Scintillation counting of the filters enabled glucose accumulation to be calculated as shown in equation 2.1; values were expressed as pmol/mg protein/4 seconds.

Equation 2 1. Calculation of glucose accumulation by BBM vesicles.

$$\frac{(\text{DPM of incubated samples} - \text{DPM background}) \times \text{glucose concentration}}{\text{DPM of initial counts} \times (\text{protein concentration} \times 2)}$$

Where :-

- DPM of incubated samples = the accumulation of glucose by BBM vesicles incubated with uptake solution containing varying glucose concentrations.
- Background = non-specific retention of radioactivity on the filters (DPM).
- Glucose concentration (μM) = glucose concentration of the uptake solution taking into account the specific activity of the ^{3}H D-glucose.
- Protein concentration of BBM vesicle suspension (mg/ml), x 2 (to account for 50% dilution of vesicles with uptake solution).
- Initial counts = the DPM of the uptake solution (in thousands).

Table 2.1 demonstrates the raw data and glucose uptake values (calculated using equation 2.1) of a typical uptake experiment using BBM vesicles prepared from kidneys removed from control animals. The kinetic parameters of V_{max} (maximum transport capacity) and K_m (glucose concentration at half V_{max}) for SGLT-mediated glucose uptake were derived using a non-linear least squares curve-fitting program (Instat) from calculated uptake values obtained using 29.7-959.7 μM glucose. The V_{max} and K_m were derived as 3085 pmol/mg protein and 376 μM, respectively.

Table 2.1. Raw data and calculated glucose uptake from a typical experiment using control BBM vesicles.

Protein concentration x 2 (mg/ml)	Initial counts in thousands (DPM)	Glucose concentration (μM)	DPM of incubated samples	Background (DPM)	Glucose uptake (pmol/mg protein/4 sec)
8.1	321	29.7	22626	500	252
8.1	402	49	23875	500	351
8.1	476	97.2	22248	500	548
8.1	572	241	20179	500	1023
8.1	600	479.1	18287	500	1753
8.1	624	957.7	14157	500	2587
8.1	1404	20000	2896	500	4213
8.1	1419	20000 + PZ	1126	500	1089

2.2.6. Statistics

Data are presented as means ± standard error of the mean (SEM). Normal distribution was determined by Kolmogrov-Smirnov test of normality and statistical comparisons made using a one-way analysis of variance (ANOVA), with *post-hoc* comparisons performed using the Bonferroni multiple comparisons test. All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$. Each group consists of n=6-8.

2.3. Results

2.3.1. Animal parameters

Blood glucose levels were increased 3.5 fold in 2-4 week diabetic animals and 5.4 fold in 8-10 week diabetic animals when compared with control (Table 2.2). Overnight fasting of 2-4 week diabetic animals reduced blood glucose levels to that of control (Table 2.2). Although the blood glucose levels at 8-10 weeks diabetes is considerably higher than at 2-4 weeks, this level of hyperglycaemia has been reported previously in animals rendered chronically diabetic with the same STZ concentration used in this study (Thompson & Mikhailidis, 1992).

2-4 week diabetic animals lost 37.2 ± 3.6 g body weight, whilst those rendered diabetic for 8-10 weeks displayed signs of chronic diabetes, with severe weight loss, 52.3 ± 7.2 g, and the beginnings of cataract formation. In contrast, kidney and cortex weights were significantly increased by STZ-induced diabetes, with overnight fasting having no effect on these values (Table 2.2). This finding is in accordance with studies of type 1 (Osterby & Gundersen, 1975) and type 2 (Rodby, 1997) diabetic patients, where glomerular and tubuloepithelial hypertrophy are among the earliest pathological alterations of diabetes mellitus. Diabetic animals also displayed polydipsia, polyuria and hyperphagia (results not shown), all of which are characteristic of diabetes (Watkins PJ, 1983).

Table 2.2. Kidney weights and plasma data from control, 2-4 week, 8-10 week and 2-4 week fasted-diabetic animals. n=8 for each group, * P<0.05, ** P<0.001 compared with control; $^{\#}P$<0.001 compared with 2-4 wk diabetic using one-way ANOVA with Bonferroni multiple comparsions *post-hoc* test.

	Control	2-4 wk diabetic	8-10 wk diabetic	Fasted diabetic
Kidney weight (g/kidney)	0.97 ± 0.04	1.33 ± 0.06 **	1.31 ± 0.06 **	1.28 ± 0.05
Cortex weight (g/kidney)	0.44 ± 0.02	0.62 ± 0.03 *	0.69 ± 0.05 **	0.59 ± 0.03
Blood glucose (mM)	10.3 ± 0.35	36.8 ± 1.87 **	56.3 ± 3.3 **	11.5 ± 0.68 #

2.3.2. BBM vesicle validation data

Glucose uptake displayed the characteristic time-dependent overshoot that was inhibited by phlorizin (Fig. 2.1). Uptake was performed at 100 μM D-glucose in the presence of 200 mM sodium thiocyanate, at time points ranging from 1 second to 20 minutes. Between 1 and 10 seconds uptake was linear and represented active glucose transport due to the presence of an inwardly directed sodium gradient provided by the sodium thiocyante. Therefore, 4-second uptakes were performed for the subsequent uptake experiments described in this thesis. At peak uptake the sodium gradient is dissipated and glucose diffuses out of the vesicle until equilibrium is reached, where there is no net movement of the glucose into or out of the vesicles, this is therefore representative of vesicle trapped space. Uptake at each time point in the presence of 1 mM phlorizin, a competitive inhibitor of SGLT-mediated glucose uptake (Oulianova *et al.*, 2001), completely abolished the sodium-dependent overshoot. This demonstrates that uptake at 100 μM glucose is predominately sodium-dependent with the remaining component being a mixture of GLUT-mediated and passive (non-carrier mediated) glucose transport.

The contribution of passive glucose transport was established using L-glucose at 100 μM and 20 mM, and was found to contribute <2% of SGLT-mediated (0.02 nmol/mg protein/4 sec) and <1% of GLUT-mediated transport (0.013 nmol/mg protein/4 sec). These values were unaffected by STZ-induced diabetes (results not shown).

Determination of vesicle trapped space showed that the conditions described in this chapter had no significant affect on this parameter (in μl/mg protein: control: 1.96 ± 0.15 *vs.* 2-4 wk diabetic: 1.72 ± 0.32, 8-10 wk diabetic: 1.93 ± 0.15 and fasted diabetic: 1.73 ± 0.12, $P>0.5$ using a one-way ANOVA).

During the process of BBM preparation, it is inevitable that contamination with BLM and membranes of cellular organelles occurs (Murer & Gmaj, 1986). A number of marker enzymes can be used to establish the degree of contamination of the BBM vesicles with membranes from other cellular locations. For example, alkaline phosphatase, γ-glutamyltranspeptidase and aminopeptidase M are commonly used as markers for the BBM, with Na^+/K^+-ATPase and Ca^{2+}-ATPase used to demonstrate BLM, succinate-cytochrome *c*-oxidoreductase for mitochondria, glucuronidase for lysosomes and NADH-oxidoreductase lactate dehydrogenase for the cytosol (Biber *et al.*, 1981; Murer & Gmaj, 1986). In the experiments presented in this chapter the fold-enrichment of the BBM marker enzyme, alkaline phosphatase, and the BLM marker enzyme, Na^+/K^+-ATPase, was derived from the activity of the enzymes in the initial homogenate and final BBM vesicle preparation. Table 2.3 shows that BBM vesicles were enriched for alkaline phosphatase but not Na^+/K^+-ATPase. No significant difference in these enrichment values was apparent between the 4 experimental groups ($P<0.5$ using a one-way ANOVA). The enrichment of alkaline phosphatase in intestinal BBM vesicles generally yields higher values than the kidney (personal observations), however the enrichment values presented in this thesis are in accordance with those previously published (Dominguez *et al.*, 1992; Dominguez *et al.*, 1994).

Figure 2.1. Representative graph showing time dependency of glucose uptake by BBM vesicles prepared from renal cortex. The incubation buffer contained 100 μM D-glucose and 200 mM NaSCN, with or without 1 mM phlorizin (PZ).

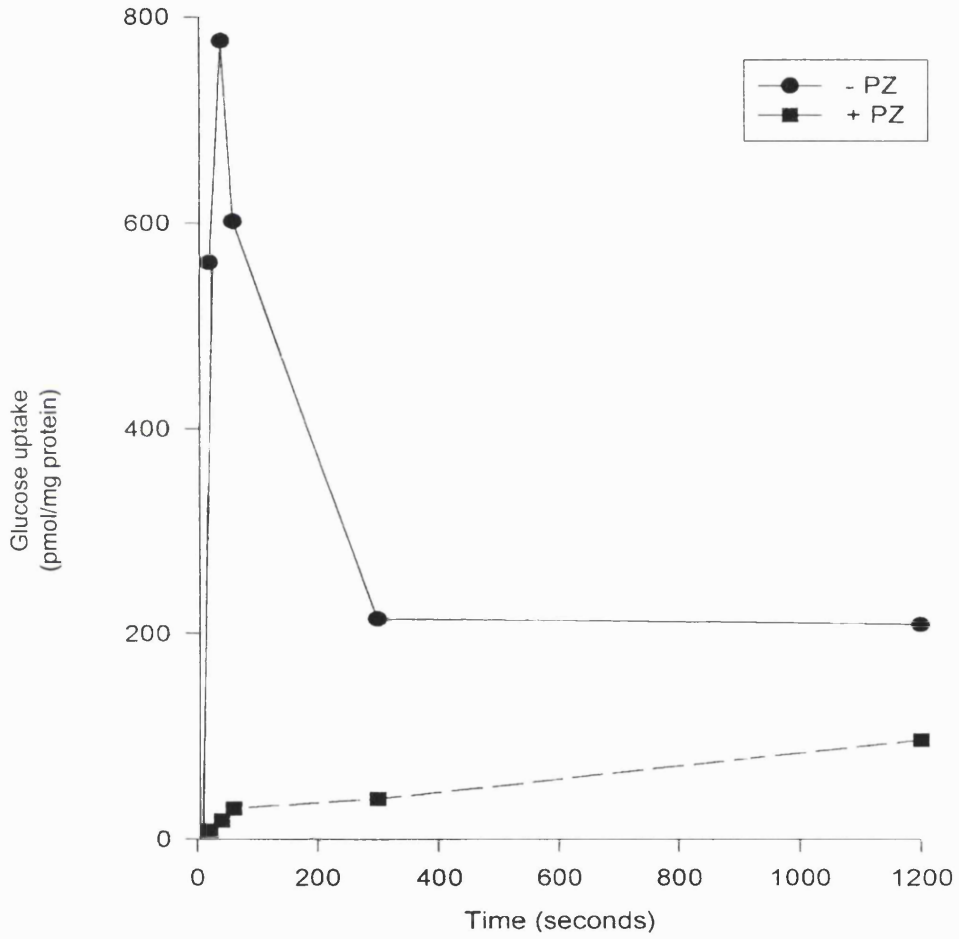


Table 2.3. Enrichment of the BBM marker enzyme, alkaline phosphatase, and the BLM marker enzyme, Na^+K^+ATPase. *n* = 6 for each condition.

	Control	2-4 wk diabetic	8-10 wk diabetic	Fasted diabetic
Alkaline phosphatase	7.1 ± 0.75	7.4 ± 1.30	6.5 ± 0.68	6.4 ± 0.33
Na^+/K^+ATPase	0.54 ± 0.11	0.69 ± 0.16	0.61 ± 0.09	0.68 ± 0.10

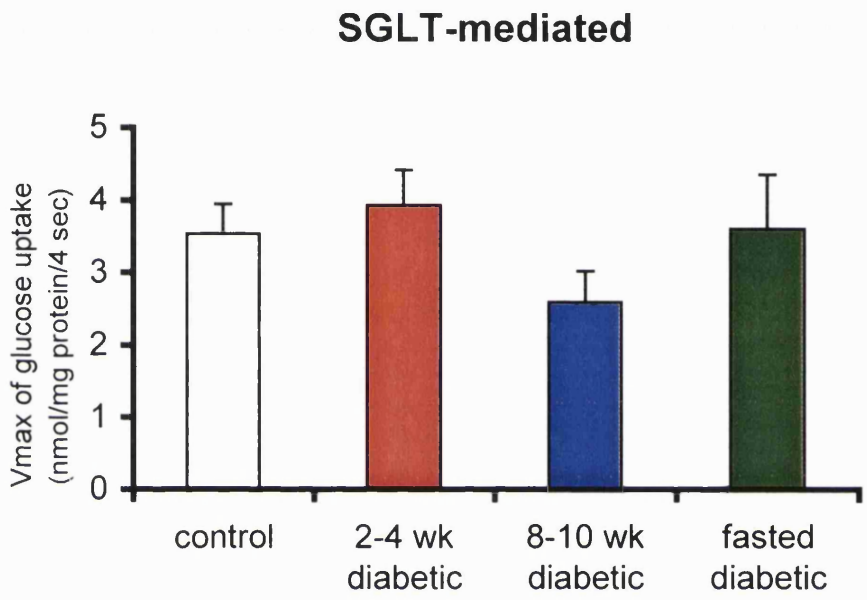
2.3.3. Streptozotocin-induced changes in renal BBM glucose transport.

Acute (2-4 week) STZ-induced diabetes had no significant effect on the V_{max} (Fig. 2.2, A) or K_m of SGLT-mediated glucose transport (K_m of control: 434 ± 68 *vs.* 2-4 wk diabetes: 585 ± 54 μM). In contrast, 2-4 week diabetes significantly increased the rate of GLUT-mediated glucose uptake by 67.5% (Fig. 2.2, B). Overnight fasting of 2-4 week diabetic animals had no effect on SGLT-mediated transport, but returned GLUT-mediated transport to that of controls (Fig. 2.2, B). This finding suggests that raised levels of glucose in the plasma or tubular fluid may be the stimulus for increased GLUT-mediated glucose transport.

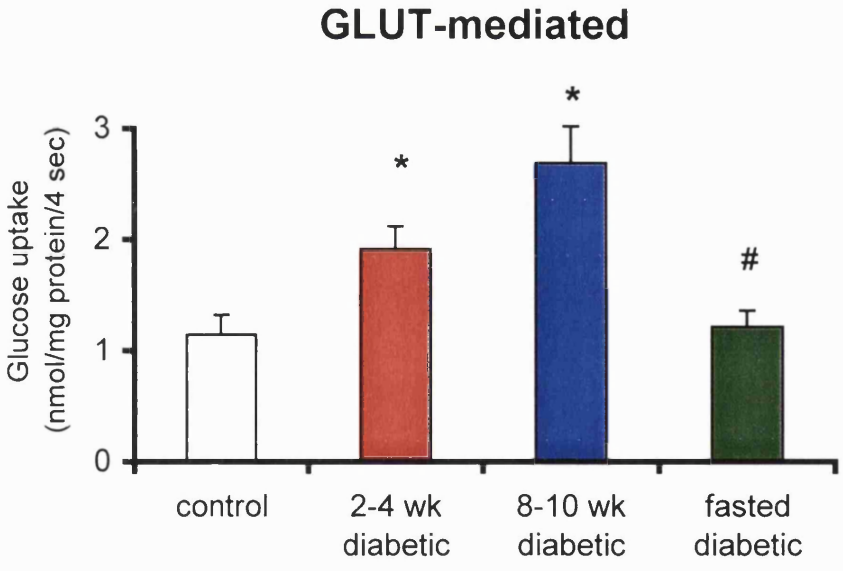
Chronic (8-10 week) diabetes had no significant effect on either the V_{max} (Fig. 2.2, A) or K_m (8-10 wk diabetes: 607 ± 61 μM) of SGLT-mediated transport, but increased the rate of GLUT-mediated glucose uptake at 8-10 weeks diabetes by 135 % (Fig. 2.2, B).

Figure 2.2. Effect of STZ-induced diabetes on **A** V_{max} of SGLT-mediated glucose uptake and **B** GLUT-mediated glucose uptake into renal BBM vesicles. $n = 6$, * $P<0.05$ vs. control; # $P<0.05$ *vs.* 2-4 wk diabetic using one-way ANOVA with Bonferroni multiple comparsions *post-hoc* test.

A



B



2.3.4. Inhibition of GLUT-mediated glucose transport

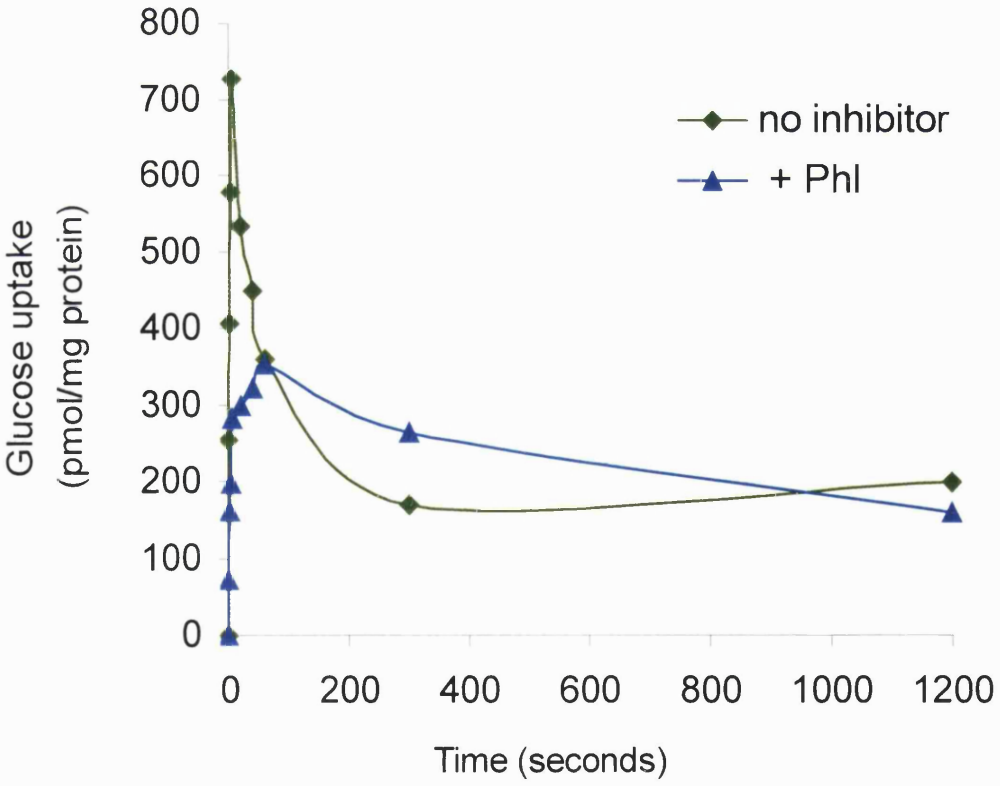
Cytochalasin B and phloretin are both reported inhibitors of facilitative glucose transport (Colville *et al.*, 1993; Kellett & Helliwell, 2000). The contribution of facilitative glucose transport in control and diabetic BBM vesicles was originally confirmed using 0.5 mM phloretin. This inhibitor reduced GLUT-mediated glucose transport in control vesicles by 33.8 ± 8.0% (n=6) and by 40.7 ± 7.4% (n=6) in diabetic BBM vesicles. Addition of 20 μM cytochalasin B, abolished GLUT-mediated glucose transport in control BBM vesicles, incubated with 20 mM glucose, by 24 ± 3.2% (n=4), and by 25.25 ± 5.6% in diabetic BBM vesicles (n=4). These data therefore demonstrate specific GLUT-mediated transport occurs under the experimental conditions described in this thesis.

However, Since both cytochalasin B and phloretin target GLUT1 and GLUT2, it was unexpected to find such a difference in their percentage inhibition. Further study established that phloretin partially inhibited the Na^+-dependent overshoot (Fig. 2.3), suggesting that phloretin is able, at least in part, to inhibit renal SGLT-mediated glucose transport.

One explanation for the inhibition of the overshoot is that the phloretin used for these experiments was impure. Therefore, its composition was established by HPLC, and was confirmed to be 99.94% phloretin (kindly performed by Dr Abd El Mohsen, Antioxidant Research Group, GKT School of Biomedical Sciences, Kings College). However, Yokota *et al.* (1983), showed that phloretin, the aglycone of phlorizin, can inhibit Na^+-dependent glucose uptake in the presence of a Na^+ gradient (Yokota *et al.*, 1983). The authors demonstrated that there was no direct effect of phloretin on the SGLT-transporter, but suggested that the inhibitory effect occurred by disruption in the coupling of the transporter protein and the sodium gradient and/or hastened dissipation of the sodium gradient. They suggested that these effects could occur through interaction with the phenol-binding site of the transporter resulting in malfunction of the protein so that glucose would be transported along its own chemical gradient in the presence of phloretin,

or due to its high hydrophobicity, may interact with membrane lipids and result in changes in membrane permeability. Additionally, Silverman *et al.* demonstrated that phloretin could displace ^{3}H-phlorizin, bound to proximal tubule cells, with cytochalasin B having no effect (Silverman & Black, 1975). These findings strongly suggest that inhibition by phloretin cannot be used as reliable measure of GLUT-mediated transport using the experimental conditions described in this thesis. Therefore, renal BBM GLUT-mediated transport was taken as cytochalasin B-sensitive rather than phloretin-sensitive.

Figure 2.3. Representative graph demonstrating inhibition of SGLT-mediated glucose transport by 0.5 mM phloretin.



2.4. Discussion

As described in chapter 1, diabetes mellitus is a chronic disease that leads to complications such as retinopathy, neuropathy, nephropathy and heart disease (Olefsky, 2001). Extensive research has demonstrated that hyperglycaemia is a key factor in the pathogenesis of the disease (Larkins & Dunlop, 1992). The results presented in this chapter have focused on the effects of hyperglycaemia on proximal tubular glucose transport using an animal model of type 1 diabetes mellitus, induced by the drug streptozotocin. This diabetogenic agent can be used to induce both insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetes mellitus. A single intravenous injection of between 40 and 60 mg/kg body weight is effective at inducing IDDM in adult rats (Ganda *et al.*, 1976). Whereas, NIDDM results from intravenous or intraperitoneal injection of 100 mg/kg, to rat pups on the day of birth (Portha *et al.*, 1974).

STZ is a relatively specific diabetogenic agent, it exerts its effects on pancreatic β-cells via uptake through GLUT2 (Hosokawa *et al.*, 2001) which, through a series of biochemical events, leads to cell death by necrosis. The main reason for β-cell death is alkylation of the DNA (Elsner *et al.*, 2000), although production of nitric oxide and reactive oxygen species have also been shown to attenuate STZ toxicity (Szkudelski, 2001). Therefore, a decrease in pancreatic insulin secretion and a subsequent rise in blood glucose concentrations result from STZ-induced β-cell death, both of which are characteristic of human diabetes. Numerous other similarities have also been described, including increased glomerular filtration rate (GFR) (Ward *et al.*, 2001), changes in the plasma and urine biochemistry, polyphagia, polydipsia and polyuria (Thompson & Mikhailidis, 1992). In addition, the pathological features of the kidney in STZ-induced diabetes display striking similarities to those seen in kidneys from human diabetics, including changes in mesangial cell morphology (Park *et al.*, 1997), general renal hypertrophy (Seyer-Hansen, 1976) and glycogen deposits (Asada *et al.*, 1997). Thus, due to the similarities described above, STZ-induced diabetes is widely used

as an animal model of type 1 diabetes and has provided valuable insights into the progression of diabetic nephropathy and other complications associated with the disease.

Previous reports have demonstrated effects of STZ-induced diabetes on both renal and intestinal glucose transport. In the small intestine, diabetes results in increased SGLT-mediated glucose transport, through changes in potential difference across individual enterocytes (Debnam & Ebrahim, 1989) and via enlarged villi, which display a higher proportion of functionally mature enterocytes (Debnam *et al.*, 1990; Fedorak *et al.*, 1989). Changes in the transport capacity of the intestinal facilitative glucose transporters have also been described in response to STZ-induced diabetes (Burant *et al.*, 1994; Debnam *et al.*, 1990). These early studies suggested this adaptation occurred exclusively at the enterocyte BLM, however, more recent reports describe alteration in the expression of GLUT1 and GLUT2 at the BBM (Boyer *et al.*, 1996; Chowrimootoo G *et al.*, 1993; Corpe *et al.*, 1996).

Studies focusing on the effect of diabetes on renal SGLT-mediated glucose transport have yielded conflicting results (Blank *et al.*, 1985; Harris RC *et al.*, 1986; Yasuda *et al.*, 1990). However, the effect on facilitative glucose transport seems to be more consistent. The expression of GLUT2 and GLUT5 (the BBM fructose transporter) is increased at the basolateral and brush border membranes, respectively (Dominguez *et al.*, 1994; Kamran M *et al.*, 1997; Vestri S *et al.*, 2001), and is accompanied by corresponding increases in mRNA expression levels (Chin *et al.*, 1997). In contrast, the levels of GLUT1 protein and its mRNA in the proximal tubule have been shown to decrease in diabetes (Chin *et al.*, 1997; Vestri S *et al.*, 2001). However, the adaptation of GLUT2 protein and mRNA levels in response to diabetes comes from studies performed on whole tubule homogenates, with the assumption that the resulting protein would be expressed at the BLM. The results presented in this chapter suggest that facilitative glucose transport is increased at the proximal tubule BBM, and that this uptake is regulated by alterations in plasma glucose levels.

BBM vesicles have been used for many years to elucidate the transport characteristics of a wide range of transporter proteins. Early reports into the purification of the brush border membrane were based on the isolation of large membrane fragments called brush border caps (Berger SJ & Sacktor, 1970). The methodology evolved, and subsequent reports for the isolation of BBM vesicles involved the separation of subcellular organelles from the BBM, using precipitation with calcium (Del Castillo & Robinson, 1982) or magnesium (Biber *et al.*, 1981). This methodology is based on the different properties of the BBM and other cellular membranes. The differences in lipid to protein ratios and carbohydrate contents result in distinct physiological properties. The BBM has a high negative charge which allows it to compensate within the membrane the two charges of the divalent cation. The other cellular membranes lack this ability to compensate, resulting in production of a coprecipitate as the divalent cations crosslink with the BLM and other cellular organelles (Murer & Gmaj, 1986). The method of double magnesium precipitation in the presence of EDTA was chosen for the isolation of renal BBM vesicles because it has been reported to improve the yield and reduce the leak permeability of the vesicles (Biber *et al.*, 1981). Although this method is reported to produce vesicles with limited contamination of other membrane fractions (Biber *et al.*, 1981) and the enrichment values presented in this chapter are consistent with those reported previously (Dominguez *et al.*, 1992; Dominguez *et al.*, 1994), no BBM preparation is totally free from contamination. In some instances cross-contamination may be acceptable, as long as the contaminating membranes do not contain high activities of the same or similar transport system. The results presented in this chapter demonstrate alterations in BBM GLUT-mediated glucose transport, which is a transport system, considered to occur exclusively at the BLM. Therefore, the findings presented may be a consequence of BLM contamination and in fact represent STZ-induced changes in BLM transport capacity. As with all data obtained using BBM vesicles, it is critical to be able to make comparisons with data obtained using intact epithelium. Indeed, alterations in the expression pattern of GLUT2 at the proximal tubule BBM have been confirmed using

immunohistochemistry and are reported in chapter 3, therefore suggesting that the results presented are not simply a consequence of the methodology.

The renal transport maximum (Tm) for glucose is increased in patients with type 1 diabetes (Mogensen, 1971). Although exact values for Tm in animals with STZ-induced diabetes have not been determined, the capacity for glucose reabsorption is increased (Carney *et al.*, 1979; Noonan *et al.*, 2001). In addition to this increased glucose reabsorption, proximal tubules from diabetic rats have also been shown to have increased rates of glucose metabolism (Kleinzeller & McAvoy, 1986), which is accompanied by increased expression of the enzyme hexokinase (Kleinzeller & McAvoy, 1986) and those involved in the pentose phosphate pathway (Sochor *et al.*, 1979). Interestingly, changes in glucose metabolism in mesangial cells has been linked to glomerulosclerosis (Dunlop M, 2000; Heilig *et al.*, 1995b) and has been shown to occur, not as a consequence of increased glucose transport *per se*, but as a result of increased GLUT1 expression (Heilig *et al.*, 2001). Indeed, alterations in proximal tubular glucose metabolism have also been suggested to be involved in hyperglycaemia induced changes in this tubular segment (Phillips *et al.*, 1999b). Thus it appears that increased glucose uptake and metabolism by the kidney during diabetes may have detrimental effects. Although the kidneys are classically thought to play a minor role in glucose homeostasis, recent reports have provided strong evidence that this is not the case (reviewed in (Stumvoll *et al.*, 1997)). Renal glucose utilization has been shown to account for 20-30% of plasma glucose disposal in the post-absorptive state (Cersosimo *et al.*, 1994; McGuinness *et al.*, 1993). Postprandial hyperglycaemia appears to have no significant affect on glucose utilization. In contrast, during pathological hyperglycaemia, it has been proposed that increased glucose uptake, glycogen storage and lactate formation, may represent mechanisms by which the kidney helps to maintain plasma glucose concentrations (Cersosimo *et al.*, 1997). Thus, the increased glucose uptake described in this chapter following STZ-induced diabetes may represent an adaptation that aims to normalize plasma glucose concentrations, albeit with potentially detrimental effects. The normalisation of the rate of GLUT-mediated uptake following overnight fasting of the

diabetic animals, which display plasma glucose levels similar to that of the controls, further substantiates this hypothesis. *In vivo* microperfusion experiments would enable STZ-induced changes in proximal tubule glucose transport to be examined at the level of the individual nephron. Additionally, such experiments would enable the relative contribution of the sodium-dependent and independent transport systems to be examined, using the specific inhibitors employed in the vesicle experiments described in this chapter.

At present it is unknown whether adaptations in renal facilitative glucose transport occurs in response to short term changes in plasma glucose levels. Kellett *et al.* have demonstrated that exposure of the intestine to increased luminal glucose concentrations for 15 minutes results in increased GLUT-mediated glucose transport, via insertion of GLUT2 protein into the enterocyte BBM (Kellett & Helliwell, 2000). They hypothesise that before a meal the expression level and intrinsic activity of GLUT2 at the BBM is low, following food ingestion the local concentrations of glucose increase and glucose uptake occurs predominately via SGLT1. This sodium dependent uptake has been shown to activate PKC-βII, followed by the insertion of GLUT2 protein into the BBM, thus providing a mechanism that allows maximal nutrient absorption following assimilation of a meal. Although postprandial hyperglycaemia has no effect on renal glucose utilization *per se*, an increase in the rate of glucose reabsorption has been demonstrated (Cersosimo *et al.*, 1997). Classically it would be assumed that this increased reabsorption occurs via sodium-dependent glucose uptake into the proximal tubule cell and its subsequent release in the peritubular circulation. However, in light of the findings of Kellett *et al.* an additional sodium-independent mechanism may exist to maximize renal glucose reabsorption in the presence of postprandial hyperglycaemia. Experiments to induce short term hyperglycaemia, by intravenous infusion of glucose, would establish if the mechanisms of renal glucose reabsorption is altered in a similar manner to that shown for the small intestine following food ingestion.

Thus, the results presented in this chapter demonstrate that both acute and chronic STZ-induced diabetes increases facilitative glucose reabsorption across the renal BBM. However, it is important to note that this diabetogenic agent has been reported to have toxic effects on the kidney (Bennett & Pegg, 1981; Kraynak *et al.*, 1995). Since fasting of diabetic animals abolishes the increased BBM glucose transport, it is unlikely that the results presented in this chapter are a consequence of STZ toxicity, however, this needs to be confirmed using other animal models of diabetes. Alloxan is another diabetogenic agent, but is generally considered to be less specific than STZ and consequently even light overdosing can result in premature death, which most likely occurs as a consequence of renal failure (Szkudelski, 2001). Therefore, it would be more appropriate to study models of diabetes where the disease occurs spontaneously. There are two commonly studied animal models of spontaneous type 1 diabetes, the non-obese diabetic (NOD) mouse and the biobreed (BB) rat, both of which occur through autoimmune destruction of the pancreatic β-cells (Allen & Braverman, 1996; Segev *et al.*, 1997). These spontaneously diabetic animal models display renal functional and structural abnormalities that resemble human diabetic nephropathy (Tochino, 1987; Velasquez *et al.*, 1990), therefore they could be used to confirm that pathological hyperglycaemia induces alteration in renal glucose reabsorption. The renal abnormalities associated with diabetes appear to be similar in both type 1 and type 2 diabetes. Therefore, it would be of interest to establish whether alteration in renal glucose transport occurs in animal models of type 2 diabetes. The most characterised mouse model of type 2 diabetes is the db/db mouse; it has been shown to have many similar features of human diabetic nephropathy and has therefore been used extensively to investigate the mechanisms that lead to diabetic renal disease (Sharma *et al.*, 2003). Renal abnormalities have also been identified in Goto-Kakizaki (GK) rats and in Obese Zucker rats, both of which are models of type 2 diabetes (Phillips *et al.*, 1999a). Perhaps most noteworthy is the study by Kamran *et al.* which demonstrated that GLUT2 protein and mRNA levels were increased in the proximal tubules of diabetic Zucker rats (Kamran M *et al.*, 1997). Although the functional consequence of this increased

expression was not examined it was proposed that increased expression of GLUT2 at the BLM would maintain a higher trans-tubular glucose flux of glucose during diabetes. In light of the findings presented in this chapter the increased GLUT2 expression demonstrated in Zucker rats may reflect altered glucose handling not only at the BLM but also the BBM.

2.5. Conclusions

In conclusion, both acute and chronic STZ-induced diabetes increases facilitative glucose transport across the proximal tubule BBM, without detectable changes in sodium-dependent glucose uptake. Reduction in plasma glucose levels eliminates the increased rate of glucose transport, suggesting that increased glucose reabsorption is dependent on the glycaemic status of the animal. This finding alters the current view of the process of glucose transport across the renal BBM during hyperglycaemia and may prove be a contributing factor in the pathogenesis of diabetic nephropathy. The experiments described in the subsequent chapter aimed to investigate the glucose transporters involved in this adaptation.

Chapter 3.

The effects of streptozotocin-induced diabetes on renal facilitative glucose transporter expression and distribution.

3.1. Introduction

The experiments described in chapter 2 demonstrated that streptozotocin-induced diabetes increases GLUT-mediated glucose transport across the renal brush border membrane. However, previous studies using control animals (Dominguez *et al.*, 1992; Heilig *et al.*, 1995a) have failed to detect GLUT transporters at the renal BBM, with the exception of GLUT5 (Sugawara-Yokoo *et al.*, 1999). Studies investigating the effect of diabetes on the renal expression of GLUT1, GLUT2 and GLUT5 have demonstrated alterations in the mRNA and protein expression of all three isoforms. GLUT1 mRNA and protein levels decrease in proximal tubules isolated from diabetic rats (Chin *et al.*, 1997; Dominguez *et al.*, 1994), whilst GLUT2 (Chin *et al.*, 1997; Dominguez *et al.*, 1994; Kamran M *et al.*, 1997) and GLUT5 (Asada *et al.*, 1997; Chin *et al.*, 1997) mRNA and protein is increased. These changes in facilitative glucose transport expression is accompanied by an increase in transtubular flux of glucose (Dominguez *et al.*, 1994). However, the experiments examining the effect of diabetes on GLUT protein expression have used tubule preparations isolated by microdissection or a Percoll gradient, rather than using BLM or BBM vesicles. Thus, the interpretation of these results is based on the assumption that the transporters are exclusively expressed at the BLM. One study has examined the protein expression of the renal facilitative glucose transporters during diabetes using immunohistochemistry (Asada *et al.*, 1997). The authors conclude that at the BBM there is increased GLUT5 expression, whereas GLUT2 expression is increased at the BLM. However, the images presented in this paper were taken at such a low power it is hard to be convinced of exclusive expression at either membrane, even in the case of GLUT5. Additionally, the methodology used snap frozen tissue rather than that fixed *in vivo*, a procedure that has recently been shown to be critical for the detection of GLUT2 at the enterocyte BBM (Affleck *et al.*, 2003; Kellett *et al.*, 2002).

The experiments described in this chapter were therefore designed to optimise the technique of immunohistochemistry for the analysis of renal glucose transporters, using a variety of tissue fixation methods and

antibodies raised against distinct regions of the GLUT2 protein. Once optimum conditions were established the technique was used to determine the membrane location of the renal GLUT transporters during conditions of euglycaemia and hyperglycaemia. Western blotting of BBM vesicles was also used to assess the contribution of the different GLUT isoforms to the changes in glucose transport seen in STZ-induced diabetes, that were reported in chapter 2.

3.2. Materials and Methods

3.2.1. Induction of diabetes (as 2.2.1)

3.2.2. BBM vesicle preparation

BBM vesicles were prepared as described in chapter 2, section 2.2.3. Vesicles remaining from the uptake studies were snap frozen in liquid nitrogen and stored at –70°C for western blotting.

3.2.3. Western blotting

Affinity purified antibodies were kindly provided by Professor S. Baldwin (School of Biochemistry and Molecular Biology, University of Leeds). In brief, synthetic peptides corresponding to residues 477-492 (GLUT1), 507-522 (GLUT2) and 490-502 (GLUT5) of the rat glucose transporter sequences were synthesized and conjugated via an additional N-terminal cysteine residue to ovalbumin. The conjugates were emulsified with complete Freund's adjuvant and 100 μg injected subcutaneously into New Zealand white rabbits. A month later, the rabbits were boosted with a further 50 μg of conjugated peptide in incomplete Freund's adjuvant and then two weeks later the animals were bled. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. The blood was allowed to clot and the serum separated by centrifugation at 1000 x g for 10 minutes. GLUT antibodies were then affinity purified by passage of the antiserum

through a column of peptide immobilised on Sulfollink gel (Pierce, Chester, UK) followed by elution in 50 mM diethylamine-HCl and dialysis into PBS.

For Western blotting, BBM samples (20 μg protein) were solubilised in Laemmli sample buffer containing 5% sodium dodecyl sulphate (SDS) and electrophoresed on a 10% SDS polyacrylamide gel using 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS running buffer (pH 8.3) (Biorad, Hertfordshire, UK) at 20 mAmps per gel. The proteins were transferred to nitrocellulose membranes by electrophoretic blotting for 1 hour at a constant current of 1 mA/cm^2 (Trans-blot semi-dry transfer cell, Biorad Hertfordshire, UK) using transfer buffer containing 10% (v/v) methanol, 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS (pH 8.2-8.4). Non-specific protein-binding sites were blocked with PBS-T (phosphate buffered saline (pH7.4) containing 0.1% Tween 20) and 5% (w/v) fat-free milk powder for 2 hours at room temperature. The membranes were incubated with GLUT1 (1:1000 dilution), GLUT2 (1:1500) or GLUT5 (1:1000) antibodies diluted in PBS-T for 16 hours at 4°C. The membranes were washed (2 x 15 minutes) with PST-T containing 1% fat-free milk powder and incubated with a swine anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:2000 dilution) (Amersham Pharmacia Biotech UK Limited, Bucks, UK) for 2 hours at room temperature and finally washed again with PBS-T (2 x 15 minutes). Bound antibodies were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech UK Limited, Bucks, UK) and visualised and quantified using a Fluor-S MultiImager System (Biorad, Hertfordshire, UK). Band density was expressed in arbitrary units.

3.2.4. Localisation of GLUT transporters using immunohistochemistry.

3.2.4.1. Tissue preparation for immunohistochemistry without *in vivo* fixation.

Both kidneys were removed from three-week STZ-treated and weight matched control Sprague-Dawley rats, anaesthetised with intraperitoneal pentobarbitone sodium (Sagatal, Rhone-Merieux, Harlow, UK, 90 mg/kg).

The kidneys were rapidly decapsulated, embedded in OCT cryoprotectant (BDH, Poole, UK), then snap-frozen in isopentane that had been pre-cooled in liquid nitrogen. Samples were stored at -70°C until use. Cryostat sectioning and immunohistochemistry were performed as described in 3.2.4.4.

3.2.4.2. Preparation of periodate-lysine-paraformaldehyde (PLP) fixative

The periodate-lysine-paraformaldehyde (PLP) fixative was prepared as described previously (McLean & Nakane, 1974). In brief, 200 mM lysine-HCl was dissolved in distilled water and mixed with an equal volume of 100 mM sodium phosphate buffer to give a solution containing 100 mM lysine, 50 mM phosphate (pH 7.4). Freshly prepared 8% paraformaldehyde was diluted 1:3 with the lysine-phosphate solution to give a final concentration of 2% paraformaldehyde. Prior to use, periodate was added to the lysine-phosphate-paraformaldehyde solution to give a final concentration of 10 mM.

3.2.4.3. Surgical protocol for *in vivo* fixation

Control and three-week streptozotocin treated animals (see section 2.2.1.) were anaesthetised with intraperitoneal pentobarbitone sodium (Sagatal, Rhone-Merieux, Harlow, UK, 90 mg/kg), and the aorta cannulated with polyethylene tubing. The left kidney was perfused with 10 mls of Hanks Balanced Salt Solution (Sigma, Dorset, UK) to remove blood, followed by perfusion with 40 mls of PLP fixative. The kidney was perfused using a hand operated syringe, at a rate just sufficient to stop blood re-entering the renal artery. The left kidney was removed, decapsulated, embedded in OCT and snap-frozen in isopentane pre-cooled in liquid nitrogen. Samples were stored at -70°C until use. In some instances the Hanks balanced salt solution was supplemented with 7 mM glucose, this value being within the range for circulating blood glucose levels of control animals.

3.2.4.4. Immunohistochemistry

Cryostat sections (7 μm) were mounted onto polylysine-coated slides (BDH, Poole, UK), air-dried for 1 hour and then stored at -70°C until use. Sections were allowed to reach room temperature and then washed for 5 minutes in 100 mM PBS (pH 7.4) followed by treatment with 0.3% methanolic H_2O_2 for 15 minutes to eliminate endogenous peroxidase activity. Sections were washed (3x10 minutes) with 100 mM PBS and then blocked for 30 minutes with 10% normal goat serum (NGS) in 100 mM PBS (Sigma, Poole, UK), containing 1% bovine serum albumin (BSA) and 1% Triton X-100. Sections were briefly washed and incubated overnight at 4°C with GLUT1, GLUT2 or GLUT5 antibody at 1:100 dilution, in 100 mM PBS containing 1% NGS, 0.1% BSA and 0.1% Triton X-100 (PBGST). Following overnight incubation, sections were washed (3x10 minutes, 100 mM PBS) and then incubated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratory, Burlingame, CA) at 1:500 dilution in PBGST for 1 hour at room temperature. Subsequently, sections were washed (3x10 minutes, 100 mM PBS) and then incubated with ExtrAvidin® (Sigma, Poole, UK) at 1:1000 dilution in PBGST for 1 hour at room temperature. Sections were washed (3x10 minutes) using 500 mM Tris/HCl buffer (pH7.4) and then incubated in a mixture of 0.1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), in 500 mM Tris buffer (pH 7.4) with 0.1% H_2O_2. When the desired intensity was reached the reaction was stopped by 3 brief washes with cold Tris/HCl. Slides were counterstained, dehydrated and cleared, followed by cover slipping using DPX (BDH, Poole, UK). Negative controls were performed whereby the primary antibody was omitted from the protocol and using primary antibody that had been pre-absorbed with a 10 fold excess to antigenic peptide.

3.2.4.5. Immunofluorescence using cyanine-2 (Cy2) for confocal imaging.

Cryostat sections (7 μm) were mounted onto polylysine-coated slides (BDH, Poole, UK) and then stored at -70°C until use. Sections were allowed to

reach room temperature and then washed for 5 minutes in 100 mM PBS (pH 7.4), followed by blocking for 30 minutes with 10% normal goat serum (NGS) in 100 mM PBS (Sigma, Poole, UK), containing 1% bovine serum albumin (BSA) and 1% Triton X-100. Sections were briefly washed and incubated overnight at 4°C with GLUT2 antibody at 1:100 dilution, in 100 mM PBS containing 1% NGS, 0.1% BSA and 0.1% Triton X-100 (PBGST). Following overnight incubation, sections were washed (3x10 minutes, 100 mM PBS) and then incubated in the dark with Cy2–labelled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Cambridgeshire, UK) at a 1:100 dilution in PBGST for 30 minutes at room temperature. Subsequently, sections were washed (3x10 minutes, 100 mM PBS) and mounted with Mowoil overnight at 4°C. Cell fluorescence was recorded by excitation at 488 nm with an Omnichrome series 43 (PerkinElmer, Bucks, UK) argon ion laser system using an Olympus IX70 Microscope fitted with a x100 oil immersion objective. Images were acquired with a charged coupled device camera (PerkinElmer, Buckinghamshire, UK) cooled to –35°C and controlled with the Ultraview 4.0 software (PerkinElmer, Bucks, UK). Z-scans (5.0 μm thickness) were performed with 0.5 μm spacing between each slice. The images obtained were produced as an overlay of 1 to 3 images selected from the Z-stack as the most representative of the entire section. Dr Nicolas Carvou (Department of Physiology and Centre for Nephrology, Royal Free & University College Medical School) provided assistance with the confocal imaging.

3.2.5. Statistics

Western blotting data are presented as mean ± SEM. Since the data did not fit a normal distribution, effects were determined using the Kruskal-Wallis ANOVA by ranks test, with *post-hoc* comparisons performed using the Dunns multiple comparisons post test. All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$. Each group consists of $n=6$-8.

3.3. Results

3.3.1. Western blotting results

Western blotting for detection of GLUT1, GLUT2 and GLUT5 was carried out in order to determine which transport proteins are responsible for the increased GLUT-mediated glucose uptake described in Chapter 2. All three transporters were detectable in BBM vesicles at 54, 58 and 49 kDa, respectively (Fig. 3.1) which corresponds to the known molecular weights of these proteins (Boyer *et al.*, 1996; Dominguez *et al.*, 1992; Miyamoto *et al.*, 1994). There was no significant difference in GLUT1 protein levels in BBM vesicles prepared from control, diabetic and fasted diabetic kidneys (Fig. 3.2). In contrast, 2-4 and 8-10 week STZ-induced diabetes resulted in a marked increase in the expression of GLUT2 at the BBM ($P<0.001$ and $P<0.01$, respectively, compared with control). Overnight fasting of diabetic animals abolished the diabetes-induced increase in GLUT2 protein expression ($P<0.05$ compared with 2-4 week diabetes). GLUT5 protein expression was also significantly increased in 2-4 week diabetes ($P<0.05$), and although the protein appears to be increased in 8-10 week and fasted diabetic animals when compared with control, (Fig. 3.1) this did not reach statistical significance. The biphasic effect of diabetes on GLUT5 protein expression is consistent with published data. Chin *et al*, demonstrated that GLUT5 mRNA levels were increased at 30 and 90 days post-STZ treatment, but was not different to control levels after 180 days (Chin *et al.*, 1997).

Figure 3.1. Detection of GLUT1, GLUT2 and GLUT5 by western blotting using BBM vesicles prepared from renal cortex of control, 2-4 wk and 8-10 wk STZ-induced diabetic rats and overnight fasted, 2-4 wk diabetic rats.

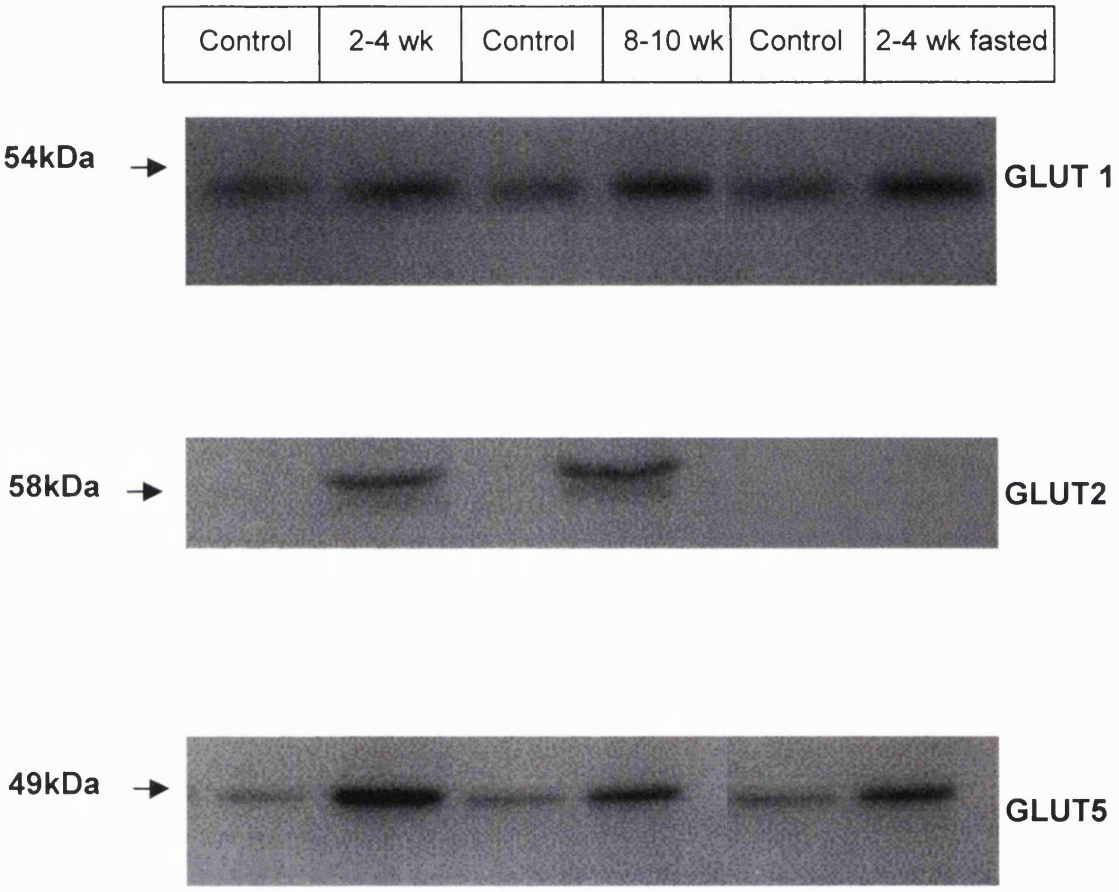
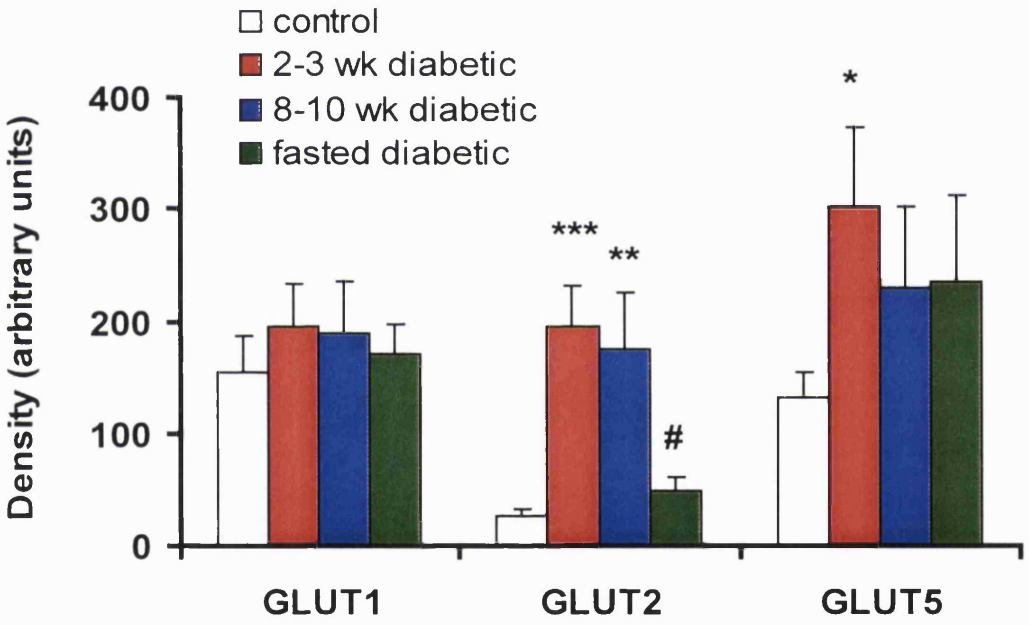


Figure 3.2. Quantification of levels of GLUT1, GLUT2 and GLUT5 from bands produced by western blotting of cortical BBM vesicles prepared from normal, 2-4 week diabetic, 8-10 week diabetic and overnight fasted, 2-4 week diabetic rats. Results are expressed as Mean + SEM. Values were obtained from western blots for each GLUT isoform carried out on 6 vesicle preparations. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control; #*P*<0.05 compared with 2-4 week diabetic using the Kruskal-Wallis ANOVA by ranks test, with *post-hoc* comparisons performed using the Dunns multiple comparisons post test.



3.3.2. Immunohistochemistry

3.3.2.1. Immunohistochemistry performed on unfixed tissue

Immunohistochemistry performed on snap-frozen kidney for the facilitative glucose transporters revealed an expression pattern that was consistent with previous reports (Dominguez *et al.*, 1992; Sugawara-Yokoo *et al.*, 1999). GLUT5 was found to be expressed exclusively at the BBM, whilst GLUT1 and GLUT2 were localised to the BLM. However, western blotting of BBM vesicles clearly demonstrates a shift in the expression pattern of GLUT2 at the BBM during diabetes. An explanation for this failure to detect GLUT2 at the BBM by immunohistochemistry may be that the antibody is unable to recognise its antigenic site. To overcome this potential problem GLUT2 antiserum was raised in rabbits, against a synthetic peptide corresponding to the exoplasmic region (amino acids 40-55) of the rat GLUT2 sequence (Thorens *et al.*, 1988). Peptide synthesis and antibody production was kindly performed by Dr Bala Ramesh (Department of Biochemistry and Molecular Biology, Royal Free & University College Medical School).

Western blots using the antiserum raised against the exoplasmic loop of GLUT2 produced a single band with a molecular weight of 68 kDa (results not shown). However, Thorens and co-workers detected a protein of 57 kDa using an antibody raised against the same peptide sequence (Thorens *et al.*, 1988). Further investigation showed that western blotting using antiserum pre-absorbed with 0.5-2 μg of synthetic peptide, or pre-immune serum, also produced a band of 68 kDa. This indicates that the antiserum contained no specific antibodies directed against the GLUT2 transporter.

3.3.2.2. Immunohistochemistry performed on kidneys fixed *in vivo* with periodate-lysine-paraformaldehyde.

Published findings by Helliwell *et al.* (Helliwell *et al.*, 2000b), demonstrates that GLUT2 is rapidly shuttled into or out of the BBM of enterocytes and *in vivo* fixation enables the detection of the GLUT2 protein at the BBM of this cell type (Affleck *et al.*, 2003; Kellett *et al.*, 2002). Therefore immunohistochemistry was performed on sections obtained from kidneys fixed *in vivo* with PLP.

Immunohistochemistry performed on kidneys that had been fixed *in vivo* with 2% PLP showed the expected basolateral staining of GLUT2 in the S1 segment of the proximal convoluted tubule (PCT) in control animals (Fig. 3.3, panel E). However, staining on both basolateral and brush border membranes of the same region was evident in animals that had been diabetic for three weeks (panel B). In agreement with vesicle uptake and western blotting data, GLUT2 was not apparent at the BBM of proximal tubules from diabetic animals that had been fasted overnight (panel C). Antibody specificity was confirmed using sections probed with antibody pre-absorbed with an excess of antigenic peptide (panel D).

PLP fixative has been reported to preserve antigenicity as effectively as paraformaldehyde, and cellular ultrastructure as effectively as glutaraldehyde (McLean & Nakane, 1974). It was proposed that stabilisation of carbohydrate structures occurs due to the interaction of periodate and lysine. In these reactions, periodate oxidises carbohydrates to form aldehyde groups and lysine, a divalent amine, then cross links the carbohydrate containing molecules by interacting with these aldehyde groups (McLean & Nakane, 1974). Further studies by Hixson *et al.* failed to confirm this mechanism of cross-linking but suggested a complex interaction between periodate oxidised plasma membrane glycoproteins and polymeric complexes of lysine and formaldehyde occurs (Hixson *et al.*, 1981). Regardless of the mechanism involved, *in vivo* PLP fixation has enabled

GLUT2 to be detected at the BBM of the S1 segment of the proximal tubule from diabetic animals and is therefore critical for the detection of GLUT2 at the renal BBM.

The staining pattern of GLUT1 and GLUT5 in control and 3-week diabetic kidneys fixed with 2% PLP was also examined (Fig 3.4). Under both control (panel A) and diabetic (panel B) conditions GLUT1 displayed intracellular, as well as membrane staining, in the S3 segment of the proximal tubule. GLUT5 protein was located exclusively at the BBM of the S3 segment in both conditions (panel C and D). In accordance with the western blotting data, GLUT5 staining appeared more intense in the diabetic proximal tubules (panel D).

3.3.2.3. Confocal imaging of kidney fixed *in vivo* with PLP

Figure 3.5 shows the expression of GLUT2 in proximal tubules from control, diabetic and overnight-fasted diabetic animals as shown by confocal imaging. These experiments were performed using the same C-terminal GLUT2 antibody used for the conventional immunohistochemistry, but aimed to demonstrate, with more precision, the localisation of the protein in the three conditions. Confocal images reveal the same pattern of staining for GLUT2 as shown by conventional immunohistochemistry, but makes evident the cellular localisation of the protein in more detail. In control proximal tubules GLUT2 is localised exclusively at the BLM. Staining in the diabetic proximal tubule reveals that the protein is expressed at both the BLM and BBM. Perhaps most interesting are the confocal images of the proximal tubules from fasted diabetic animals. It can be seen that the protein is no longer expressed at high levels at the BBM, but appears to be redistributed back to the BLM, possibly via transcytosis.

Figure 3.3. Localisation of GLUT2 protein in kidneys from control (A), 3-week diabetic (B) and overnight fasted, 3 week diabetic rats (C). GLUT2 is localised at the BLM in control and overnight fasted kidneys (arrows), but can be detected at both the BLM (arrows) and BBM (arrowheads) of diabetic kidneys. Antibody specificity was confirmed using sections probed with antibody pre-absorbed with a 10-fold excess of antigenic peptide (D). Low magnification image demonstrating that GLUT2 protein is expressed in the outer cortex of the kidney (E). Scale bar (A-D) = 50 μm, (E) = 500 μm.

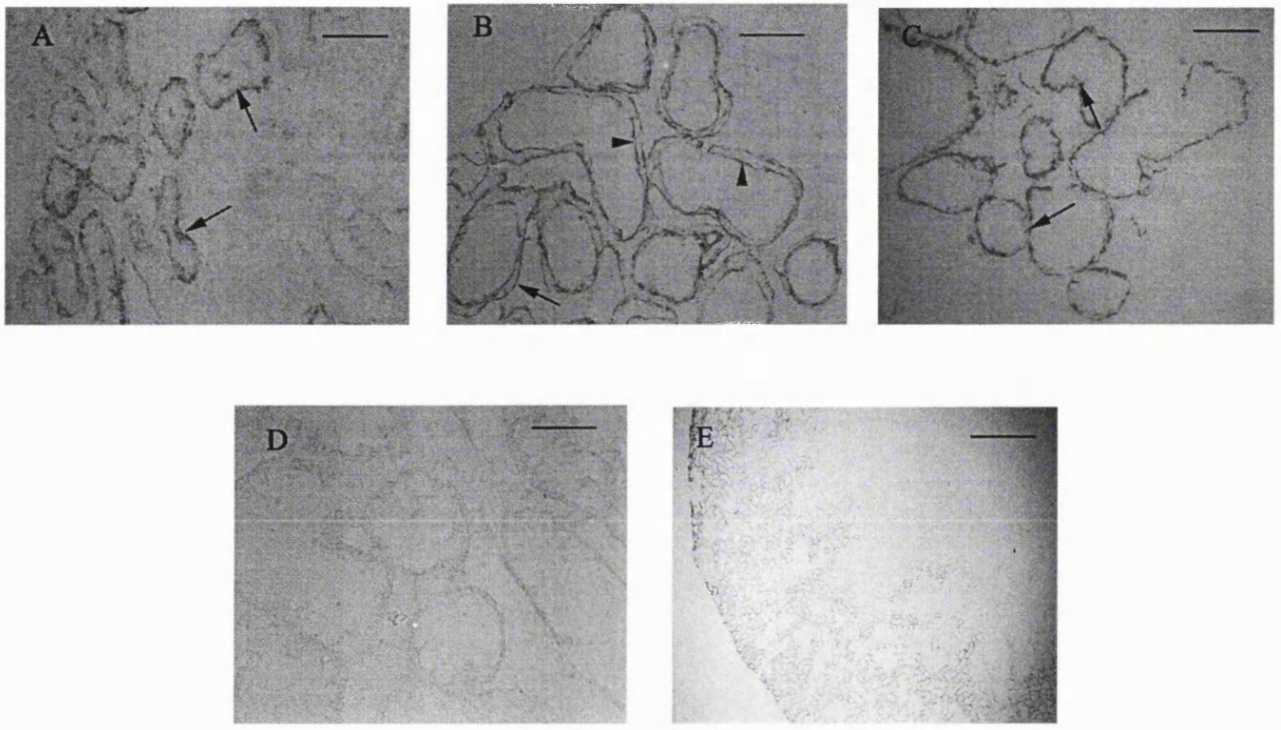


Figure 3.4. Localisation of GLUT1 (A and B) and GLUT5 (C and D) protein in kidneys from control (A and C) and 3-week diabetic rats (B and D). In both control and diabetic kidneys, GLUT1 displays intracellular as well as membrane staining (arrows), whilst, GLUT5 can be detected exclusively at the BBM (arrowheads). Scale bar = 50 μm.

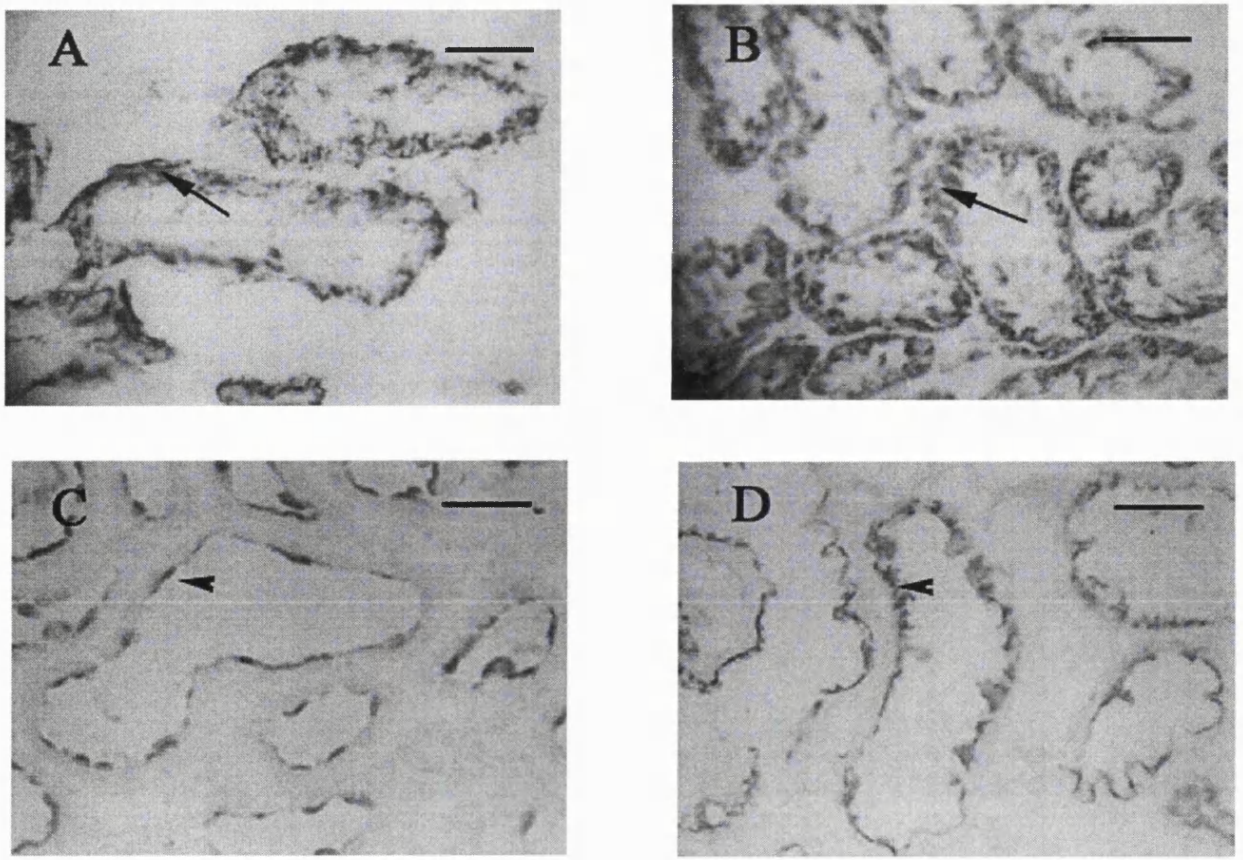
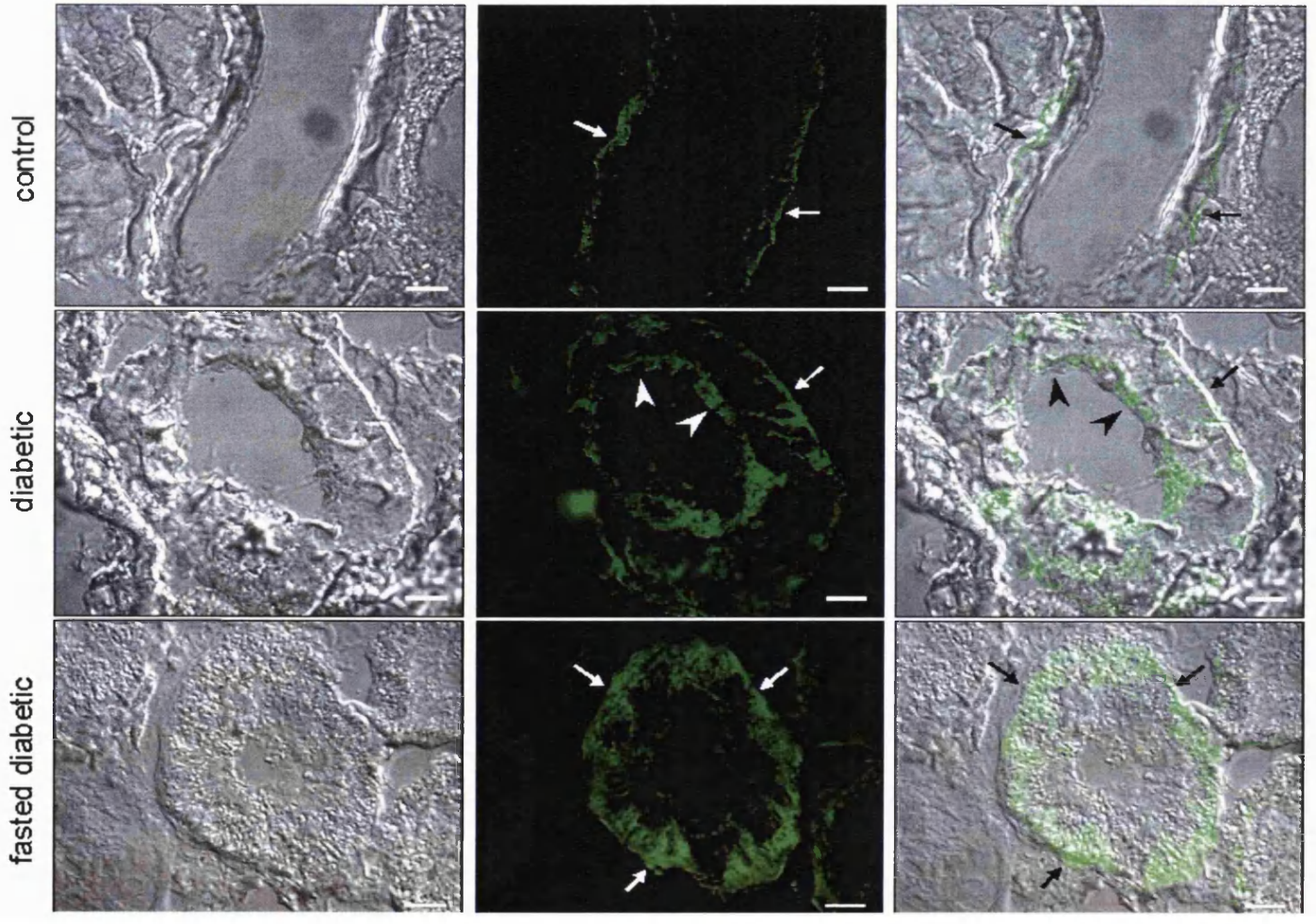


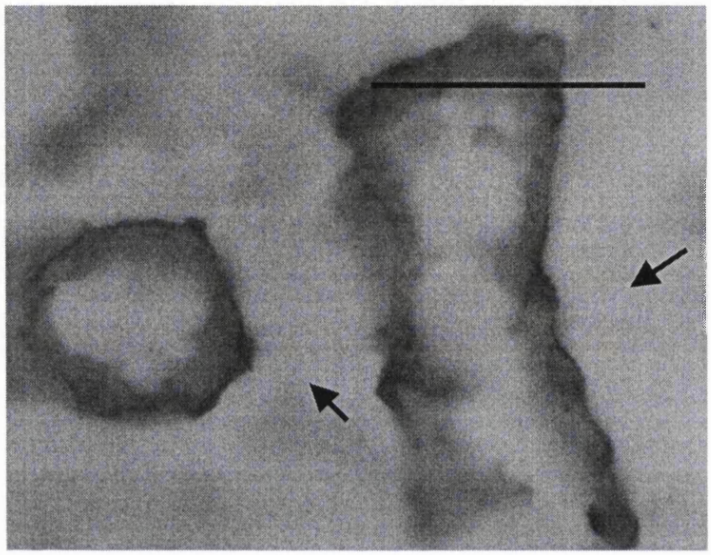
Figure 3.5. Confocal imaging of GLUT2 protein in kidneys from control, 2-4 week diabetic and overnight fasted, 2-4 week diabetic rats. The images obtained were produced as an overlay of 1 to 3 images selected from the Z-stack as the most representative of the entire section. GLUT2 was localised at the BLM (arrows) in control kidneys, but was detected at both the BLM and BBM (arrowheads) of diabetic kidneys. Overnight fasting of diabetic rats showed intracellular and BLM staining of GLUT2. Scale bar = 10 μm



3.3.2.4. Immunohistochemistry performed on kidneys perfused with 7 mM glucose prior to *in vivo* fixation with PLP

Helliwell *et al.* have previously demonstrated that GLUT2 is rapidly translocated into and out of the enterocyte BBM in response to circulating glucose levels (Helliwell *et al.*, 2000b), and that even under conditions of euglycaemia the transporter is present at the BBM (Kellett *et al.*, 2002). However, immunohistochemistry and western blotting of renal tissue has failed to detect this protein at this membrane location, except during conditions of hyperglycaemia. To ensure that removal of blood from the kidneys prior to fixation did not result in redistribution of BBM GLUT2, the Hanks Balanced Salt Solution was supplemented with 7 mM glucose. This glucose concentration is representative of the circulating blood glucose levels of control rats. Figure 3.6 shows GLUT2 staining is only apparent at the BLM of control proximal tubules following perfusion with the supplemented Hanks buffer. This demonstrates that GLUT2 is localised either exclusively to the renal BLM during conditions of euglycaemia or that the levels are so low they can not be detected by immunohistochemistry.

Figure 3.6. Immunohistochemistry demonstrating the BLM localisation (arrow) of GLUT2 in control proximal tubules, the left section shows a tubule cut transversely, whilst the right section shows a longitudinal view. Kidneys were perfused with 7 mM glucose prior to fixation with 2% PLP. Scale bar = 50 μm



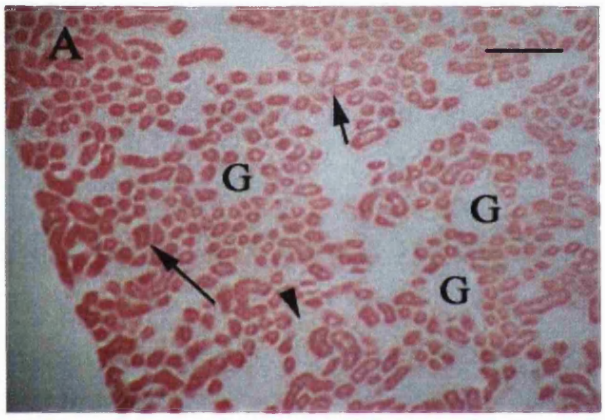
3.3.2.5. Identification of tubular structures using eosin and Vector Red.

In order to confirm the segment specific localisation of the GLUT proteins, sections were probed with Vector Red (Vector Labs LTD, Peterborough, UK) to stain for endogenous alkaline phosphatase, a marker of the BBM, which allows identification of the proximal tubules (Fig. 3.7) (Yusufi *et al.*, 1994). GLUT2 was localised to proximal tubules in the outer cortex (Fig 3.3, panel E), that showed intense staining for alkaline phosphatase (Fig. 3.7), thus representing the S1 segment of the proximal tubule. GLUT1 and GLUT5 staining was apparent in deeper cortical segments, in tubules that displayed less intense staining for alkaline phosphatase (Fig. 3.7), indicative of the S2 and S3 segment of the proximal tubule (Yusufi *et al.*, 1994).

Counter-staining of cryostat sections with eosin (Fig. 3.8) revealed that *in vivo* fixation with PLP reduces tubule collapse, which is a common feature of sections cut from snap frozen kidneys, without prior fixation. Additionally, the thickness of the cytoplasm shows that the pattern of GLUT2 staining in proximal tubules from diabetic kidneys (Fig. 3.3, panel B) is representative of the two membranes on either side of the cytoplasm.

The BBM staining pattern of GLUT5 shows a difference compared with that of GLUT2, with GLUT5 displaying classical BBM staining and GLUT2 appearing to show staining of a smooth BBM. Figure 3.8 demonstrates that outer cortical proximal tubules, the S1 segment, have smooth membranes at both the BLM and BBM, whereas, inner cortical proximal tubules, S3 segments, show classical, convoluted BBM.

Figure 3.7. Low (A) and high (B) magnification images of cryostat sections stained for endogenous alkaline phosphatase using Vector Red. Panel A demonstrates the renal cortex is comprised of a large proportion of alkaline phosphatase positive tubules (arrows), whilst glomerular (G) and tubular structures with no BBM (arrowheads) are unstained. Panel B demonstrates alkaline phosphatase positive tubules stain with different intensities, with the outer cortical nephrons exhibiting intense staining (long arrows), whilst in the deeper cortical nephrons the staining is less intense (short arrows). Scale bar A = 100 μm, B = 50 μm.



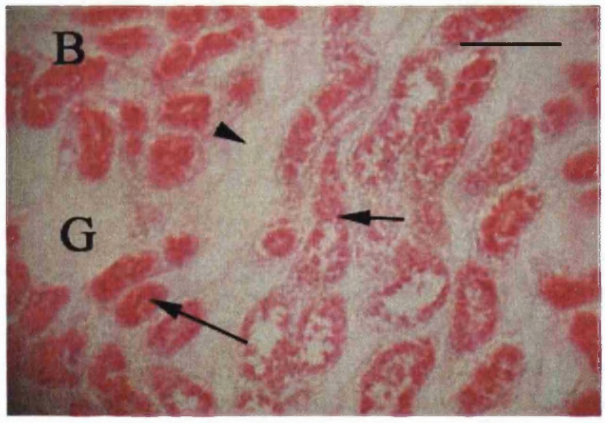
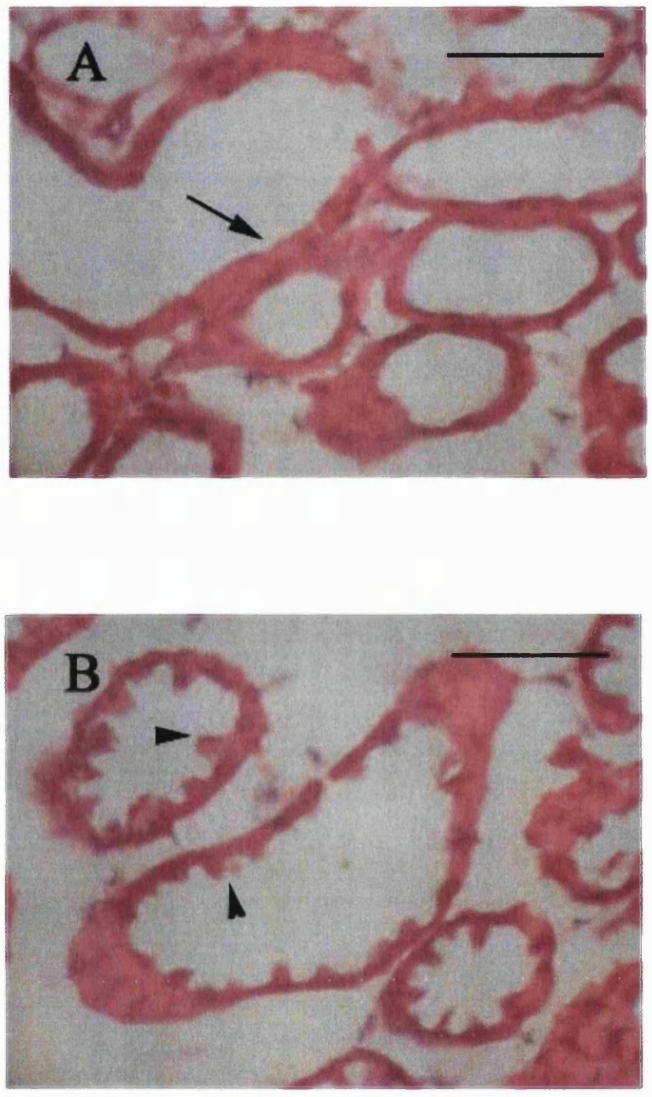


Figure 3.8. Cryostat sections (7 μm) counter-stained with eosin. Panel A shows outer cortical proximal tubules with apparently smooth BBM (arrows). Panel B displays inner cortical proximal tubules with classical BBM features (arrowheads). Scale bar = 50 μm.



3.4. Discussion

As discussed in chapter 2, facilitative transport represents an important route for glucose uptake across the renal BBM during diabetes. Overnight fasting of diabetic rats reduced blood glucose levels to those of controls and abolished the increase in GLUT-mediated transport. The results presented in this chapter, show that the increase in glucose uptake during diabetes is accompanied by significantly raised levels of GLUT2 and GLUT5 protein, but not of GLUT1, at the proximal tubule BBM. Overnight fasting completely abolished the increase in GLUT2 protein expression, yet only had a modest effect on the levels of GLUT5. The observation that GLUT2 is not detectable at the BBM of proximal tubules from control or diabetic animals after an overnight fast, suggests that the protein is shuttled into and out of the BBM in response to changes in plasma or luminal glucose concentrations. Indeed, confocal imaging of GLUT2 in diabetic animals that have been subjected to an overnight fast clearly shows that the protein is no longer located at the BBM. The strong intracellular staining suggests that the protein is being redistributed back to the BLM.

This finding is consistent with the newly proposed mechanism of intestinal glucose transport, where it has been shown that GLUT2 protein is rapidly recruited (within 15 minutes) to the enterocyte BBM in response to increased luminal glucose concentrations (Kellett & Helliwell, 2000). Additionally, STZ-induced diabetes results in an increase in the protein levels of GLUT2 and GLUT5 at the BBM of enterocytes (Chowrimootoo G *et al.*, 1993; Corpe *et al.*, 1996). Recruitment of GLUT2 to the enterocyte BBM in response to raised luminal glucose levels has been shown to be regulated by activation of PKC-βII (Helliwell *et al.*, 2000b). It has been proposed that uptake of glucose by the sodium-dependent transporter, SGLT1, is required for the activation of this PKC isoform and the subsequent insertion of GLUT2 into the enterocyte BBM (Helliwell *et al.*, 2000b). Glucose induced activation of the extracellular signal-regulated kinase (ERK) or the p38 mitogen activated protein kinase (MAP kinase) pathways (Helliwell *et al.*, 2000a) or increased

entry of extracellular calcium, occurring as a result of changes in intracellular osmolarity (Kellett & Helliwell, 2000) may be the factors responsible for PKC-βII activation. Since the conventional mechanisms of renal and intestinal glucose transport display striking similarities (Debnam & Unwin, 1996), it is not unexpected that the mechanisms involved in adapting to high glucose concentrations appears to be comparable in the two cell types. Further investigation into the intracellular signalling pathways involved in promoting GLUT2 to the proximal tubule BBM are likely to further demonstrate the similarities in the regulatory mechanisms of glucose transport in the two epithelial cell populations.

It has been reported previously that the substrates for GLUT2 and GLUT5 can positively regulate the expression and activity of these transporter proteins. GLUT2 is regulated by both glucose and fructose, whilst GLUT5 is regulated by fructose only (Burant & Saxena, 1994; Mesonero *et al.*, 1995). This data is consistent with the proposal that GLUT2 can transport both fructose and glucose (Burant & Bell, 1992; Colville *et al.*, 1993) whereas, *in vivo* GLUT5 only transports fructose (Burant *et al.*, 1992; Inukai *et al.*, 1995). The finding that renal BBM levels of GLUT2 are increased during diabetes and this increase is abolished by overnight fasting, suggests that levels of this transporter are adapted in response to local glucose concentrations. However, the modulation of GLUT5 protein levels appears to be distinct from that of GLUT2. During diabetes the expression of GLUT5 is increased but overnight fasting failed to completely normalise its expression. This increase in GLUT5 levels may therefore be a consequence of increased intracellular fructose concentrations produced as a consequence of increased polyol pathway activity (Larkins & Dunlop, 1992).

The earliest pathological alterations associated with diabetes mellitus are glomerular and tubuloepithelial hypertrophy, which precede and may contribute, to the renal abnormalities that occur at the later stages of the disease, such as glomerulosclerosis and tubulointerstitial fibrosis (Norman & Fine, 1999; Wolf & Thaiss, 1995). These abnormalities result from increased

accumulation of extracellular matrix components such as fibronectin and collagen (Steffes *et al.*, 1989), which has been demonstrated to be a consequence of hyperglycaemia (Larkins & Dunlop, 1992; Nishikawa *et al.*, 2000; Wolf & Thaiss, 1995). Hyperglycaemia-induced mesangial cell damage has been linked to increased expression of GLUT1 protein, leading to elevated intracellular glucose accumulation (Heilig *et al.*, 1995b). It has been shown that transforming growth factor β (TGF-β), a cytokine that is elevated in the glomeruli of diabetic animals and diabetic patients with nephropathy, can induce increased GLUT1 mRNA and protein expression (Inoki *et al.*, 1999). However the mechanisms by which TGF-β enhances GLUT1 expression are unclear. It is possible that TGF-β may act by altering the glycosylation of GLUT1, a mechanism that occurs in fibroblasts displaying increased GLUT1 activity (Mogyorosi & Ziyadeh, 1999). Alternatively, TGF-β may decrease GLUT1 mRNA degradation and increase its transcription rate, a phenomenon which has been demonstrated to occur in response to hyperosmolarity (Hwang & Ismail-Beigi, 2001). Increased activity of GLUT1 during hyperglycaemia could also be due to retention of the protein in the plasma membrane, as a consequence of increased stability or reduced endocytosis.

The causative factors involved in the hyperglycaemia-induced damage of proximal tubule cells have received relatively little attention. Previous studies focusing on the effect of diabetes on proximal tubular glucose reabsorption by SGLT1 have yielded conflicting results (Blank *et al.*, 1989; Harris *et al.*, 1986; Yasuda *et al.*, 1990). However, increased influx of glucose across the BBM by SGLT proteins, has been attributed to mass action effects, due to the higher filtered load of glucose presented to the proximal tubule (Dominguez *et al.*, 1994). Subsequent efflux of glucose from the cell during diabetes then occurs by increased BLM levels of GLUT2 (Dominguez *et al.*, 1994). Studies that have demonstrated increased mRNA or protein levels of this transporter during diabetes have used whole proximal tubule preparations or *in situ* hybridization (Chin *et al.*, 1997; Dominguez *et al.*, 1994), and may therefore not exclusively represent increases in GLUT2

protein expression at the BLM. The results presented in this chapter suggests that GLUT2 protein levels increase at the BBM, as well as the BLM, and that this transporter may be responsible for the increase in glucose influx into the proximal tubular epithelial cells during diabetes. This increased glucose accumulation within the cells may initiate a complex series of biochemical events, resulting in tubulointerstitial damage.

Under normal conditions epithelial transport functions are dependent on the development and maintenance of a precise polarised distribution of proteins and lipids. Specific targeting of proteins within the cell is a complex process, which is initially determined by amino acid sequences within transmembrane and cytosolic proteins. It has been demonstrated that proteins that are sorted to the basolateral domain often contain a critical tyrosine or double leucine residue within the cytoplasmic amino acid sequence (Gut *et al.*, 1998). Apical sorting appears to be dependent on motifs present in the extracellular domain or the region responsible for membrane anchoring (Yeaman *et al.*, 1999). N-glycosylation has also been proposed to be a determinant in apical sorting, however, many proteins can be targeted to the apical membrane in the absence of N-glycosylation (Yeaman *et al.*, 1999). Overall the determinants for sorting to the apical membrane appear to be more diverse than those required for targeting proteins to the basolateral membrane. Sorting of proteins to specific membranes may also be restricted by other factors including the surrounding amino acid sequence, the proximity of the target sequence to the transmembrane domain and protein phosphorylation or glycosylation (Brown, 2000; Yeaman *et al.*, 1999). Additionally, it has been demonstrated that proteins may be transported indirectly via the basolateral membrane where they are rerouted to the apical membrane by endocytic and transcytotic processes (Simons & Wandinger-Ness, 1990).

Interestingly some proteins, including aquaporin-1 (AQP-1) can be delivered to both the apical and basolateral membrane. It is unknown if such proteins contain motifs that allows sorting to both the BLM and BBM or if there is an absence of a specific signalling motif (Brown, 2000). Additionally, it has

been demonstrated that basolateral proteins with inactivated basolateral targeting domains can be expressed at the apical membrane, indicating that targeting to the BLM is the dominant pathway (Brown, 2000). For example, mutation of the anion exchanger, AE1, causes mistargeting of the protein from the basolateral to the apical membrane, resulting in distal tubular acidosis (Mostov *et al.*, 2003). It has been suggested that the dominance of basolateral sorting may be a consequence of the basolateral determinants having a higher affinity for their sorting machinery than apical targeting signals. Alternatively, the specific motifs for apical or basolateral sorting may be spatially separated, with the proteins encountering the motif for basolateral sorting before that of the apical signal (Gut *et al.*, 1998).

Transepithelial glucose transport occurs as a consequence of the distinct profile of BBM and BLM glucose transporter protein expression, which displays striking similarity in the enterocyte and proximal tubule cell. It is widely accepted that Na^+-dependent glucose transporters are expressed at the BBM, as is the fructose transporter GLUT5. GLUT1 and GLUT2 are considered to be proteins that are exclusively targeted to the BLM (reviewed in (Debnam & Unwin, 1996; Thorens, 1996). The GLUT transporters show considerable sequence and structural homology (Baldwin, 1993) although significant isoform differences occur that determine their substrate specificity and membrane localisation. However, the exact mechanisms involved in this selective translocation have not been fully clarified. Employing glucose transporter chimeras Czech *et al.* have suggested that the COOH-terminal cytoplasmic sequence of GLUT1 and GLUT4 is important for the cellular localisation of these transporters (Czech *et al.*, 1993). This finding was further substantiated by the fact that a protein termed GLUT1 C-terminal binding protein (GLUT1CBP) specifically interacts via a PDZ domain with the C-terminal of GLUT1 (Bunn *et al.*, 1999). The protein was also found to interact with cytoskeletal motor proteins and therefore may be involved in vesicle trafficking or anchoring of GLUT1 to its specific membrane domain (Bunn *et al.*, 1999). However, Asano *et al.* demonstrated that the region consisting of amino acids 300-327 of GLUT1 was essential for targeting of the protein to the BLM. This region is representative of the whole of the 7^{th}

transmembrane domain, part of the exoplasmic domain between transmembrane domains 7 and 8 and a portion of the intracellular loop (Asano *et al.*, 1992). A later study by this group using chimeras of GLUT1 and GLUT5 determined that the intracellular loop of the GLUT5 transporter played a pivotal role in the apical sorting of this transporter (Inukai *et al.*, 1997). This study used a chimera which contained two reciprocal swapping sites; the translated protein contained the N-terminus to transmembrane domain 6 (TM6) of GLUT5, the intracellular loop domain of GLUT1 and the TM7 to C-terminus of GLUT5, and was found to be expressed at the BLM (Inukai *et al.*, 1997). Although convincing, these results would have been strengthened by the production of an additional chimera, containing the intracellular loop of GLUT5, with the remaining regions of GLUT1, to demonstrate conclusively that the intracellular loop was indeed responsible for targeting of facilitative glucose transporters to specific membrane domains. The cell types used for the expression of the chimeras, or the density at which they were expressed may explain differences between the results of these two groups. However, the later paper by Asano *et al.* employed the Caco-2 cell line, and investigated the difference in the sorting of the apical protein GLUT5 and the basolateral protein GLUT1, and may therefore be of more relevance to this study than the results of Czech *et al.* Overall, from the results of these two independent groups it could be assumed that the COOH terminal is responsible for targeting of GLUT transporters to the BLM, whilst the intracellular loop is required for specific targeting to the apical domain. Indeed it has been suggested that GLUT transporters harbour one or more motifs that direct the protein to its specific cellular location (Joost & Thorens, 2001). At present there is no evidence in the literature that the membrane specific translocation of GLUT2 in polarised epithelial cells has been investigated. Establishing the motifs responsible for GLUT2 targeting during euglycaemia would advance the understanding of GLUT2 trafficking *per se* and enable the mechanisms involved in targeting of the protein to the BBM during conditions of hyperglycaemia to be elucidated.

Targeting of protein to specific membrane domains involves three distinct processes. Apical and basolateral proteins are sorted into separate transport

vesicles in the *trans*-Golgi network (TGN) depending on the specific amino acid sequence. These protein containing vesicles are then trafficked to their specific membrane where docking and fusion with the membrane occurs (Yeaman *et al.*, 1999). The cell cytoskeleton is responsible for movement of proteins from the TGN to specific cellular domains. This consists of different types of filamentous structures, including microtubules (which are formed from tubulin monomers), actin (which is composed of globular actin monomers) and intermediate filaments (that contain long chains of amino acids) (Brown & Breton, 2000). Microtubules deliver newly synthesised and recycled proteins to the cell surface, by interaction of the transport vesicles with ATPase motor proteins, such as the dynelin and kinesin familiy. These motor proteins use the energy created by ATP hydrolysis to move the protein rich vesicles along the microtubules (Brown, 2000). Experimental evidence using agents that disrupt either the actin cytoskeleton or microtubules, suggests that the latter are involved in "long range" transport of protein rich vesicles, whereas actin filaments are involved in the final steps of vesicle docking with the cell membrane. Additionally it has been proposed that actin acts as an intracellular scaffold that retains proteins in their specific membrane domain (Brown, 2000). PDZ-binding proteins form another actin-based scaffolding system, which maintains proteins in specific membrane domains. These PDZ-binding proteins are maintained at specific membranes by interaction with actin, where they can then form complexes with proteins containing PDZ-motifs within their amino acid sequence (Brown, 2000). It has been proposed that PDZ-binding proteins are involved in the maintenance of functionally interdependent proteins within a specific cellular domain, thus increasing the efficiency of their interactions (Brown & Breton, 2000).

The process of vesicle recognition, docking and fusion is highly specific in order to prevent protein insertion with the incorrect membrane domain. The SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) hypothesis was proposed in the early 1990's (Sollner *et al.*, 1993), and postulates that the protein rich vesicles contain addressing proteins (v-SNAREs) which interact with specific receptors on the target membrane (t-

SNAREs). Two t-SNARE proteins have been identified in epithelial cells, syntaxin 3 is localised exclusively to the BBM, whilst syntaxin 4 is found only at the BLM (Mostov *et al.*, 2003). It was proposed that these proteins provide a mechanism that confers specificity of protein insertion into the target membrane (Yeaman *et al.*, 1999). However, this hypothesis has undergone considerable debate and it is now suggested that GTPases of the Rab protein family, are involved in the specific docking and anchoring of protein-rich vesicles, whilst, SNARE proteins are responsible for the later stages of membrane fusion (Chen & Scheller, 2001; Mayer, 1999; Pelham, 2001).

Defective targeting and trafficking of proteins has been linked to a number of diseases, which have been grouped under the name of 'sorting disorders' (Brown & Breton, 2000). One such disease is cystic fibrosis, which is associated with reduced expression of the protein CFTR (cystic fibrosis transmembrane conductance regulator), a cAMP-activated chloride channel of lung epithelia (Stein *et al.*, 2002). Appropriate trafficking of the CFTR protein is dependent on two C-terminal domains and a PDZ-binding motif (Stein *et al.*, 2002). Reduced protein expression during cystic fibrosis is largely due to ineffective folding of the protein leading to its retention and degradation in the rough endoplasmic reticulum (RER) (Qu *et al.*, 1997). However, 10% of CFTR mutations result from deletion or mutation of the C-terminus of the protein sequence (Stein *et al.*, 2002). These defects lead to impaired chloride secretion and result in the alterations in lung epithelia function characteristic of the disease.

Defective targeting of membrane components has also been demonstrated to lead to changes in renal function. Nephrogenic diabetes insipidus (NDI) is characterised by polyuria and polydipsia and ultimately leads to impaired renal function (Oksche & Rosenthal, 1998). This disease can be inherited as an autosomal recessive or dominant trait (Marr *et al.*, 2002), or acquired through administration of pharmacological agents, such as lithium, which is used for the treatment of affective disorders, such as manic depression (Marples *et al.*, 1995). The molecular basis of the disease has recently been

investigated and has been shown to occur as a consequence of reduced insertion of the water channel, aquaporin 2 (AQP2), into the apical membrane of the principle cells of the collecting duct. AQP2 is regulated by cAMP induced insertion of the protein into the apical membrane, where binding of the anti-diuretic hormone, arginine-vasopressin (AVP), to its V2 receptor (AVPR2) situated at the basolateral membrane, leads to an increase in intracellular cAMP levels and subsequent phosphorylation and redistribution of the protein to the BBM (Marr *et al.*, 2002). Acquired NDI appears to occur due to reduced responsiveness of AVP-induced insertion of AQP2 into the apical membrane (Marples *et al.*, 1995), whereas inherited NDI is a consequence of mutations in the AQP2 gene, leading to misfolding of the translated protein and its subsequent retention in the ER (Marr *et al.*, 2002).

Therefore, the finding that GLUT2 is expressed at the BBM of the proximal tubule during STZ-induced diabetes may be due to a variety of factors. These may include loss of polarity of the epithelial cells, mutation in the basolateral signalling mechanism or as a consequence of activation of intracellular signalling cascades mediating apical expression. Since activation of a variety of major intracellular signalling pathways have been shown to increase enterocyte BBM expression of GLUT2 (Helliwell *et al.*, 2000a), this mechanism seems to be the most likely candidate for the control of renal BBM GLUT2 expression. However, the shuttling mechanisms involved in the redistribution of GLUT2 in enterocytes have not been investigated. It is unclear at present if GLUT2 is redistributed from the BLM to the BBM, by transcytosis or if a mechanism exists that permits direct targeting of the protein from the TGN. Interestingly, it has been shown that transcytosis of BLM membrane proteins to the apical membrane can occur as a result of increased intracellular calcium levels (Mostov *et al.*, 2003). High glucose concentrations can raise intracellular calcium levels in a number of cell types including the proximal tubule (Symonian *et al.*, 1998). Indeed it has been shown that basal levels of calcium are increased in the proximal tubules of rats treated with STZ (Marcinkowski *et al.*, 1997). Therefore expression of GLUT2 at the BBM during conditions of

hyperglycaemia may be a consequence of transcytosis of GLUT2 protein from the BLM, in response to glucose induced changes in intracellular calcium concentration. Experiments could be performed using green fluorescent protein (GFP)-tagged GLUT2 according to the method of Schulein *et al.* (Schulein *et al.*, 1998). Using real-time confocal microscopy the cellular distribution of GLUT2 could be examined when cells are exposed to high glucose concentrations or following PKC activation and increased intracellular calcium concentration.

3.5. Conclusion

The finding that GLUT2 can be detected by immunohistochemistry at the BBM of proximal tubule cells from diabetic animals is consistent with the vesicle uptake and western blotting data, and confirms that contamination of BLM is not responsible for the results presented in chapter 2. The fact that GLUT2 has not been localised previously to this membrane may be a consequence of the dependence of this shuttling mechanism on ambient plasma glucose levels (Helliwell *et al.*, 2000b). Therefore, it is likely that GLUT2 provides a dominant low affinity, high capacity pathway for proximal tubule glucose reabsorption across the BBM during diabetes.

Chapter 4.

Involvement of the protein kinase A (PKA) and protein kinase C (PKC) signalling pathways in the differential regulation of SGLT and GLUT-mediated glucose transport.

4.1. Introduction

Cyclic adenosine 3',5'-monophosphate (cAMP) is an intracellular secondary messenger, involved in signal transduction following receptor stimulation. However, extracellular cAMP has more recently been shown to influence cellular function, including that of the proximal tubule. For example, the nucleotide mediates the actions of parathyroid hormone (PTH) on phosphate excretion (Friedlander *et al.*, 1992) and is involved in the increased excretion of sodium, phosphate and water in response to pancreatic glucagon (Ahloulay *et al.*, 1996). These responses occur via two distinct mechanisms. Binding of PTH to its basolaterally located receptors in the proximal convoluted tubule (PCT), induces an increase in intracellular cAMP, followed by efflux of the nucleotide across the apical membrane. This nephrogenous cAMP is then degraded by membrane bound ecto-5'-nucleotidases to adenosine, which in turn inhibits phosphate reabsorption in the proximal straight tubule (PST) (Friedlander & Amiel, 1995). Although this group has provided compelling evidence for the involvement of extracellular cAMP in the regulation of renal phosphate transport, a role for intracellular accumulation of cAMP in the regulation of phosphate transport has also been established (Cole *et al.*, 1987; Lederer *et al.*, 1998). In contrast, the effect of cAMP on the renal handling of sodium, phosphate and water in response to glucagon occurs as a consequence of liver-derived cAMP. Circulating glucagon stimulates the production of cAMP from the liver by the binding of the hormone to adenylate cyclase coupled receptors, resulting in an increase in intracellular cAMP and its subsequent release into the circulation (Bankir *et al.*, 1997). This circulating cAMP is then freely filtered by the kidney (Broadus *et al.*, 1970) where it has been shown to influence proximal tubular function, particularly in the PST (Ahloulay *et al.*, 1995; de Rouffignac *et al.*, 1991).

Little is known about the regulatory mechanisms involved in the control of renal glucose transport. However, studies using the *Xenopus* oocyte expression system (Hirsch *et al.*, 1996) and those performed using intestinal enterocytes, imply that cAMP is involved in the regulation of SGLT-mediated

glucose transport. Thus, it has been reported that increased levels of cAMP promote glucose transport across the enterocyte brush border membrane (BBM) (Sharp & Debnam, 1994b), with increased expression of the sodium-dependent glucose transport, SGLT1 (Brown M *et al.*, 1997), together with an increase in the membrane electrochemical gradient (Sharp & Debnam, 1994b) being the likely mechanisms. These observations, together with the suggestion that cAMP alters the potential difference at the brush border membrane of renal proximal tubule cells (Lipkowitz & Abramson, 1989), raises the possibility that cAMP is involved in the regulation of renal glucose transport.

At the time of experimentation little was known about the intracellular signalling events involved in the regulation of the sodium-independent glucose transporters at the BBM of epithelial cells. However, recent publications have described the involvement of the protein kinase C (PKC) signalling pathway in the control of facilitative glucose transport at the intestinal BBM. Helliwell *et al.* have demonstrated that perfusion of rat jejunum *in vitro* with phorbol 12-myristate 13-acetate (PMA), a PKC agonist, results in enhanced fructose transport. This increased transport is correlated with an increase in GLUT2 protein expression at the BBM and increased activation of PKC-βII (Helliwell *et al.*, 2000b). Additionally, this group has provided evidence that BBM transport by both GLUT2 and GLUT5, is rapidly modulated by the extracellular signal-regulated kinase (ERK) and p38 MAP kinase pathways and by the phosphatidylinositol (PI) 3-kinase-dependent pathway (Helliwell *et al.*, 2000a). The authors conclude that activation and cross-talk between these pathways is involved in the regulation of glucose and fructose transport in response to glucose in the intestinal lumen and also during pathophysiological conditions, such as diabetes (Kellett *et al.*, 2002).

The aim of the experiments described in this chapter was to elucidate the intracellular signalling events involved in regulating renal SGLT and GLUT-mediated glucose transport.

4.2. Materials and Methods

4.2.1. Incubation of cortical suspension

The study used male Sprague-Dawley rats of weight 230-260g allowed *ad libitum* access to food (diet RM1, SDS Ltd, Witham, Essex, UK) and water up to the time of experimentation. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Animals were anaesthetised with intraperitoneal pentobarbitone sodium (Sagatal, Rhone-Merieux, Harlow, UK, 90 mg/kg) and the kidneys excised and placed in ice-cold 154 mM NaCl. Cooled kidneys were then placed individually onto an ice-cold surface, the renal capsule removed and the kidney sliced into 2 mm sections. The cortex and medulla were clearly distinguishable allowing accurate dissection of the cortical region only. Cortical fragments from 6 kidneys were pooled and then divided into two equal aliquots. These were subsequently lightly homogenised in 30 ml of buffer containing 300 mM mannitol, 12 mM Tris HCl and 5 mM EGTA (pH 7.4) using 6 strokes of a hand held glass-Teflon homogeniser. Paired incubations were carried out by adding either 2 mM cAMP (native) or 2 mM dibutyryl cAMP (membrane permeable) in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) (inhibits phosphodiesterase) to one suspension, the other receiving 2 mM mannitol to act as an osmotically balanced control. Some incubations with cAMP included the addition of 200 μM probenecid (blocks membrane transfer of cAMP) or 20 μM colchicine (disrupts microtubules). Paired incubations were also carried out using 1 μM phorbol 12-myristate (PMA), a protein kinase C activator or 1 μM thapsigargin, an agent known to cause the release of calcium from intracellular stores, with the vehicle acting as the control. Suspensions were incubated at 37°C in a shaking water bath, whilst being gassed with 100% oxygen. After 30 minutes, tubules were separated from the suspension by centrifugation (2 minutes at 500 rpm) and BBM vesicles prepared from the precipitate. All chemicals were purchased from Sigma (Dorset, UK).

4.2.2. BBM vesicle preparation (as 2.2.3.) and uptake studies (as 2.2.4.)

4.2.3. Western blotting (as 3.2.3.)

4.2.4. Statistics

Results are expressed as mean ± SEM of 6 vesicle preparations. Statistical comparisons were made using either a Student's unpaired or paired *t* test (or a Wilcoxon matched-pairs test when data were found not to fit normal distribution). All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

4.3. Results

4.3.1. Viability of cortical preparation

Light microscopy of the cortical tubule preparation (Fig 4.1) indicated the presence of small clumps of tubules, consisting mainly of proximal tubules often with glomeruli still attached. Viability of the preparation was confirmed by the ability of the tubule cells to exclude Trypan Blue dye for periods of upto 1 hour (results not shown).

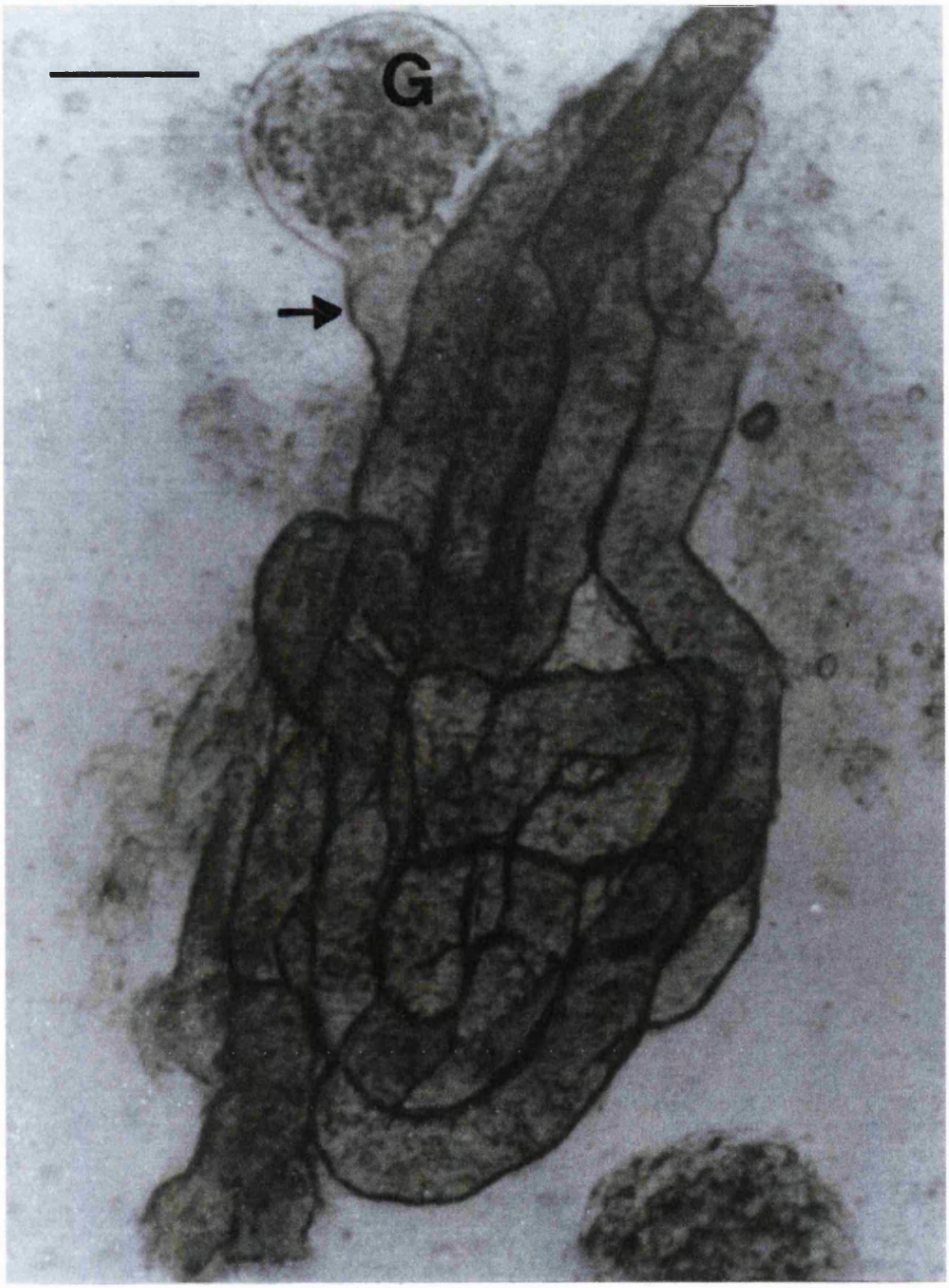
4.3.2. BBM vesicle validation

Validation of BBM vesicles was carried out as described in 2.2.3.1 and 2.2.4.1. Vesicles displayed the characteristic time-dependent overshoot that could be inhibited by phlorizin. Vesicle trapped space was unaffected by incubation with cAMP, PMA and thapsigargin (in μl/mg protein: control: 2.08 ± 0.21 *vs.* experimental: 2.45 ± 0.20, $n = 14$, $P>0.1$, unpaired *t* test). Vesicles were enriched with alkaline phosphatase (control: 6.5 ± 0.38 vs. experimental: 6.71 ± 0.32, $n = 30$, $P>0.5$ unpaired *t* test) but not Na^+/K^+-ATPase (control: 0.88 ± 0.15 vs. experimental: 0.79 ± 0.13, $n = 15$, $P>0.5$ unpaired *t* test).

4.3.3. Regulation of SGLT-mediated glucose transport by cAMP

Incubation of the cortical tubule suspension for 30 minutes with either cAMP or dibutyryl cAMP significantly increased the V_{max} of SGLT-mediated glucose transport (Fig 4.2, A), but had no effect on GLUT-mediated uptake (Fig 4.2, B). Since 'native' membrane-impermeable cAMP and its membrane-permeable analogue, dibutyryl cAMP, both increase SGLT-mediated glucose transport, it implies that the nucleotide has both intracellular and extracellular actions. The finding that probenecid, a recognised inhibitor of membrane cAMP transport (Strewler, 1984), failed to abolish the cAMP induced increase in SGLT-mediated glucose transport (Fig 4.2, A), suggests that the extracellular action of the nucleotide may occur through binding to specific membrane receptors. cAMP receptors (cARs) have been cloned and characterised in lower eukaryotes, and have been classed as members of the superfamily of 7 transmembrane domain G-protein coupled receptors, that includes receptors for secretin and parathyroid hormone (Bankir L *et al.*, 2002; Klein *et al.*, 1988). Additionally, G-protein coupled receptor has been cloned from higher eukaryotes, that displays 23% homology to cARs (Josefsson & Rask, 1997). Although not yet identified in mammals, the observation that cAMP specifically binds to BBM isolated from rabbit renal cortex (Insel P *et al.*, 1975), and endogenous cAMP influences proximal tubular sodium and phosphate transport (Ahloulay *et al.*, 1996), has lead Bankir and colleagues to propose the existence of renal cAMP receptors (Bankir L *et al.*, 2002).

Figure 4.1. Light microscopic appearance of the cortical preparation. A proximal tubule together with an attached glomerulus (G) can be seen. Arrow indicates the beginning of the S1 segment. Scale bar = 50 μm.



The ability of the nucleotide to increase SGLT-mediated glucose transport within 30 minutes indicates that *de novo* protein synthesis is not required for the cAMP induced increase in glucose transport. The observation that colchicine, an agent that disrupts the microtubular network (Pavelka & Gangl, 1983), inhibits the cAMP induced increase in sodium-dependent glucose transport ($P>0.2$, Wilcoxon matched-pairs test) (Fig 4.2, A), implies that the nucleotide promotes trafficking of SGLT protein to the renal BBM. Western blotting of BBM vesicles prepared following 30 minute exposure to cAMP revealed consistently higher levels of SGLT1 protein, but not of GLUT1, GLUT2 or GLUT5 (Fig 4.3). Quantification of these western blots showed that the expression of SGLT1 protein was increased by 124% following cAMP incubation compared with control ($P<0.05$, paired *t* test) (Fig 4.4) This finding is in keeping with SGLT1 expression studies carried out in *Xenopus* oocytes, which showed that activation of the PKA signalling pathway, by 8-Bromo-adenosine 3',5'-cyclic monophosphate (8-Br-cAMP), increased the level of SGLT1 protein in the plasma membrane (Hirsch *et al.*, 1996). Additionally, a more recent study has demonstrated that *in vitro* exposure of rat jejunum to forskolin, an agent that stimulates endogenous cAMP levels through the activation of adenylate cyclase (Lipkowitz & Abramson, 1989), results in an increase in the expression of SGLT1 at the enterocyte BBM (Williams & Sharp, 2002).

Figure 4.2. Effect of 30 minutes exposure of renal cortex to 2 mM dibutyryl cAMP (db-cAMP) and 2 mM cAMP on **A** the V_{max} of SGLT-mediated glucose transport and **B** GLUT-mediated glucose transport. Data from co-incubation with 20 μM colchicine (+ col) and 200 μM probenecid (+ prob) are also shown. Values are expressed as Mean ± S.E.M and are representative of 6 vesicle preparations per group. **P*<0.05 compared with control (open bars) using a paired *t* test.

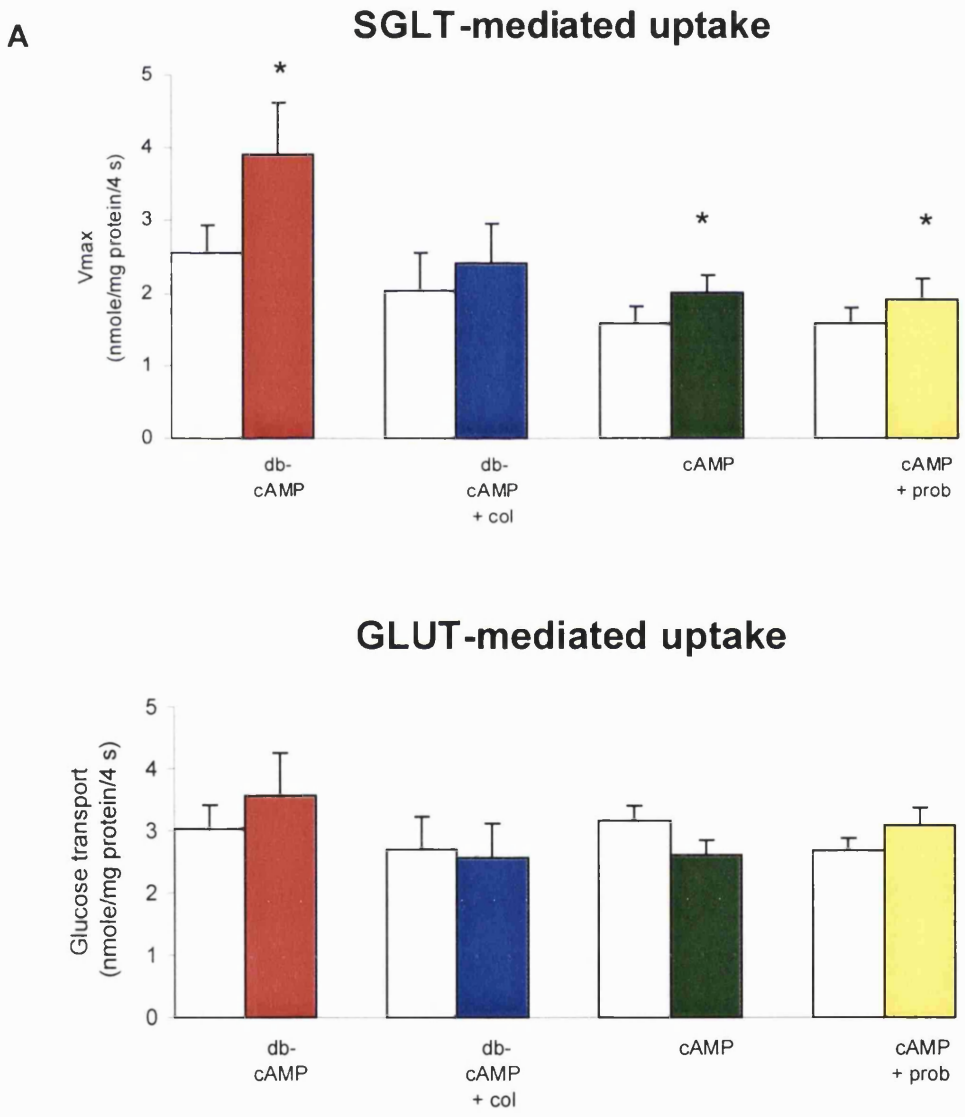


Figure 4.3. Detection of GLUT1, GLUT2, GLUT5 and SGLT1 by western blotting using BBM vesicles prepared from renal cortex exposed to 2 mM cAMP for 30 minutes at 37°C. Representative of n = 6.

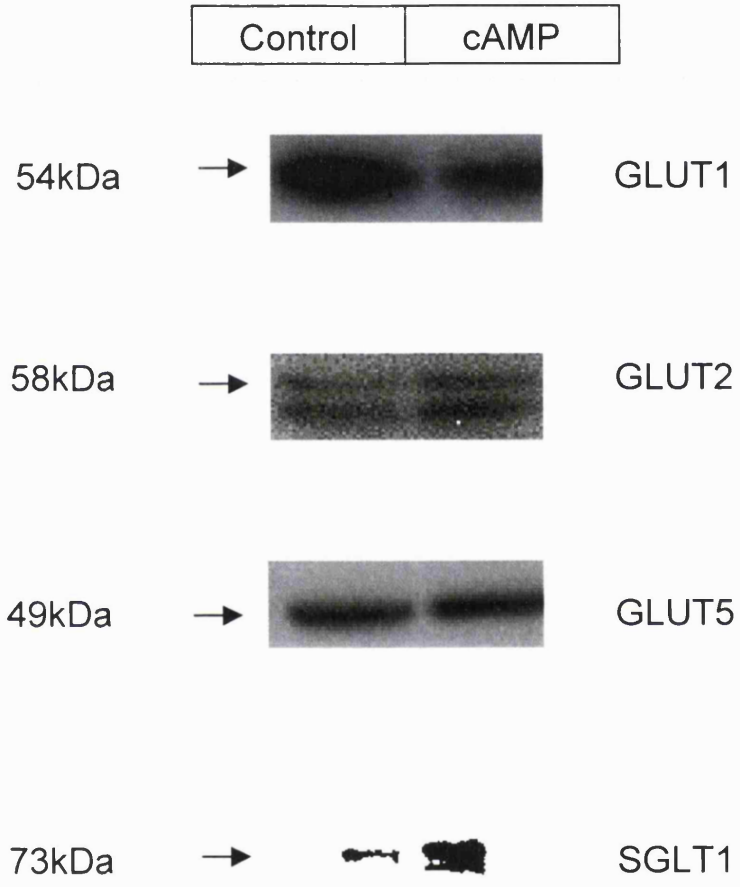
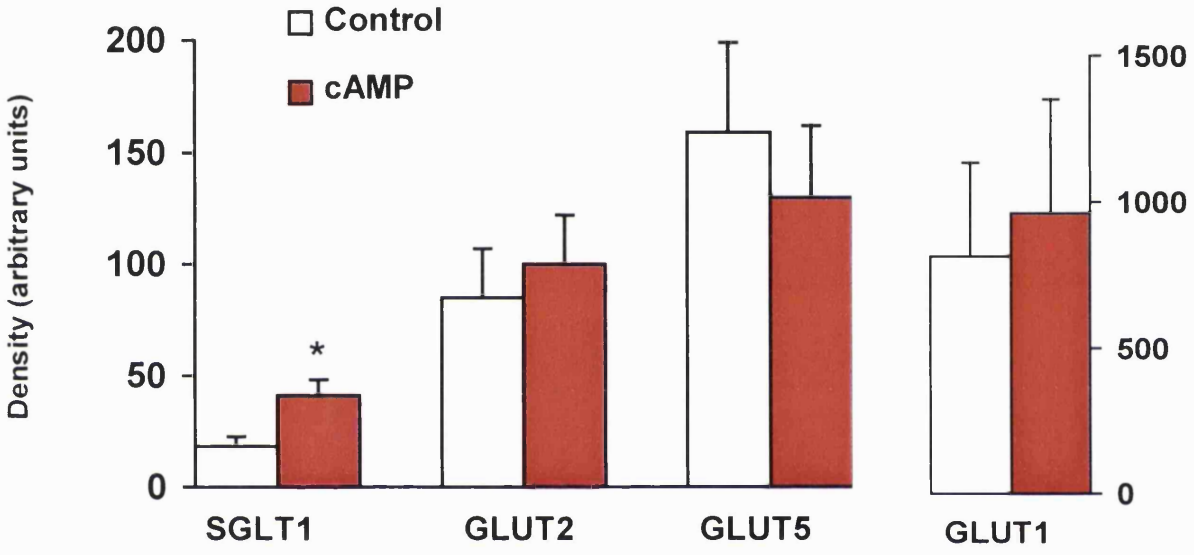


Figure 4.4. Quantification of levels of GLUT1, GLUT2, GLUT5 and SGLT1 protein in BBM vesicles prepared following exposure to 2 mM cAMP. Results were obtained from western blots carried out on 6 vesicle preparations, and expressed as Mean ± SEM. *P<0.05 compared with control (open bars) using a paired t test.



4.3.4. Regulation of GLUT-mediated transport by the PKC signalling pathway

In contrast to cAMP, the V_{max} for SGLT-mediated transport was unaffected by cortical exposure to 1 μM PMA or 1 μM thapsigargin (Fig 4.5, A). GLUT-mediated uptake was however significantly enhanced by 45% and 118% following 30 minute incubation with 1 μM PMA and 1 μM thapsigargin, respectively ($P<0.05$) (Fig 4.5, B). Exposure to 500 nM PMA increased GLUT-mediated uptake by 41% but this failed to reach statistical significance (control: 1.37 ± 0.23 *v's* PMA: 1.94 ± 0.19 $P=0.06$). This increase in GLUT-mediated transport is consistent with the findings of Helliwell *et al.* who demonstrated that activation of the PKC signalling pathway is involved in the regulation of intestinal fructose transport (Helliwell *et al.*, 2000b).

Western blotting of BBM vesicles prepared following exposure of the cortical tubule suspension to PMA or thapsigargin, revealed no change in GLUT transporter expression levels (Fig 4.6). Therefore, unlike the affect of cAMP on SGLT transporter levels, activation of PKC or release of calcium from intracellular stores does not influence GLUT transporter expression. In the absence of increased protein expression an increase in the intrinsic activity of one or more of the facilitative glucose transporters already present in the BBM, may be responsible for the increase in GLUT-mediated uptake. Indeed, this mechanism of modulating transport activity has been reported previously for facilitative glucose transporters (Au *et al.*, 2002; Helliwell *et al.*, 2000a).

Thapsigargin has been shown to specifically increase cytosolic free calcium levels, by the direct discharge of intracellular stored calcium, without the activation of PKC or inositophospholipids (Jackson *et al.*, 1988). In contrast, PKC activation by phorbol esters has been associated with a rise in intracellular calcium levels in a variety of cell types, an effect which has been proposed to be a consequence of membrane depolarization or activation of calcium channels (Symonian *et al.*, 1998). Therefore, the finding that both

Figure 4.5. Effect of 30 minutes exposure of renal cortex to 1 μM PMA and 1 μM thapsigargin on **A** the Vmax of SGLT-mediated glucose transport and **B** GLUT-mediated glucose transport. Values are expressed as Mean ± S.E.M and are representative of 6 vesicle preparations per group. *P<0.05 compared with control (open bars) using a paired t test.

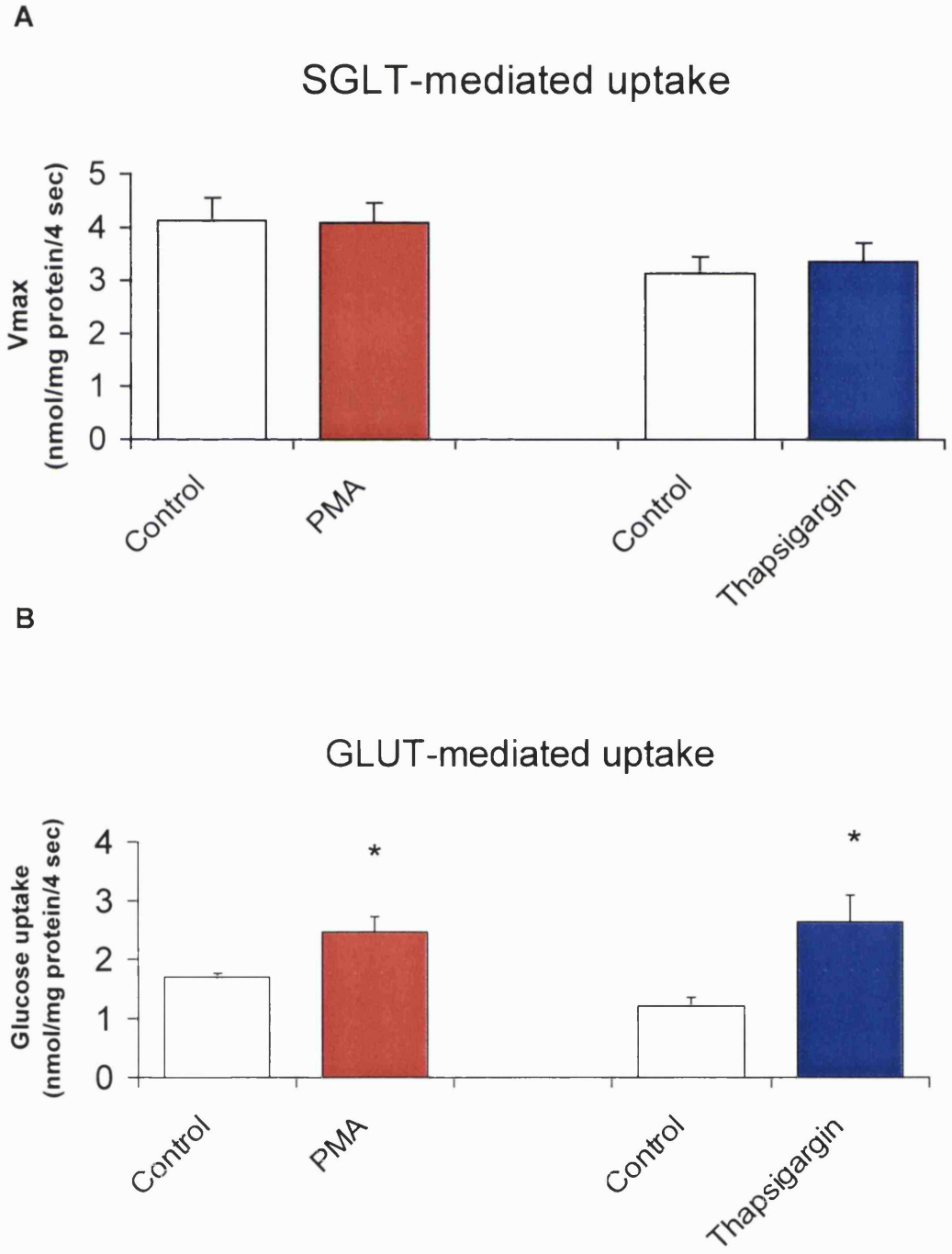
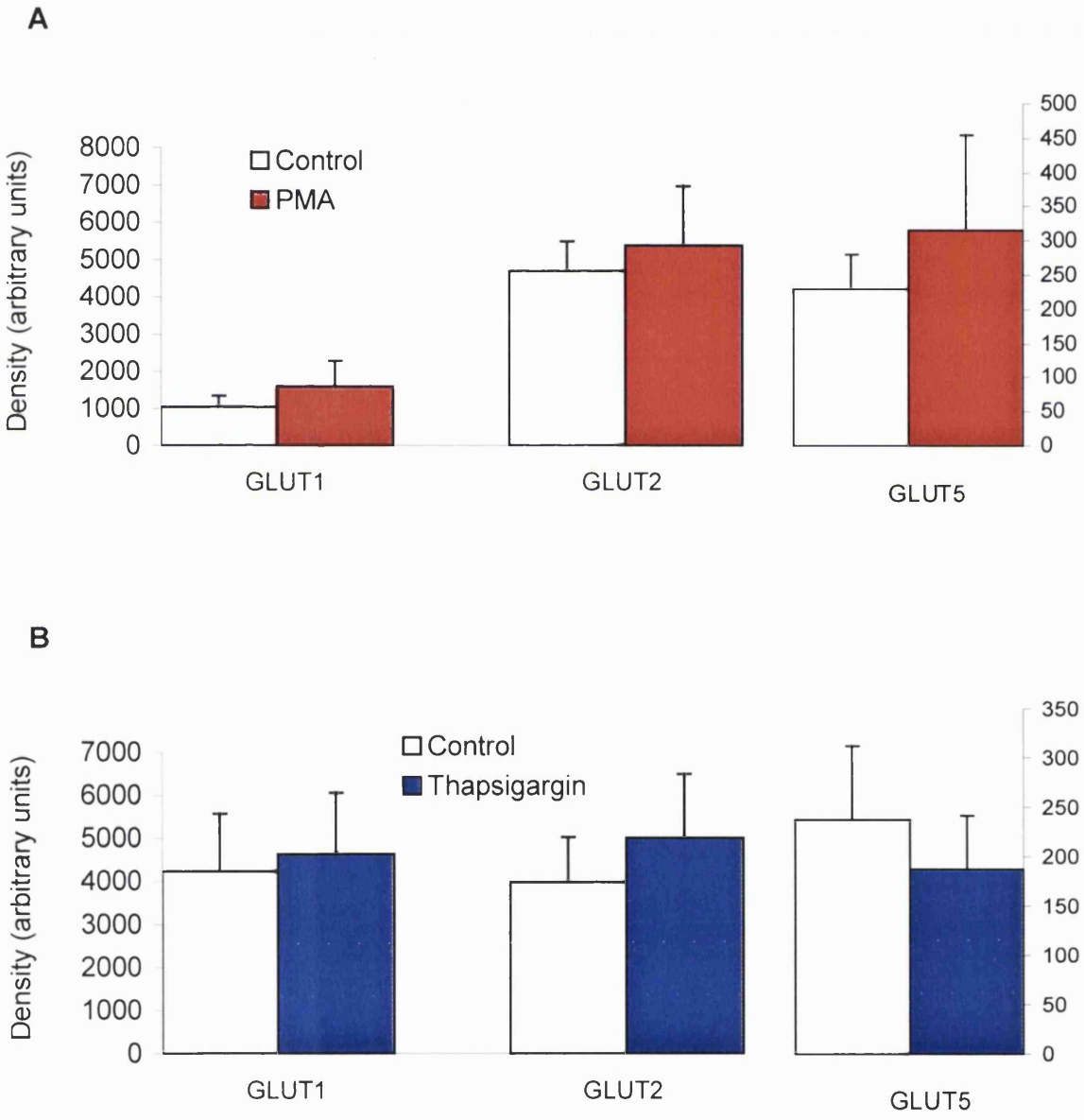


Figure 4.6. Quantification of levels of GLUT1, GLUT2 and GLUT5 protein in BBM vesicles prepared following exposure to **A** 1 μM PMA and **B** 1 μM thapsigargin. Results were obtained from western blots carried out on 6 vesicle preparations, and expressed as Mean ± SEM.



activation of PKC and increased levels of intracellular calcium increase GLUT-mediated glucose transport may be a consequence of interaction between these two signalling pathways. In this context, activation of phospholipase C (PLC) produces two intracellular signalling messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of intracellular calcium, whilst, DAG activates PKC (Rhee S.G & Dennis E.A, 1997). Therefore, PLC activation maybe a key factor involved in the regulation of facilitative glucose transport and warrants further investigation.

4.4. Discussion

Very little is known about the cellular mechanisms involved in the regulation of renal glucose transport. However, studies carried out using enterocytes isolated from the small intestine, where the transport of glucose displays striking similarities to that of proximal tubule cells, have provided compelling evidence for the involvement of the PKA and PKC signalling pathways in the regulation of SGLT-mediated and GLUT-mediated glucose transport, respectively. The results presented in this chapter reveal that the regulatory mechanisms of renal glucose transport are indeed similar to those described for the small intestine.

Short-term exposure of a proximal tubule suspension to 'native' membrane-impermeable cAMP or its membrane-permeable analogue, dibutyryl cAMP, increased the V_{max} for SGLT-mediated glucose transport, but did not influence GLUT-mediated transport. This increase in the capacity of SGLT-mediated transport implies either enhanced activity of transporter proteins already present in the membrane or recruitment of additional transporters to the BBM. Western blotting of BBM vesicles prepared following exposure to cAMP revealed consistently higher levels of SGLT1 protein, and colchicine, an agent reported to disrupt the microtubule network (Pavelka & Gangl, 1983), abolished the cAMP-induced increase in SGLT-mediated glucose transport. Therefore, the nucleotide specifically enhances renal sodium-dependent glucose transport by the rapid insertion of additional protein into the BBM. This finding is in keeping with SGLT1 expression studies carried

out in *Xenopus* oocytes (Hirsch *et al.*, 1996) and the kinetic properties of the transporter. This isoform is a high-affinity, low capacity transporter, and hence would be saturated at physiological glucose concentrations (Clancey & Lever, 2000). Therefore an alteration in the number of transporters at the cell surface, either by translocation of existing transporters or increased translation, would be necessary to increase glucose transport rates when physiological glucose concentrations are exceeded. In this context, Wright *et al.* have estimated that in oocytes, intracellular transport vesicles contain 10-20 SGLT1 proteins. They calculated that the net basal rate of exocytosis of these vesicles is 10000 per second, and that this rate is increased 100-fold following activation of PKA (Wright *et al.*, 1997). Interestingly, long-term exposure of LLC-PK1 cells (a porcine renal epithelial cell line with a proximal tubule phenotype) to cAMP for 4 days results in stabilisation of SGLT1 mRNA levels (Peng & Lever, 1995a), by binding of a cytoplasmic protein to a uridine-rich sequence (URE) in the 3'-untranslated region (UTR) of the SGLT1 mRNA (Peng & Lever, 1995b). Further investigation revealed that the protein involved in this stabilization was a 38 kDa nucleocytoplasmic protein, which binds to the SGLT1 UTR in a cAMP-dependent manner. This binding is initiated in the nucleus and accompanies the message to the cytoplasm, thus providing continuous protection of the mRNA from cellular decay (Lee *et al.*, 2000).

In contrast to the intestine, at least two SGLT isoforms are expressed in the renal proximal tubule. Approximately 90% of filtered glucose is reabsorbed in the early S1 segment of the proximal tubule by the low-affinity sodium-dependent transporter, SGLT2, whilst the remaining glucose presented to the later portions of the tubule are reabsorbed by the high affinity transporter, SGLT1 (Kanai *et al.*, 1994). The studies described in this chapter are unable to establish an affect of cAMP on SGLT2 transporter expression due to the lack of a commercially available antibody.

An interesting observation from this set of experiments is that the nucleotide appears to have both intracellular and extracellular actions on renal glucose transport. Probenecid, a recognised inhibitor of membrane cAMP transfer

(Strewler, 1984), failed to abolish the cAMP induced increase in SGLT-mediated glucose transport, suggesting that the extracellular action of the nucleotide may occur through binding to specific membrane receptors. In this context, cAMP has been demonstrated to specifically bind to BBM isolated from rabbit renal cortex (Insel P *et al.*, 1975) and more recently the existence of renal cAMP receptors has been proposed (Bankir L *et al.*, 2002). It is also of interest that both liver-derived (Bankir L *et al.*, 1997) and nephrogenous cAMP (Friedlander & Amiel, 1995) can influence proximal tubular function. Although this study did not investigate further the role of extracellular cAMP in the regulation of renal glucose transport, it would be of interest to determine whether the source of extracellular cAMP influences its effect on renal glucose transport.

Short-term exposure of proximal tubular cells to PMA or thapsigargin increased GLUT-mediated but not SGLT-mediated glucose transport. An effect on protein levels of the facilitative transporters was not evident but increased transport may be a consequence of alteration in one or more of the transporters intrinsic activities. This finding demonstrates that short term activation of the PKC pathway specifically alters GLUT-mediated transport but does not influence transporter levels, which is in contrast to the action of cAMP/PKA activation on SGLT-mediated transport. This striking difference in the response of the two types of glucose transporters may be a consequence of targeting of the protein to its specific membrane location. In polarized epithelial cells, SGLT1 protein is expressed at the BBM. Targeting of the protein to this membrane has been shown by two independent groups to involve a short amino acid sequence within the N-terminal domain of SGLT1 (Suzuki *et al.*, 2001; Wright *et al.*, 1998). In oocytes, regulation of functional SGLT1 levels occurs by regulated endocytosis and exocytosis of the protein between intracellular compartments and the plasma membrane (Hirsch *et al.*, 1996). Indeed, recently it has been established that in Caco-2 cells, the major fraction of SGLT protein resides in the intracellular compartment where it is associated with microtubules. The authors propose that this pool of intracellular SGLT1 protein is not en route to the membrane

but in fact represents an intracellular reserve pool ready for rapid insertion into the apical membrane (Kipp *et al.*, 2003).

In contrast, GLUT1 and GLUT2 proteins have until recently been considered to be solely expressed at the BLM of epithelial cells (Dominguez *et al.*, 1992; Thorens, 1996). Although, evidence now suggests a functional role of these transporters at the BBM (Boyer *et al.*, 1996; Chowrimootoo G *et al.*, 1993; Kellett & Helliwell, 2000), the exact mechanisms involved in the delivery to this membrane have not been established. One possible mechanism is that the GLUT1 and GLUT2 protein pool at the BLM undergoes transcytosis to the BBM. This redistribution of the GLUT proteins to the BBM would take considerably longer than the rapid insertion of SGLT1 from the intracellular pool at the BBM terminal web, and may therefore explain the lack of changes in BBM GLUT protein expression following 30 minute treatment with PMA or thapsigargin.

Regulation of renal GLUT-mediated glucose transport by PKC signalling is in keeping with the recently reported regulation of intestinal glucose transport. Helliwell *et al.* (2000) have demonstrated that activation of the PKC signalling pathway, by PMA, is involved in the regulation of intestinal GLUT-mediated transport, with recruitment of GLUT2 to the enterocyte BBM being responsible for the PMA induced increase in glucose transport (Helliwell *et al.*, 2000b). The authors hypothesis that sodium-coupled glucose entry activates PKC-βII and induces structural changes in the apical surface of the enterocyte, possibly as a consequence of increased intracellular calcium concentrations, which leads to GLUT2 recruitment to the BBM (Kellett, 2001). These findings differ to that observed in the kidney, since increased renal GLUT-mediated uptake was not accompanied by increased GLUT protein expression, but this difference may be a consequence of time and experimental protocol. Helliwell *et al.* have also provided evidence that other major signalling pathways, such as ERK, MAP kinase and PI 3-kinase pathways, are involved in the regulation of intestinal glucose transporters. Activation of these pathways lead to changes in both transporter levels and

intrinsic activities of GLUT2 and GLUT5 (Helliwell *et al.*, 2000a). It would therefore be intriguing to establish if these other signalling pathways can influence renal glucose transport.

The results presented in chapter 2 demonstrate that STZ-induced diabetes alters renal GLUT-mediated glucose transport, with recruitment of GLUT2 to the BBM in response to plasma glucose levels, being the factor responsible. The results described in this chapter reveal that GLUT-mediated transport is increased by activation of the PKC and calcium signalling pathways. Interestingly, proximal tubule cells isolated from the kidneys of STZ-diabetic animals display increased activation of a variety of PKC isoforms (Ha *et al.*, 2001; Whiteside & Dlugosz, 2002) and elevated levels of intracellular calcium (Marcinkowski *et al.*, 1997). These affects can also be mimicked by incubation of renal cells in high glucose concentrations (Ha & Lee, 2000; Symonian *et al.*, 1998). There is strong evidence to suggest that diabetes-induced mesangial cell damage occurs as a result of increased glucose uptake, by GLUT1, which correlates with increased PKC activation and fibronectin and collagen accumulation (Heilig *et al.*, 1995b). Therefore, in mesangial cells, it has been proposed that increased GLUT1 expression occurs as a result of glucose induced PKC activation, leading to a variety of cellular events that culminate in cellular damage characteristic of diabetic nephropathy (Heilig *et al.*, 2001). Thus, glucose induced activation of GLUT2 expression at the proximal tubule BBM, may be a consequence of alterations in PKC activation, leading to cellular damage in this nephron region. Microdissection of proximal tubules isolated from the kidneys of STZ-diabetic rats and those subjected to an overnight fast, followed by quantitative RT-PCR for the various PKC isoforms, may provide information on the involvement of this signalling pathway in BBM GLUT2 expression.

4.5. Conclusion

In conclusion, the PKA and PKC signalling pathways differentially regulate sodium-dependent and facilitative glucose transport, respectively. The mechanisms of regulation following short term activation are also distinct,

with the increase in SGLT-mediated uptake being a consequence of increased transporter expression, whilst, increased GLUT-mediated uptake probably occurs through changes in transporter activity.

As described in chapter 2, streptozotocin-induced diabetes increases facilitative glucose transport across the renal BBM. Taken together with the results presented in this chapter and those from other laboratories, it could be hypothesised that changes in glucose transport during diabetes occurs as a consequence of activation of the PKC signalling pathway.

Chapter 5.

Detection of glucagon and glucagon-like peptide-1 receptor in the rat proximal tubule: Potential role for glucagon in the control of renal glucose transport during streptozotocin-induced diabetes.

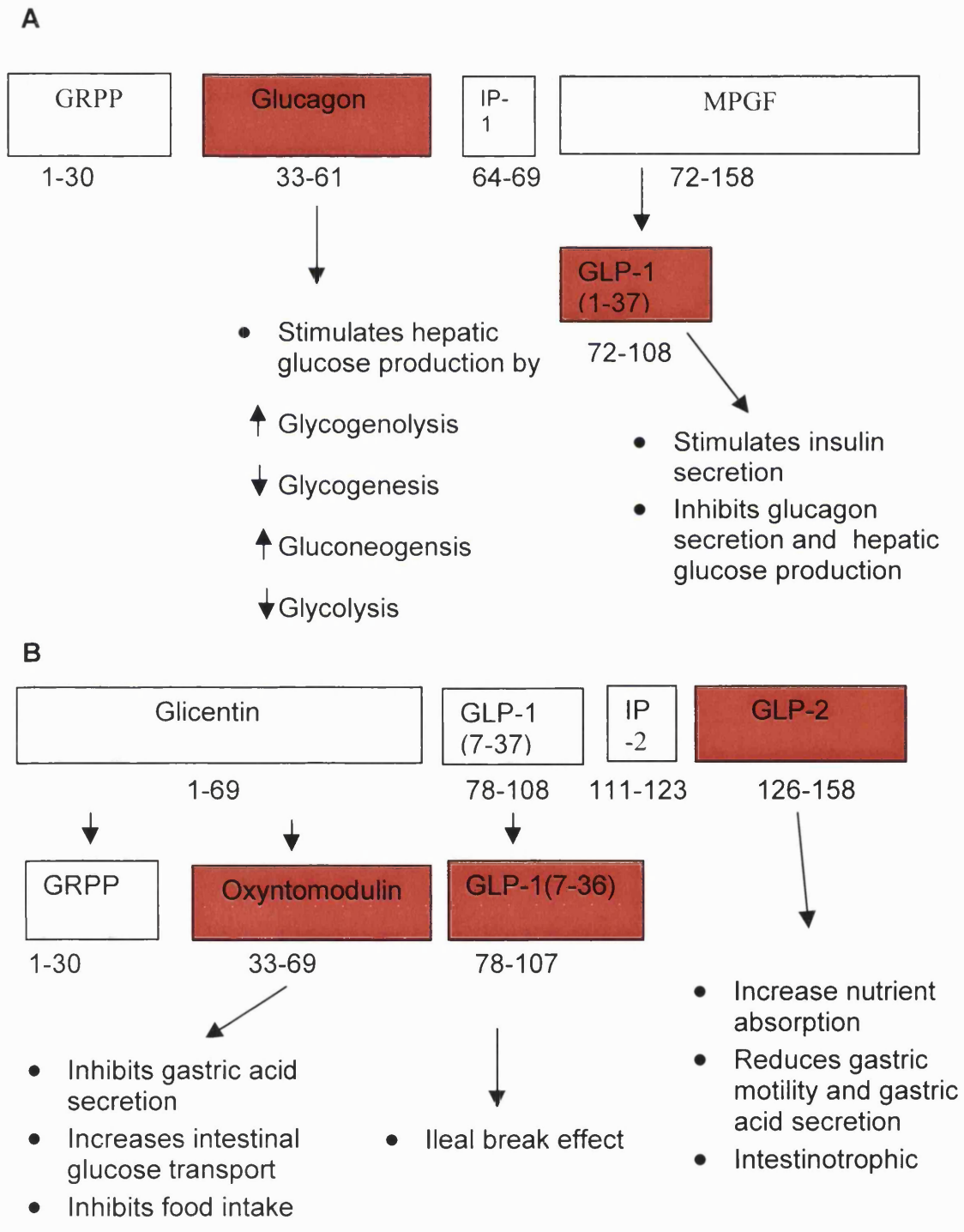
5.1. Introduction

5.1.1. Synthesis of glucagon and its structurally related peptides

The preproglucagon gene has been mapped to chromosome 2 (Tricoli *et al.*, 1984) and is expressed in the pancreatic α-cells and in the L cells of the distal jejunum, ileum and colon (Hoyt *et al.*, 1996; Larsson & Moody, 1980; Smith, 1983). Translation of this gene generates preproglucagon, which is then reduced to proglucagon. Cleavage of proglucagon, a polypeptide consisting of 160 amino acids, occurs by limited proteolysis, in a tissue-specific manor, by a family of enzymes known as the prohormone convertases (Steiner, 1998). In the pancreas, cleavage results in the production of bioactive pancreatic glucagon, consisting of 29 amino acids, glicentin-related pancreatic polypeptide (GRPP) and the major proglucagon fragment (MPGF). Processing of the proglucagon gene product in the pancreas also produces trace amounts of glucagon-like peptide-1 (GLP-1), which has been proposed to induce local actions within the islets (Kieffer & Habener, 1999). In the L-cells of the intestine, post-translational processing of proglucagon yields glicentin, oxyntomodulin, which consists of 37 amino acids, GLP-1 and glucagon-like peptide-2 (GLP-2) (Perfetti & Merkel, 2000) (Fig. 5.1), with the glucagon-like peptides inferring the greatest biological activity.

Cleavage of proglucagon in both the intestine and pancreas yields GLP-1 (7-37), a biologically inactive peptide that requires further processing to produce biologically active GLP-1 (7-36 amide) (Fehmann *et al.*, 1995). This peptide has a short plasma half-life of approximately 5 minutes, due to rapid degradation by the enzyme dipeptidyl peptidase (DPP) IV, which results in the cleavage of the two N-terminal amino acid residues of GLP-1 (7-36 amide) to produce biologically inactive GLP-1 (9-36 amide) (Holst, 1994). In contrast, GLP-2 is secreted as a biologically active peptide and although like GLP-1 it is degraded by DPP IV, it has a longer plasma half-life of 7.5 minutes (Hartmann *et al.*, 2000).

Figure 5.1. Processing of proglucagon in the pancreas **(A)** and intestine **(B)** and the proposed biological functions of the resulting peptides. Adapted from Perfetti and Merkel (Perfetti & Merkel, 2000). GRPP, glicentin-related pancreatic polypeptide; MPGF, major proglucagon fragment; IP-1 and 2, intervening peptides 1 and 2; GLP, glucagon-like peptide.



5.1.2. Physiological function

The major function of pancreatic glucagon is to maintain blood glucose levels during hypoglycaemia. The peptide is secreted in response to fasting and insulin-induced hypoglycaemia, and increases circulating blood glucose levels via the stimulation of hepatic glucose production (Jiang & Zhang, 2003) and the mobilisation of glucose from peripheral tissues (Kieffer & Habener, 1999). Binding of glucagon to its hepatic receptor affects both glycogen and glucose metabolism. It promotes glycogenolysis, resulting in increased glycogen breakdown and inhibits glycogenesis. Glucagon influences hepatic glucose metabolism by increasing glucose production via gluconeogenesis and decreases glucose storage by a reduction in the rate of glycolysis. The overall effect of these changes in glycogen and glucose metabolism is to increase the glucose pool available for hepatic glucose release into the circulation (Jiang & Zhang, 2003).

Oxyntomodulin, also termed enteroglucagon or glucagon 37, is cleaved from proglucagon in intestinal L cells in response to nutrient ingestion. Oxyntomodulin has been shown to stimulate adenylate cyclase activity in isolated rat liver plasma membranes, although this response is less potent than that seen with pancreatic glucagon (Bataille *et al.*, 1982). However a later study by Stumpel *et al.* demonstrated that oxyntomodulin was incapable of stimulating hepatic glucose release (Stumpel *et al.*, 1998). It is now accepted that oxyntomodulin's main sites of action are the stomach and intestine, where it has a physiological role in the regulation of gastric acid secretion (Gros *et al.*, 1992; Le Quellec *et al.*, 1992) and sodium-dependent glucose absorption (Collie *et al.*, 1997; Stumpel *et al.*, 1997; Stumpel *et al.*, 1998), respectively. Additionally, recent studies have demonstrated a role for oxyntomodulin in the inhibition of food intake, via actions on the central nervous system (Cohen *et al.*, 2003; Dakin *et al.*, 2001).

The major source of circulating GLP-1 arises from the intestinal L cells, with the stimulus being nutrient ingestion. The primary biological role of this peptide is to lower blood glucose levels during feeding. GLP-1 instigates its

hypoglycaemic action through numerous complementary systems. In the intestine GLP-1 has been shown to regulate nutrient intake via the ileal brake effect, whereby it inhibits upper gastrointestinal motility and gastric acid secretion. This reduces the availability of nutrients for absorption therefore diminishing the glycaemic fluctuations that occur after food ingestion (Wettergren *et al.*, 1993; Wettergren *et al.*, 1997; Wettergren *et al.*, 1998). In conjunction with these intestinal effects, GLP-1 targets the pancreatic β-cells, where it stimulates insulin gene transcription and glucose-dependent insulin release and has an inhibitory effect on glucagon secretion (Drucker *et al.*, 1987). Additionally the peptide is involved in short term regulation of feeding behaviour through activation of GLP-1 receptors located in the central nervous system (Drucker, 2002a).

The principle target of GLP-2 is the gastrointestinal tract, its effects include expansion of the mucosal epithelium, most noticeably the jejunum (Drucker *et al.*, 1996; Tsai *et al.*, 1997b). This intestinotrohpic effect has been attributed to an increase in villus height and mucosal surface area, as a result of increased crypt cell proliferation and a decreased rate of enterocyte apoptosis (Drucker *et al.*, 1996; Tsai *et al.*, 1997a). The peptide has also been shown to inhibit gastric acid secretion (Wojdemann *et al.*, 1999), reduce gastric motility (Wojdemann *et al.*, 1998) and influence enterocyte hexose transport. Vascular infusion of GLP-2 induces an increase in SGLT-mediated glucose transport and SGLT-1 protein expression at the enterocyte BBM within 1 hour (Cheeseman, 1997), and increases GLUT2-mediated efflux across the enterocyte BLM (Cheeseman & O'Neill, 1998). Interestingly, it has recently been demonstrated that GLP-2 also promotes the insertion of GLUT2 at the enterocyte BBM (Au *et al.*, 2002). Based on these findings it has been proposed that luminal glucose stimulates the release of gastric inhibitory peptide (GIP) from the K cells of the jejunum. GIP in turn stimulates GLP-2 production in the L cells of the ileum, culminating in increased enterocyte expression of the glucose transporter proteins and enhanced glucose absorption (Cheeseman & O'Neill, 1998). Numerous studies have demonstrated a beneficial effect of GLP-2 on the gut

mucosa following experimental intestinal injury, making it a potential therapeutic candidate for gastrointestinal diseases (reviewed in (Drucker, 2002a; Drucker, 2002b)).

5.1.3. Physiological functions in the kidney

The proximal tubule plays a key role in the removal of glucagon and glucagon-like peptides from the circulation. Early studies into the renal handling of glucagon demonstrated that following glomerular filtration the peptide is rapidly degraded by enzymes located on the proximal tubule BBM, with the resulting metabolites being locally reabsorbed (Peterson *et al.*, 1982; Peterson *et al.*, 1986). Later studies have implicated a similar process in the renal catabolism of GLP-1 and GLP-2 (Ruiz-Grande *et al.*, 1993; Tavares *et al.*, 2000), with the BBM ectopeptidase, dipeptidyl-peptidase-IV, being the key enzyme in the endoproteolysis of GLP-1 (Thum *et al.*, 2002).

Circulating glucagon has been linked to the increase in glomerular filtration rate and renal blood flow following a protein-rich meal, to re-feeding natriuresis following starvation (Unwin *et al.*, 1990), and to increases in phosphate excretion (Murer *et al.*, 2000). These effects of glucagon may be due to a direct action on the glomerulus and proximal tubule, but they might also be indirect, mediated by the hepatic release of circulating cyclic AMP (Ahloulay *et al.*, 1995; Bankir L *et al.*, 2002; Butlen & Jard, 1972). In contrast, the effects of glucagon on calcium, magnesium and proton transport in the thick ascending limb (TAL) and collecting duct (CD) are the result of a direct stimulation of glucagon receptors coupled to adenylate cyclase and cyclic AMP generation (Ahloulay *et al.*, 1995; Bailly *et al.*, 1980; Bailly & Amiel, 1982; Butlen & Morel, 1985). However, although glucagon inhibits sodium and phosphate reabsorption along the proximal tubule (de Rouffignac *et al.*, 1991), there is still no evidence for a direct action on this nephron segment, or for the presence of glucagon-sensitive adenylate cyclase.

GLP-1 has been shown to influence renal hemodynamics and proximal tubule function. Intravenous infusion of the peptide increases GFR, urine flow rate and sodium excretion. The diuretic and natriuretic effects of GLP-1 are thought to be a consequence of reduced sodium reabsorption in the proximal tubule (Moreno *et al.*, 2002). At present a specific effect of GLP-2 on renal function has yet to be established.

5.1.4. The glucagon receptor

5.1.4.1 Sites of expression

The rat glucagon receptor was cloned in the early 1990's by two independent groups and was found to be a member of a large family of GTP binding protein-coupled receptors that have seven transmembrane domains (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993). Cloning of the human glucagon receptor identified a receptor that displayed 82% homology to that cloned from rats (MacNeil *et al.*, 1994), and has subsequently been mapped to chromosome 17, at 17q25 (Lok *et al.*, 1994). Studies investigating the tissue distribution of the rat glucagon receptor have demonstrated that the mRNA is expressed abundantly in the liver, kidney, heart, spleen and adipose tissue, with lower levels detected in the stomach, small intestine, skeletal muscle and brain (Dunphy *et al.*, 1998; Hansen *et al.*, 1995; Svoboda *et al.*, 1994).

5.1.4.2 Regulation

Studies investigating the regulation of the glucagon receptor have demonstrated that increased intracellular cAMP levels, resulting from incubation of pancreatic islet cells with forskolin or glucagon for 48 hours, leads to a reduction in the glucagon receptor mRNA (Abrahamsen *et al.*, 1995; Abrahamsen & Nishimura, 1995). In contrast, 24-hour exposure of human embryonic kidney cells to forskolin resulted in an increased expression of glucagon receptor mRNA and protein binding sites, however this did not correlate with increased receptor function (Ikegami *et al.*, 2001). Although these results appear to be contradictory, both demonstrate that glucagon-stimulated cAMP accumulation leads to receptor desensitisation

and a decreased responsiveness to its agonist. Conversely, incubation of cultured cells at high glucose concentrations or with agents that inhibits cAMP accumulation, results in enhanced glucagon receptor mRNA expression (Abrahamsen *et al.*, 1995; Abrahamsen & Nishimura, 1995; Burcelin *et al.*, 1998).

5.1.4.3 Signalling properties

Early studies into the cellular events induced by glucagon binding, implicated activation of adenylate cyclase and a subsequent rise in intracellular cAMP as the receptor's signalling pathway (Goldfine *et al.*, 1972; Houslay, 1979; Rodbell *et al.*, 1971). However, numerous studies in different cell types, including cultured renal cells (Hansen *et al.*, 1998; Kurstjens *et al.*, 1990), have identified a second signalling pathway involving raised intracellular calcium concentrations (Li *et al.*, 1997; Wakelam *et al.*, 1986). This increase in calcium is a consequence of activation of phospholipase C (PLC) and the subsequent activation of inositol triphosphate (IP_3) leading to release of calcium from IP_3 sensitive cellular stores (Hansen *et al.*, 1998). Wakelam *et al.* proposed the existence of two receptor populations, with different affinities for glucagon and distinct intracellular signalling events (Wakelam *et al.*, 1986). In contrast, reports have suggested that independent activation of the two signalling pathways occurs by activation of the same receptor population (Jelinek *et al.*, 1993; Kurstjens *et al.*, 1990); with low concentrations of glucagon eliciting an increase in inositol triphosphate and high concentrations evoking raised cAMP levels (Li *et al.*, 1997). The differences in the results discussed above may well reflect species and cell type variation, or may be a result of alternative splicing of the receptor mRNA or differences in post-translational modification. In this context, a structurally different, tissue specific, variant of the glucagon receptor has been reported in the MDCK cell line, which is derived from canine distal tubule cells (Iwanij, 1995). In rat tissues, a 62 kDa protein has been detected using monoclonal antibodies, which shows the same molecular weight regardless of tissue type under investigation (Iwanij & Vincent, 1990). However, in the MDCK cell line and membranes isolated from the canine kidney, a receptor with a molecular

weight of 74 kDa is detectable, which is an estimated 12kDa larger than that detected in canine liver membranes (Iwanij, 1995). Additionally, using RT-PCR, Maget *et al.* have reported the presence of several alternatively spliced glucagon receptor mRNAs, which appear to occur in a tissue specific manor (Maget *et al.*, 1994).

5.1.4.4 Binding properties

Studies into the motifs responsible for the binding of glucagon to its receptor have implicated regions within the long extracellular N-terminal domain (Carruthers *et al.*, 1994; Hjorth *et al.*, 1994) and the first extracellular loop (Buggy *et al.*, 1995; Unson *et al.*, 1996). Using a variety of approaches Unson *et al.* have provided evidence that glucagon binds to the N-terminal domain of its receptor and then subsequently interacts with the central portion of the first extracellular loop. The authors propose that the loop acts as a glucagon binding pocket to provide stabilisation of the ligand-receptor interaction, thus allowing receptor activation (Unson *et al.*, 1996; Unson *et al.*, 2002). Studies by this group, using chimeric proteins lacking intracellular loops 2 and 3, have established that these motifs are responsible for both the glucagon-mediated increase in intracellular cAMP and calcium levels (Cypess *et al.*, 1999).

5.1.5. GLP-1 and GLP-2

5.1.5.1. Sites of expression

The receptors for GLP-1 (Dillon *et al.*, 1993; Thorens, 1992; van Eyll *et al.*, 1994) and GLP-2 (Munroe *et al.*, 1999) have been cloned and characterised. They display approximately 40% homology to the glucagon receptor (Jelinek *et al.*, 1993), contain 7 transmembrane domains and have been classified as members of the glucagon subfamily of GTP binding protein-coupled receptors (Munroe *et al.*, 1999; Thorens, 1992). Human GLP-1 receptor has been localised to chromosome 6, at 6p21 (Stoffel *et al.*, 1993), whereas GLP-2 has been mapped to chromosome 17, at 17p13.3 (Munroe *et al.*, 1999). GLP-1 receptor mRNA has been detected in the small and large

intestine, pancreas, liver, lung, brain and kidney (Bullock *et al.*, 1996; Campos *et al.*, 1994; Dunphy *et al.*, 1998). Studies using intravenous infusion of radiolabelled GLP-2 detected binding of this peptide in the GI tract, predominately the jejunum, liver and kidneys. Binding in the small intestine could be displaced by unlabelled GLP-2, suggesting the presence of specific GLP-2 receptors. In contrast, binding in the liver and at the BBM of proximal tubules could not be displaced by unlabelled compound, suggesting these tissues are involved in the clearance of GLP-2 from the circulation (Thulesen *et al.*, 2000). Studies investigating the distribution of GLP-2 receptor mRNA have detected high levels of expression in the stomach and small and large intestine, with lower levels expressed in the brain and lung. In keeping with the observations of Thulesen *et al.*, the mRNA for GLP-2 receptor was undetectable in the kidney and liver and numerous other peripheral tissues (Munroe *et al.*, 1999; Yusta *et al.*, 2000).

5.1.5.2. Regulation

Agonist-induced desensitisation of GLP-1 receptor occurs within 15 minutes of exposure to GLP-1 and results in a 50% reduction in receptor binding sites (Widmann *et al.*, 1995). Activation of the protein kinase C pathway has been demonstrated to induce a reduction in the expression of GLP-1 receptor mRNA and protein and may therefore be involved in the desensitisation process (Fehmann *et al.*, 1996; Widmann *et al.*, 1996). In contrast to the stimulatory effect of glucose on the glucagon receptor, incubation of cultured rat islet cells in high glucose concentrations results in a 40% reduction in the expression of GLP-1 receptor (Abrahamsen & Nishimura, 1995). Increased intracellular cAMP has no effect on GLP-1 receptor mRNA expression yet the same conditions induced a reduction in the expression of the glucagon receptor (Abrahamsen & Nishimura, 1995). Therefore, although the glucagon and GLP-1 receptors are closely related, they appear to be differentially regulated. Due to the relatively recent cloning of the GLP-2 receptor, the mechanisms involved in its regulation have yet to be determined.

5.1.5.3. Signalling properties

Binding of GLP-1 to its receptor increases intracellular cAMP levels and causes the mobilisation of calcium from intracellular stores (Dillon *et al.*, 1993; van Eyll *et al.*, 1994). Wheeler *et al.* demonstrated that stimulation of the receptor resulted in an increase in intracellular calcium and phosphoinositol turnover, indicative of PLC activation (Wheeler *et al.*, 1993). In contrast, a later study by Gromada *et al.* demonstrated that the increase in intracellular calcium was independent of the phospholipase C / IP_3 pathway, since the PLC inhibitor, U73122, had no effect on GLP-1-induced increase in intracellular calcium. The authors demonstrated that the GLP-1-induced mobilisation of calcium from intracellular stores occurred through the activation of ryanodine sensitive calcium channels (Gromada *et al.*, 1995). These conflicting results may be a consequence of the cell type used for the expression of the GLP-1 receptor, the species of GLP-1 receptor or the concentration of GLP-1 used in these studies. The experimental approach employed by Walsh *et al.,* which used isolated rat mucosal cells to study the signalling properties of GLP-2 receptor (Walsh *et al.*, 2003), may provide a definitive insight into the signalling events that occur following GLP-1 receptor activation in the intestine.

In contrast to the signalling properties of glucagon and GLP-1 receptors, numerous studies have demonstrated that GLP-2 receptors are coupled solely to adenylate cyclase. (Munroe *et al.*, 1999; Walsh *et al.*, 2003; Yusta *et al.*, 1999).

5.1.5.4. Binding properties

The receptors for GLP-1 and GLP-2 display a high affinity for their ligands, with reported Kd values of 0.6 nM and 0.57 nM, respectively (Munroe *et al.*, 1999; Thorens, 1992). The GLP-2 receptor has been shown to be highly specific for its native ligand, and does not bind peptides with a related structure (Munroe *et al.*, 1999). In contrast, numerous reports in the early literature have provided evidence that glucagon and oxyntomodulin can bind

to and activate GLP-1 receptors (Gros *et al.*, 1993; Moens *et al.*, 1998; Schepp *et al.*, 1996). Oxyntomodulin is considered to be a weak GLP-1 receptor agonist (Fehmann *et al.*, 1994), whilst it has been reported that glucagon is a full agonist, but is 200-fold less potent than GLP-1 in stimulating the GLP-1 receptor (Graziano *et al.*, 1993). It is however noteworthy, that superphysiological concentrations of glucagon and oxyntomodulin are required for GLP-1 receptor binding and activation. Therefore activation of GLP-1 receptor by structurally related peptides is unlikely to occur at physiologically relevant concentrations.

Studies by independent research groups using different experimental approaches have provided evidence that the large N-terminal extracellular domain of GLP-1 receptor is important for receptor-ligand interaction (Gelling *et al.*, 1997; Graziano *et al.*, 1996; van Eyll *et al.*, 1996; Wilmen *et al.*, 1996). Additionally, domains within the third intracellular loop are required for the effective coupling of GLP-1 receptor to adenylate cyclase (Heller *et al.*, 1996; Mathi *et al.*, 1997; Salapatek *et al.*, 1999). A recent study has confirmed this finding and demonstrated that distinct domains within the third intracellular loop are responsible for the activation of different G-protein subtypes (Hallbrink *et al.*, 2001). To date there is no information regarding the motifs responsible for ligand-binding or activation of the GLP-2 receptor.

5.1.6. Abnormalities in glucagon and glucagon-like-peptide during diabetes

During the 1970's it was proposed that diabetes was not a simple consequence of relative or absolute insulin deficiency but occurred due to abnormalities in the insulin/glucagon ratio (Raskin & Unger, 1978; Sakurai *et al.*, 1975; Unger, 1978; Unger & Orci, 1975). Furthermore, all forms of diabetic and non-diabetic hyperglycaemia have been associated with hyperglucagonaemia (Unger & Orci, 1975). It is now widely accepted that hyperglucagonaemia plays an important role in initiating and maintaining hyperglycaemia when combined with delayed or reduced insulin secretion (Jiang & Zhang, 2003), consequently, glucagon and the glucagon receptor

are being investigated as therapeutic targets for the treatment of diabetes. Such strategies include the use of glucagon-neutralising antibodies (Brand *et al.*, 1994; Brand *et al.*, 1996), antagonistic peptide glucagon analogues (Ahn *et al.*, 2001; Hruby, 1982) and non-peptide glucagon receptor antagonists (Cascieri *et al.*, 1999; de Laszlo *et al.*, 1999; Madsen *et al.*, 1998).

Mutations in the human glucagon receptor have been associated with type 2 diabetes in the French and Sardinian population (Hansen *et al.*, 1996; Tonolo *et al.*, 1997). However, genetic screening of other populations have failed to detect (Huang *et al.*, 1999; Ogata *et al.*, 1996) or have demonstrated extremely low incidence of this genetic polymorphism (Ambrosch *et al.*, 1999; Shiota *et al.*, 2002), suggesting it is unlikely to be generally involved in the pathogenesis of type 2 diabetes.

Abnormalities in the plasma levels of the glucagon-like-peptides also occur in diabetes. The intestinal mucosal hyperplasia associated with diabetes has been attributed to an increase in circulating levels of GLP2 (Fischer *et al.*, 1997; Thulesen *et al.*, 1999). In contrast, postprandial levels of circulating GLP-1 have been shown to be significantly lower in both type 1 and type 2 diabetic patients compared with control individuals (Lugari *et al.*, 2000; Vilsboll *et al.*, 2001). Based on this finding it has been hypothesised that reduced plasma levels of GLP-1 may contribute to the inappropriate levels of insulin secretion in type 2 diabetic patients (Vilsboll *et al.*, 2001). As described earlier, GLP-1 confers its hypoglycaemic effects through actions on the intestine, pancreatic cells and the central nervous system. The combined effects of this peptide make it an ideal candidate for the treatment of type 2 diabetes. Studies have shown that intravenous, subcutaneous and oral administration of GLP-1 to type 2 diabetic patient's lowers blood glucose levels and improves glycaemic control (Kieffer & Habener, 1999). One potential drawback to the use of GLP-1 as an anti-diabetogenic agent is its short plasma half-life (Holst, 1994). Therefore, studies have focussed on the development of DPP-resistant forms of GLP-1 (Deacon *et al.*, 1998b; Doyle *et al.*, 2001; Gallwitz *et al.*, 2000), the co-administration of DPP-IV inhibitors (Deacon *et al.*, 1998a; Holst & Deacon, 1998) and the use of GLP-1 receptor

agonist, such as extendin-4 (Egan *et al.*, 2002; Greig *et al.*, 1999; Young *et al.*, 1999), and ZP10A (Thorkildsen *et al.*, 2003).

As described above glucagon and glucagon-like peptides play an important role in the control of glucose homeostasis. During diabetes abnormal secretion of these peptides has been linked to the progression and pathogenesis of the disease. To date there are no reports of an effect of glucagon on renal glucose transport during diabetes. However, studies using intestinal enterocytes, suggest that increased secretion of pancreatic glucagon in experimental diabetes may be responsible for the enhanced Na^+-dependent, SGLT-mediated, glucose uptake that occurs across the brush border membrane (Sharp & Debnam, 1994b; Thompson & Debnam, 1986). Such findings in the small intestine raise the question of whether glucagon might also regulate renal glucose transport during diabetes.

5.2. Materials and Methods

5.2.1 Effect of glucagon on renal glucose transport

5.2.1.1. Incubation of cortical suspension

The protocol for incubating proximal tubule cells with glucagon was identical to that described in section 4.2.1. The tubule suspension was incubated for 30 minutes with 2, 10 or 20 nM pancreatic glucagon (G29) or 1 nM glucagon 37 (G37), with the vehicle added to the control suspension. At the end of the 30-minute incubation period an aliquot of the homogenised tubule suspension was snap frozen for determination of cAMP levels using a cAMP-[^{125}I] biotrak Assay (Amersham Pharmacia Biotech UK Limited, Bucks, UK). BBM vesicles were subsequently prepared for uptake studies and western blotting.

5.2.1.2. BBM vesicle preparation (as 2.2.3.) and uptake studies (as 2.2.4).

5.2.1.3. Western blotting (as 3.2.3.)

5.2.1.4. Statistics

Results are expressed as mean ± SEM of 6 vesicle preparations. Statistical comparisons were made using a Student's paired *t* test. All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

5.2.2. Microdissection study

5.2.2.1. Induction of diabetes

Three-week STZ-induced diabetic animals were used to study the effects of diabetes on the renal expression of glucagon and GLP-1 receptor mRNA. Diabetes was induced as described in section 2.2.1.

5.2.2.2. Measurement of plasma cAMP and glucagon levels

Blood obtained by cardiac puncture was placed into heparinized tubes containing 7500 KIU of the broad range protease inhibitor, Aprotonin, and centrifuged for 10 minutes at 6000g. The resulting plasma was snap frozen and stored at –70°C until use. Plasma cAMP was measured using a cAMP-[^{125}I] biotrak Assay (Amersham Pharmacia Biotech UK Limited, Bucks, UK) and plasma glucagon by a double antibody radioimmunoassay (Diagnostic Products Corporation, Gwynedd, UK), both according to the manufacturer's instructions. Mrs Nadia Payne (Department of Endocrinology, Royal Free & University College London) kindly performed the analysis of cAMP and glucagon levels.

5.2.2.3. Surgical protocol

Control and 3-week diabetic animals were anaesthetised by intraperitoneal administration of sodium pentobarbitone (90 mg/kg). The abdominal aorta was cannulated and the left kidney perfused with microdissection solution (see composition below) followed by perfusion with the same solution containing 0.24% (w/v) collagenase, 0.32 U/mg (Boehringer Mannheim, East

Sussex, UK). Kidneys were rapidly excised, decapsulated and sliced into thin corticomedullary pyramids, which were incubated at 37°C for 15 minutes in microdissection solution containing 0.15% (w/v) collagenase, gassed with 5% CO_2-95% air. The microdissection solution (pH 7.5) was prepared from sterile Hanks-balanced salt solution (Sigma, Dorset, UK) supplemented with 1 mM magnesium chloride, 1 mM sodium acetate, 1 mM lactic acid, 1 mM sodium pyruvate, 1 mM glutamine, 20 mM HEPES and 0.1% protease free BSA.

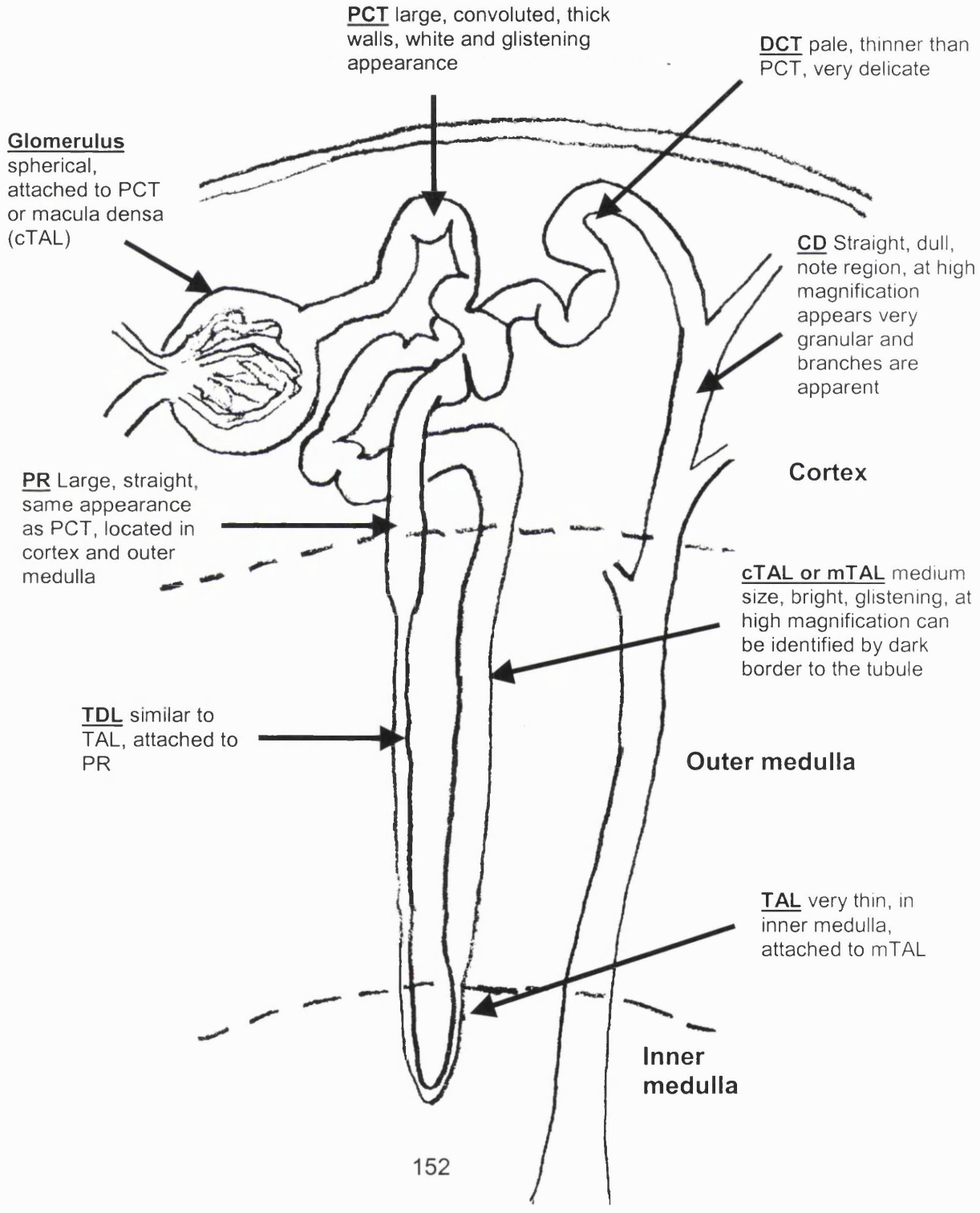
5.2.2.4. Isolation of tubular segments

After incubation, the pyramids were placed in collagenase-free solution and microdissection performed at 4°C under sterile conditions in accordance with anatomical and morphological criteria (Doucet *et al.*, 1979) (Fig. 5.2). Pools of glomeruli (20-50), or identical cortical tubular segments (5-30 mm), were transferred to sterile slides coated with 0.05% BSA and photographed to allow estimation of tubular length.

5.2.2.5. PCR protocol

For each nephron segment 10 μl of RNA was reverse transcribed with 0.5 μg Oligo(dT) 12-18 primer and a first strand cDNA synthesis kit (Superscript II RNase H^- reverse transcriptase, Life Technologies, Paisley, UK). PCR was carried out on 10% of the cDNA product (corresponding to approximately 0.5 mm of tubule) using a PCR core system I kit (Promega, Southampton, UK). cDNA volumes were adjusted to establish consistent β-actin levels between samples prior to PCR for glucagon receptor. For each reaction, denaturation at 95°C for 3 minute was followed by 25 cycles for β-actin, 33 cycles for glucagon receptor or 35 cycles for GLP-1 receptor, at 95°C for 30 seconds, 65°C, 58°C or 55 °C for 1 minute for β-actin, glucagon receptor or GLP-1 receptor, respectively, and 72°C for 1 minute. The reaction was completed

Figure 5.2. Anatomical and morphological criteria for the identification of individual nephron segments. PCT, proximal convoluted tubule, PR, pars recta, TDL, thin descending limb, TAL, thin ascending limb, cTAL and mTAL, cortical or medullary thick ascending limb, DCT distal convoluted tubule, CD, collecting duct.



by a final 5 minute extension stage at 72°C. PCR products were resolved on a 2% agarose gel containing ethidium bromide. Bands were visualized using a Multimager (Biorad, Hertfordshire, UK). PCR products were sequenced by Oswel DNA sequencing laboratory (University of Southampton).

5.2.2.6. RNA extraction

RNA was extracted from identical pools of microdissected nephron segments using an adapted guanidinum thiocyanate-phenol/chloroform method (Elalouf *et al.*, 1993). In brief, pools of identical nephron segments were transferred into 400 μl of denaturing buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% Sarcosyl and 20 μg tRNA. After immediate vortexing, 40 μl of 2 M sodium acetate (pH 4.0), 400 μl of water saturated acid phenol and 80 μl of chloroform were added sequentially, with vortexing after each addition. The suspension was cooled on ice for 15 minutes and subsequently centrifuged at 10,000 *g* for 20 minutes at 4°C. The resulting supernatant was transferred to a fresh tube containing 450 μl of isopropanol and gently mixed by inverting the tube. The suspension was stored overnight at -20°C, followed by centrifugation at 15,000 *g* for 20 minutes at 4°C. The RNA pellet was then dissolved in 100 μl of denaturing buffer and after the addition of 100 μl of ice-cold isopropanol, centrifuged at 15,000 *g* for 20 minutes at 4°C. After removal of the supernatant the RNA pellet was washed twice in 250 μl of 75% ethanol, recentrifuged at 15,000 *g* and dried under vacuum. The resulting RNA pellet was then dissolved in storage buffer, containing 10 mM Tris (pH 7.6), 1 mM EDTA, 2 mM DTT and 40 U/ml RNasin, to a concentration of 3 μl/mm tubule length and stored at -80°C until use.

5.2.2.7. PCR primers

Gene expression was measured by RT-PCR using specific intron spanning primers designed from the published sequences of rat glucagon receptor (NCBI accession number U63021, forward primer position 4527-4548, reverse position 5396-5416), GLP-1, (NM012728, forward position 1144-1165, reverse position 1375-1394) and β-actin (V01217, forward position 2170-2194, reverse position 3055-3079).

5.2.2.8. Calculations and statistics

The expression levels of glucagon and GLP-1 receptors were established by expressing the density of the PCR products for the two receptors as a ratio of that obtained for β-actin. Results were expressed as mean ± SEM; statistical comparisons were made using a Student's unpaired *t* test. . All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

5.2.3. Autoradiographic localisation of renal glucagon binding

5.2.3.1. Induction of diabetes

Three-week STZ-induced diabetic animals were used to study the effects of diabetes on the localisation and binding of glucagon to its renal receptor. Diabetes was induced as described in section 2.2.1.

5.2.3.2. Autoradiography protocol

Control and 3-week diabetic animals were anaesthetised by intraperitoneal administration of sodium pentobarbitone (90 mg/kg) and the kidneys rapidly excised. Kidneys were de-capsulated, embedded in OCT compound (BDH, Dorset, UK) then frozen in isopentane, pre-cooled in liquid nitrogen. Serial 10 μm cryostat sections (cut at -20°C) were thaw-mounted onto polysine coated slides (BDH, Dorset, UK) and air-dried for 1 hour. Slides were pre-

incubated for 15 minutes at room temperature (RT) in 100 mM Hepes (pH 7.8) containing 30 mM KCl to reduce the levels of endogenous hormone. This was followed by a 2 hour incubation at RT in buffer containing, 100 mM Hepes, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM Na Acetate, 10 mM glucose, 1 mM EDTA, 0.1% BSA, 0.025% Bacitracin, 100 x 10^6 IU/L Aprotinin and ^{125}I-glucagon ranging from 1.5 to 1000 pM. Non-specific binding was established by incubating alternate slides in the presence of 1 μM unlabelled glucagon. The reaction was terminated using 3 x 2 minute washes in ice cold 100 mM PBS (pH 7.4). Sections were dried in a stream of cold air, followed by overnight air-drying. Slides were then exposed to ^{3}H hyperfilm (Amersham, Buckinghamshire, UK) for 7 days at 4°C. Films were developed in Kodak D19 followed by fixation in Ilford Hypam and the binding density determined in the cortex and medulla using a GS-700 imaging densitometer (Biorad, Hertfordshire, UK). For high-resolution localisation studies, autoradiography was performed as described above using 500 pM ^{125}I-glucagon and 500 nM unlabelled glucagon, for total and non-specific binding, respectively. Prior to incubation with glucagon the sections were probed with Vector red (Vector Labs Ltd, Peterborough, UK) to stain for endogenous alkaline phosphatase. Following overnight drying the slides were dipped in molten nuclear emulsion (K2, Ilford, Moberly, Cheshire) and stored in the dark for 4 days at 4°C. Slides were developed and fixed as described above, and photographed using a KS300 imaging system (Imaging Associates Ltd, Oxford). Dr Michael Dashwood (Department of Clinical Pathology, Royal Free & University College London) provided advice and assistance with the autoradiography.

5.2.3.3. Calculations and statistics

Specific binding was determined by subtracting the density obtained in the presence of unlabelled glucagon (non-specific binding) from that obtained during incubation with ^{125}I-glucagon alone (total binding). Values were expressed as arbitrary units/mm^2. Stastical significance was evaluated using

a Students unpaired *t* test. All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

5.3. Results

5.3.1. Effect of glucagon on renal glucose transport

Glucose uptake experiments displayed a characteristic time-dependent overshoot, which was inhibited by phlorizin. Incubation with glucagon did not affect the fold-enrichment of alkaline phosphatase (control: 6.95 ± 2.0 *vs.* experimental: 6.68 ± 2.1, n=12, $P>0.5$) or Na^+/K^+ -ATPase (control: 0.79 ± 0.15 *vs.* experimental: 0.72 ± 0.19, n=12, $P>0.5$). Vesicle-trapped space, as determined by incubation of BBM vesicles with 100 μM ^{3}H-glucose for 15 minutes, was also unaffected by treatment (control: 2.55 ± 0.25 *vs.* experimental: 2.25 ± 0.17 μl/mg protein, $n=12$, $P>0.5$).

Since incubation of isolated enterocytes with 10 nM pancreatic glucagon rapidly stimulates SGLT-mediated glucose transport (Debnam & Sharp, 1993), this concentration was used to study the effect of glucagon on renal glucose transport. Incubation of cortical tubules with either 10 nM pancreatic glucagon (G29) or 1 nM oxyntomodulin (G37) significantly enhanced GLUT-mediated transport, by 58% and 110%, respectively (Fig. 5.3, B), but had no effect on SGLT-mediated glucose transport (Fig. 5.3, A). These results reveal that glucagon, produced by both the pancreas and small intestine, has a direct effect on proximal tubule glucose transport, even in the presumed absence of glucagon receptors in this nephron segment (Butlen & Morel, 1985).

Western blotting of BBM vesicles prepared from cortical tubule cells incubated with 10 nM glucagon showed that the hormone has no effect on the expression levels of the facilitative glucose transporters (Fig. 5.4). As discussed in chapter 4 this alteration in GLUT-mediated glucose uptake may occur through changes in intrinsic activity of one or more of the GLUT transporters expressed at the proximal tubule BBM.

In the intestine, the effect of glucagon on sodium-dependent glucose transport correlates with an increase in cellular cAMP levels. Glucagon produces a biphasic effect on enterocyte levels of cAMP, with a peak increase at 1nM (Sharp & Debnam, 1994b). From these observations Debnam *et al.* highlighted the importance of using a wide range of glucagon concentrations when studying the effect of this hormone on epithelial transport function. Therefore, the effect of 2 nM and 20 nM glucagon on renal GLUT-mediated transport was also evaluated. Figure 5.5 demonstrates that GLUT-mediated glucose transport is significantly increased following incubation of the cortical tubule suspension with 2 nM and 10 nM glucagon. At 20 nM, glucagon failed to significantly enhance renal glucose transport. In contrast to the effect of glucagon on enterocyte cAMP levels, the hormone failed to significantly stimulate cAMP accumulation in proximal tubule cell homogenates (Table 5.1).

Figure 5.3. Effect of 30 minutes exposure of a cortical tubule suspension to 10 nM pancreatic glucagon (G29) and 1 nM oxyntomodulin (G37) on **A** the Vmax of SGLT-mediated glucose transport and **B** GLUT-mediated glucose transport. Values are representative of 6 vesicle preparations per group. *P<0.005 compared with control using a paired t test.

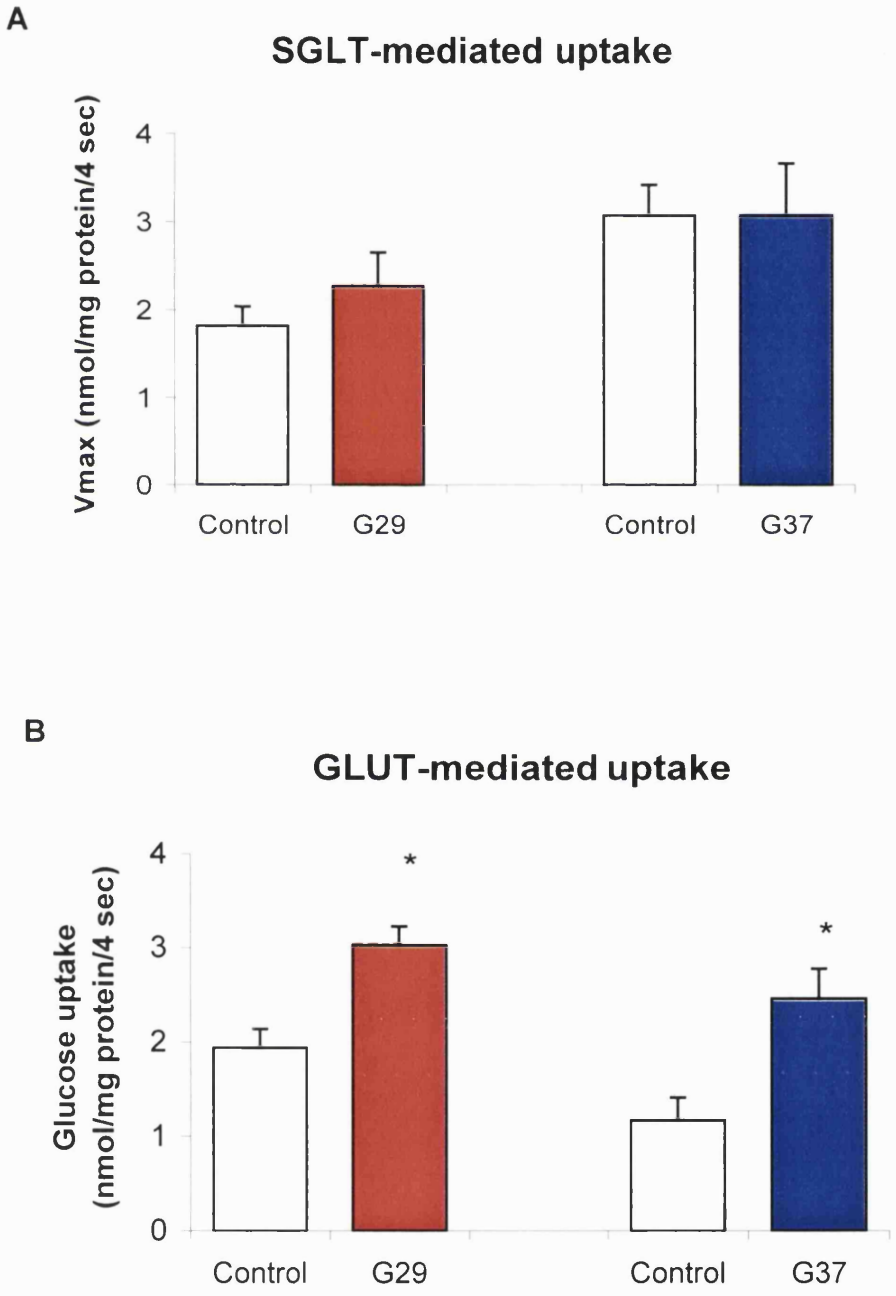


Figure 5.4. Detection and quantification of GLUT1, GLUT2 and GLUT5 protein in BBM vesicles prepared following exposure to 10 nM glucagon and its vehicle. Results were obtained from western blots carried out on 6 vesicle preparations, and expressed as mean ± SEM.

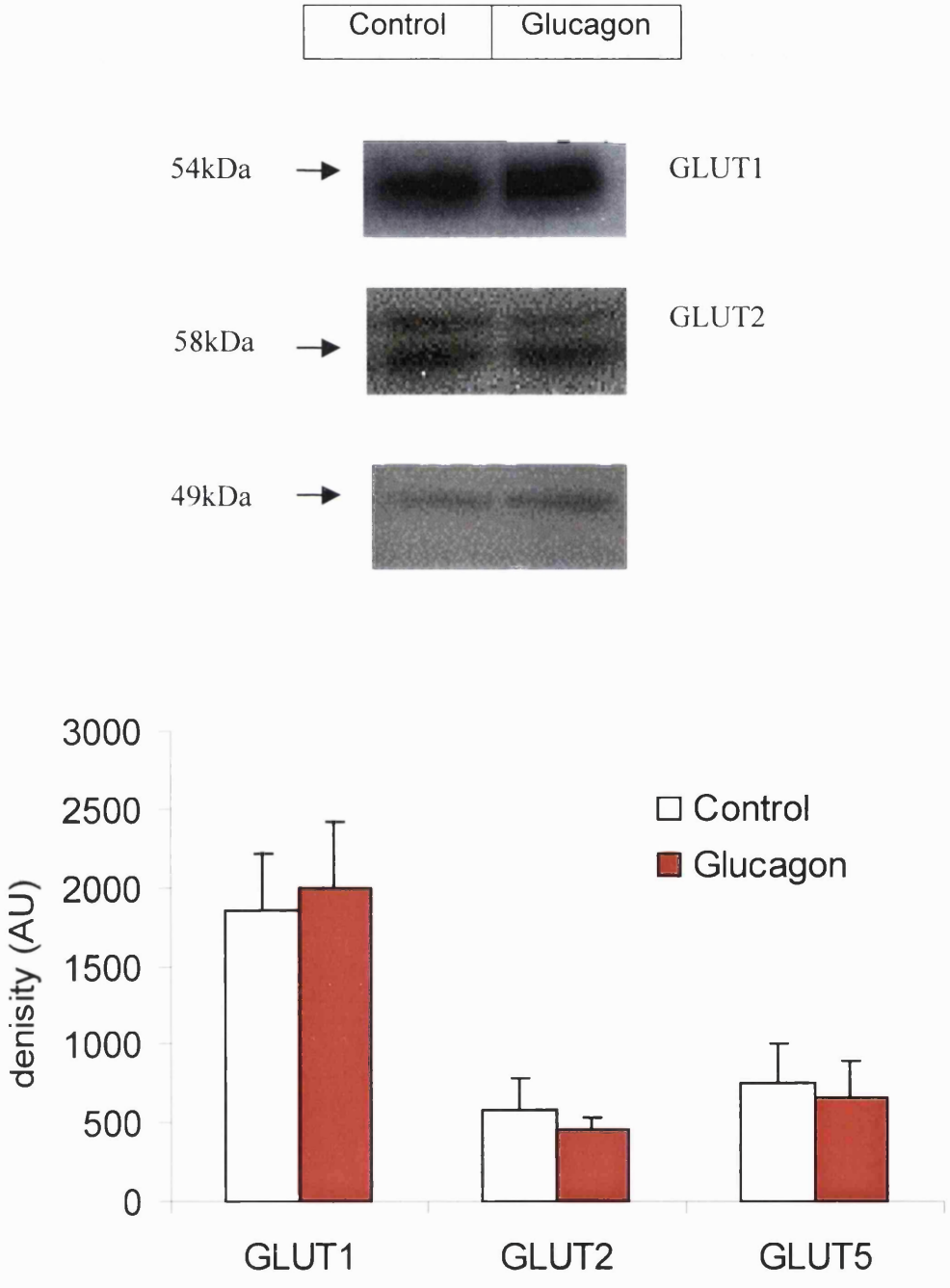


Figure 5.5. Response of GLUT-mediated glucose transport to increasing concentrations of pancreatic glucagon. Data represents 6 vesicle experiments per group. *P<0.01, **P<0.005 compared with control using a paired t test.

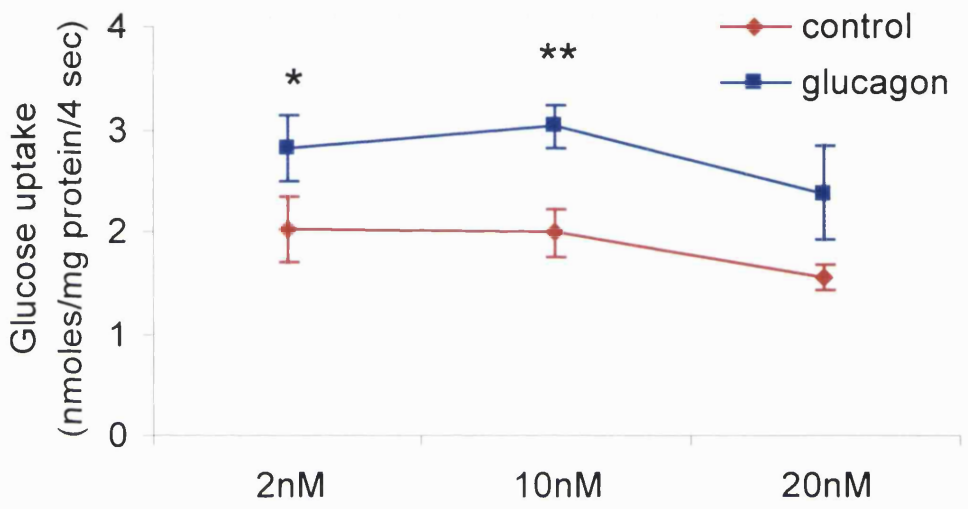


Table 5.1. cAMP levels in cortical tubule homogenates incubated with 10 nM glucagon or equivalent volume of vehicle for 30 minutes. cAMP levels were evaluated using a cAMP-^{125}I-biotrak assay. Values are representative of the mean ± SEM of 6 homogenate preparations.

Incubation condition	cAMP levels (nM)
Control	24.9 ± 7.7
Glucagon	44.3 ± 11.2

5.3.2. Microdissection results

5.3.2.1. Distribution of GR and GLP-1R in control nephron segments

As anticipated, RT-PCR of defined nephron segments revealed that glucagon receptor mRNA expression is abundant in the distal portions of the nephron (Fig. 5.6, A). However, the message for this receptor was also detected in the glomerulus, proximal convoluted and straight tubule segments. Sequencing of the PCR products from the proximal convoluted tubule (PCT) and thick ascending limb (TAL) demonstrated that the transcripts were identical; therefore the low abundance in the PCT did not represent mis-priming for a related receptor. Moreover, transcript sequences at the intron/exon boundaries revealed that alternative splice variation was not responsible for the detection of glucagon receptor mRNA expression in the PCT. Although the expression of GLP-1R mRNA in the kidney has been previously reported (Bullock *et al.*, 1996; Campos *et al.*, 1994 ; Dunphy *et al.*, 1998) the exact nephron localisation has not been established. Using RT-PCR of defined nephron segments this study demonstrates that GLP-1R is expressed exclusively in the proximal convoluted tubule (Fig. 5.6, B).

5.3.2.2. Effect of diabetes on GR and GLP-1R expression levels

The effect of STZ-induced diabetes on the renal expression of glucagon receptor and GLP-1 receptor was established using semi-quantitative RT-PCR. Initially, PCR reactions for each primer were performed using increasing numbers of cycles to ensure that all subsequent PCRs were performed within the linear range of amplification (Fig. 5.7). Based on these findings 25 cycles were used for β-actin, 33 for glucagon receptor and 35 for GLP-1R.

Figure 5.6. **A.** Representative gel from two separate RT-PCR reactions carried out for β-actin and glucagon receptor (GR). **B.** Representative gel from two separate RT-PCR reactions carried out for β-actin and GLP-1R. mRNA was prepared from isolated glomeruli (Glom), proximal convoluted tubules (PCT), proximal straight tubules (PST), cortical thick ascending limbs (cTAL) and cortical collecting ducts (CCD). Absence of contamination was confirmed by excluding the RNA (-ve) or the reverse transcriptase enzyme (RTase-). Representative of *n*=6.

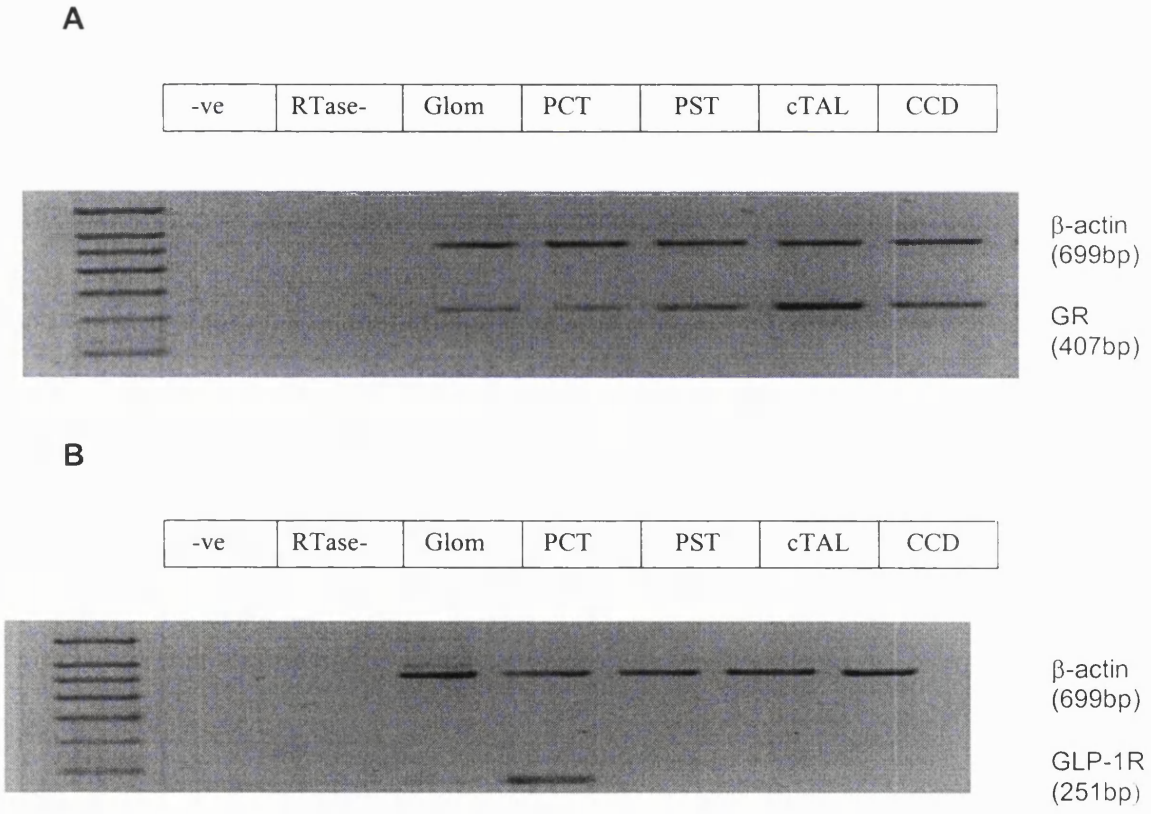
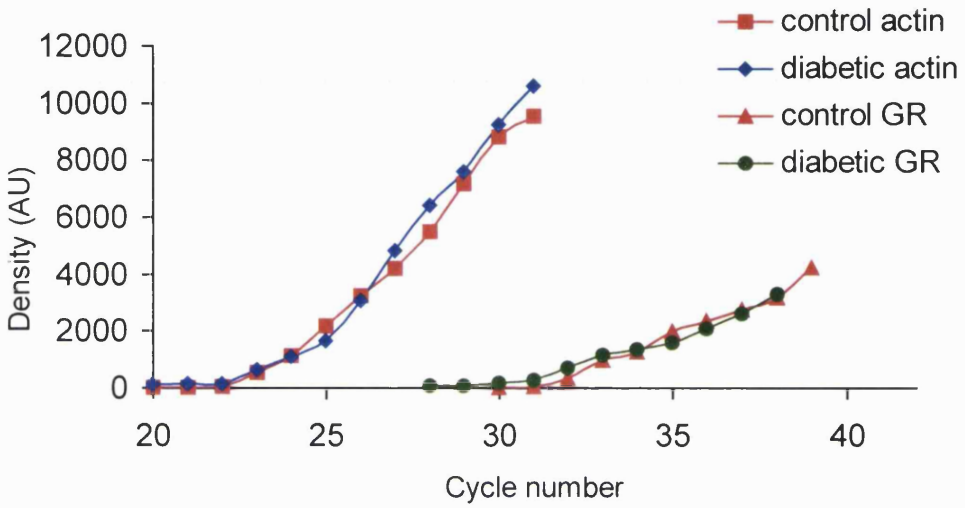


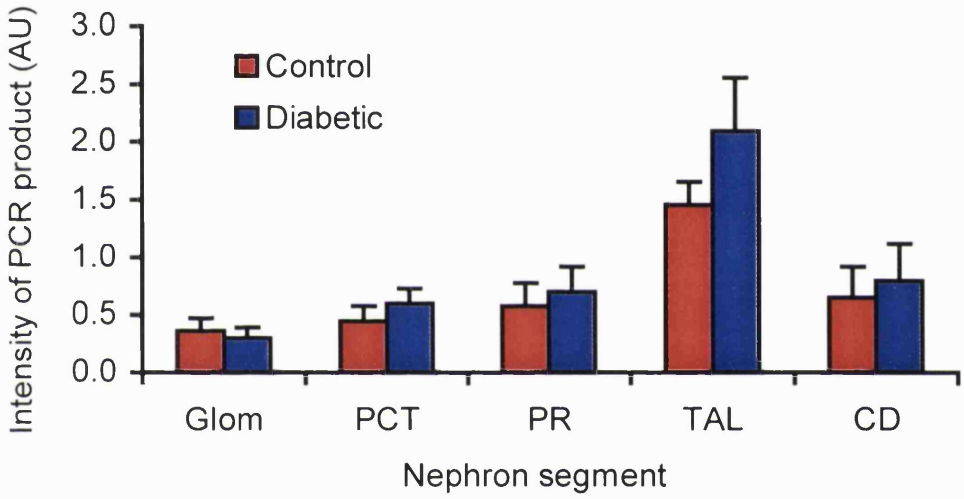
Figure 5.7. Determination of the linear range of amplification for glucagon receptor (GR) and β-actin in the PCT using increasing number of PCR cycles.



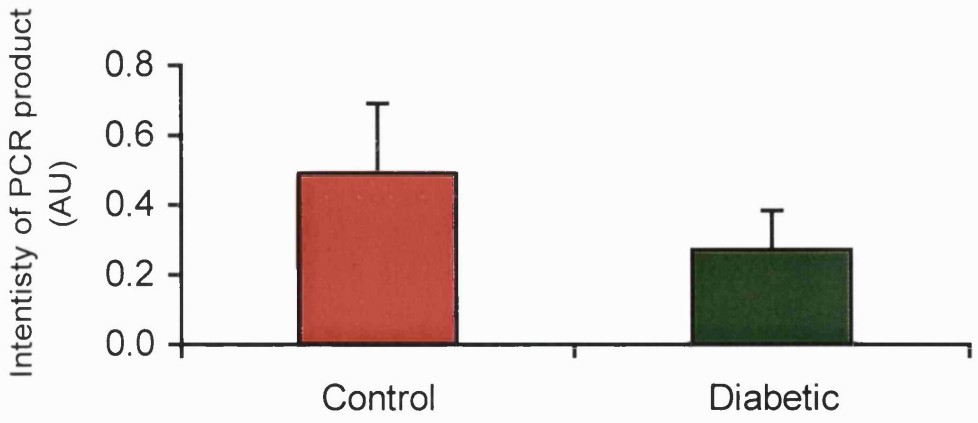
STZ-induced diabetes consistently increased the expression of the glucagon receptor mRNA in the PCT and cTAL, by 56% and 71%, respectively ($P<0.05$, $n = 5$). However, analysis of the ratio of glucagon receptor to β-actin in arbitrary units failed to reveal a significant difference between the level of expression in control and 3 week STZ-treated animals (Fig. 5.8, A). In contrast, GLP-1 receptor mRNA expression decreased by 53% ($P<0.001$, $n = 5$) in the diabetic PCT, although analysis of the arbitrary units failed to demonstrate a significant difference (Fig. 5.8, B). In addition, STZ-induced diabetes had no effect on the expression pattern of GLP-1 receptor.

Figure 5.8. Effect of 3-week STZ-induced diabetes on the expression of **A** glucagon receptor mRNA and **B** GLP-1 receptor mRNA in microdissected nephron segments. Values are expressed as arbitrary units (AU) relative to the expression of β-actin and represent RT-PCR reactions performed on segments isolated from 5 animals. Statistical comparisions were made using an unpaired *t* test.

A



B



5.3.2.3. Effect of diabetes on plasma pancreatic glucagon and cAMP levels

Hyperglucagonaemia has been proposed to play an important role in maintaining hyperglycaemia during conditions of reduced insulin secretion (Jiang & Zhang, 2003). In keeping with previous reports (Jiang & Zhang, 2003; Unger & Orci, 1975), hyperglucagonaemia was evident in the experimental model of diabetes used in this thesis (Table 5.2). In contrast, circulating levels of plasma cAMP were not significantly affected by diabetes.

Table 5.2. Plasma levels of cAMP and pancreatic glucagon in control and STZ-diabetic animals. Results are expressed as mean ± SEM, n = 6, $P<0.0001$, unpaired t test.

Plasma concentration	Control	STZ-diabetic
cAMP (nM)	51.8 ± 8	64.5 ± 4.7
Glucagon (pM)	22.3 ± 0.9	42.1 ± 3.8 *

5.3.3. Autoradiography results

Autoradiography using ^{125}I-glucagon confirmed a high degree of specific binding in the medulla of control kidneys and also demonstrated specific binding in the outer cortex, though to a lesser degree than in the medulla (Fig. 5.9). In both the cortex and medulla, non-specific binding represented approximately 40% of total binding at ^{125}I-glucagon concentrations ranging from 60-1000 pM; however at lower concentrations, non-specific binding accounted for almost 90% of total binding (results not shown). Studies to determine the localisation of ^{125}I glucagon binding employed vector red to stain for endogenous alkaline phosphatase, a marker of the BBM, which allows specific identification of proximal tubules. Total binding in the outer

cortex was apparent in all nephron segments, including those that were positively stained for alkaline phosphatase (Fig. 5.10, A). Non-specific binding was considerably lower in all nephron regions (Fig. 5.10, B) including the proximal tubule, demonstrating the presence of specific glucagon binding sites in this nephron region. Autoradiography was also used to access the effect of STZ-induced diabetes on ^{125}I-glucagon binding. In accordance with the PCR data, diabetes had no effect on specific glucagon binding in either the cortex or medulla (Fig 5.9).

Figure 5.9. Specific binding of ^{125}I-glucagon in the cortex and medulla of control and 3-week diabetic kidney sections as determined by autoradiography. Specific binding was calculated by subtracting density obtained after incubation with ^{125}I-glucagon and unlabelled glucagon from that obtained after incubation with ^{125}I-glucagon only. Data represents mean ± SEM of 4 kidney sections.

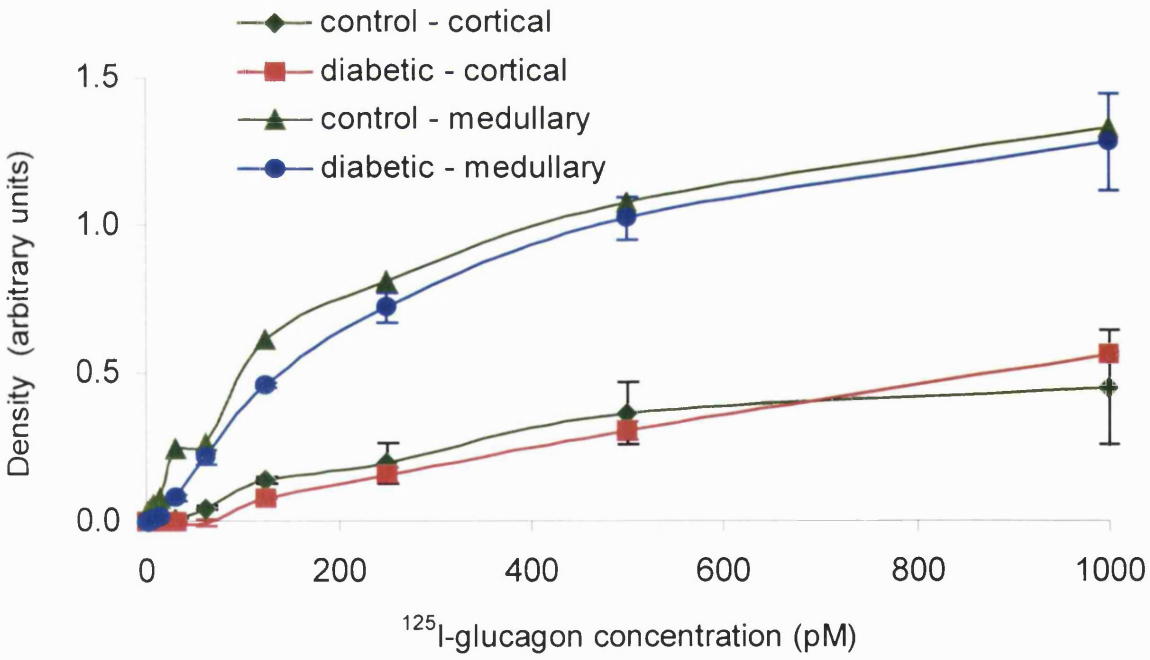
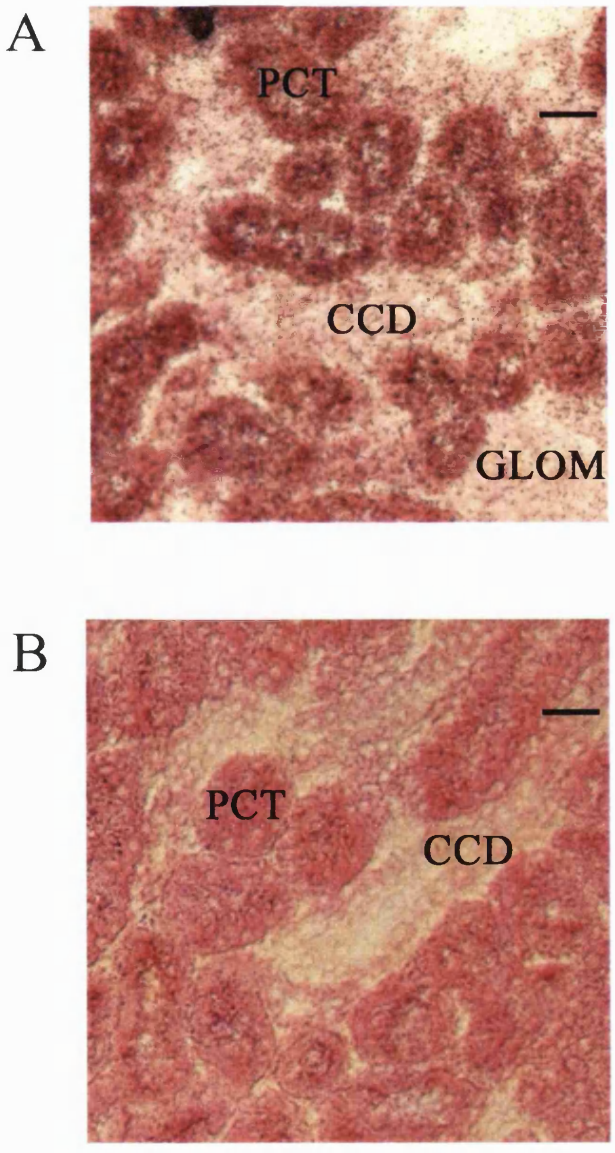


Figure 5.10 Localisation of ^{125}I-labelled glucagon binding in sections stained for alkaline phosphatase. **A** Total binding using 500 pM ^{125}I-labelled glucagon. **B** Non-specific binding using 500 pM ^{125}I-labelled glucagon in the presence of 500 nM unlabelled glucagon. These images are representative data of sections taken form four rat kidneys. Scale bar, 10 μm. PCT, proximal convoluted tubule; GLOM, glomerulus; CCD, cortical collecting duct.



5.3. Discussion

Glucagon and its structurally related peptide, GLP-1, have been reported to influence renal function. Glucagon has been shown to increase renal blood flow and glomerular filtration rate (GFR) (Unwin *et al.*, 1990), inhibit sodium and phosphate reabsorption in the proximal tubule (de Rouffignac *et al.*, 1991; Murer *et al.*, 2000), and increase calcium, magnesium and proton transport in the thick ascending limb and collecting duct (Ahloulay *et al.*, 1995; Bailly & Amiel, 1982). The influence of GLP-1 on renal function has received less attention, however, a recent study has demonstrated that the peptide can increase GFR and urine flow rate and inhibit proximal tubule sodium reabsorption (Moreno *et al.*, 2002).

The results presented in this chapter demonstrate that 30 minute exposure of cortical tubule cells to pancreatic glucagon and oxyntomodulin promotes GLUT-mediated glucose transport but has no effect on SGLT-mediated glucose transport. This finding implies a potential role for glucagon in the regulation of facilitative glucose reabsorption by the kidney. Of relevance is the finding that glucagon and the structurally related enteric hormone, glucagon-like peptide 2 (GLP-2), play a role in the acute regulation of intestinal glucose transport (Au *et al.*, 2002; Cheeseman, 1997; Debnam & Sharp, 1993; Thompson & Debnam, 1986).

Studies in the 1980's aimed to localise glucagon receptor in defined nephron regions using functional and binding studies. Bailly *et al.* examined the localisation of glucagon-sensitive adenylate cyclase activity in individual nephron segments (Bailly *et al.*, 1980). They found that compared with basal levels, glucagon maximally stimulated adenylate cyclase in the thick ascending limb, followed by the distal convoluted tubule and collecting duct. No stimulation was detected in the proximal tubules or thin segments of the loop of Henle. A later study by this group employed microdissected nephron segments to identify ^{125}I-glucagon binding sites in the rat nephron. Using a

concentration of 7.5 nM labelled hormone they showed that specific glucagon binding sites were only found in nephron segments containing glucagon-sensitive adenylate cyclase activity (Butlen & Morel, 1985). Additional studies around this time also demonstrated a role for glucagon in the regulation of sodium, potassium, calcium, magnesium and chloride reabsorption by the distal nephron (Bailly *et al.*, 1984; Di Stefano *et al.*, 1989). Renal glucose reabsorption occurs exclusively in the proximal tubule (Dominguez *et al.*, 1992), but prior to this present study, there was no evidence for a direct action of glucagon on this nephron segment or for the presence of glucagon-sensitive adenylate cyclase-coupled receptors. Therefore, the highly sensitive method of RT-PCR was used to establish the expression of glucagon receptor mRNA in defined nephron segments. As anticipated glucagon receptor mRNA was most abundant in the distal regions of the nephron, however, the transcript was also detected in the convoluted and straight regions of the proximal tubule. Autoradiography was then performed to confirm that these transcripts yielded functional glucagon receptor protein. This technique was selected because of the non-availability of a suitable antibody against the glucagon receptor protein. Specific ^{125}I-glucagon binding was evident in the cortex and medulla of whole kidney sections. High-resolution autoradiography demonstrated that specific binding in the outer cortex was at least in part due to glucagon binding sites located on the proximal tubule. Therefore, contrary to previous reports, the results presented in this chapter demonstrate the presence of glucagon receptor mRNA and specific protein binding of glucagon in the proximal tubule, suggesting glucagon affects GLUT-mediated glucose transport via activation of specific glucagon receptors located in this nephron region.

The classical model for glucose transport across renal and intestinal absorptive cells involves Na^+-dependent, SGLT-mediated, glucose uptake across the BBM, followed by efflux across the basolateral membrane via facilitative, GLUT-mediated, transport. However, the studies described in this thesis and those performed in the intestine by Kellet *et al.* have provided evidence for a role of facilitative glucose transporters at the BBM, which are dynamically regulated by PKC. Thus, the finding that cyclic AMP is involved

in the regulation of SGLT-mediated uptake at the renal BBM, whereas glucagon increases GLUT-mediated uptake (discussed in Chapter 4), suggests that glucagon receptors expressed in the proximal tubule are not coupled primarily to activation of adenylate cyclase. This interpretation would explain the failure to detect glucagon-sensitive adenylate cyclase activity in the proximal tubule (Bailly *et al.*, 1980). In support of this hypothesis, studies using cultured baby hamster kidney cells have shown that glucagon receptors can stimulate a G protein-coupled pathway, activating phospholipase C and releasing intracellular Ca^{2+} from IP_3-sensitive Ca^{2+} stores (Hansen *et al.*, 1998). Additionally, Wakelam *et al.* have also found evidence for this alternative signalling pathway in cultured hepatocyes (Wakelam *et al.*, 1986). Preliminary (unpublished) studies performed by Dr Matthew Bailey (Centre for Nephrology, Institute of Urology and Nephrology, Royal Free & University College London) have demonstrated that exposure of isolated proximal tubules to 10 nM glucagon induces a 2-fold increase in inositol triphosphate levels. These results, taken together with the observation that facilitative glucose transport is regulated by PKC and intracellular calcium levels, suggest that glucagon receptors expressed in the proximal tubule may indeed be coupled to this alternative signalling pathway.

Although it is generally accepted that oxyntomodulin mainly targets the stomach and small intestine (Collie *et al.*, 1997; Dubrasquet *et al.*, 1982; Stumpel *et al.*, 1997), the results presented in this chapter demonstrate for the first time that the peptide can influence renal transport function. Numerous reports in the early literature demonstrated that oxyntomodulin binds to both glucagon and GLP-1 receptors leading to an increase in intracellular cAMP accumulation (Butlen & Morel, 1985; Fehmann *et al.*, 1994; Gros *et al.*, 1992; Gros *et al.*, 1993; MacNeil *et al.*, 1994; Schepp *et al.*, 1996). At present it is unknown whether the effect of oxyntomodulin on renal glucose transport occurs via stimulation of glucagon or GLP-1 receptors expressed in the proximal tubule or whether the peptide exerts its affect via a distinct population of uncharacterised receptors.

As described in chapters 2 and 3, STZ-induced diabetes increases facilitative glucose transport, via insertion of GLUT2 protein at the proximal tubule BBM. This increased rate of glucose transport can be mimicked by the short-term incubation of cortical tubule cells with glucagon, raising the possibility that the hormone plays a role in the regulation of renal glucose transport during hyperglycaemia. Interestingly, it has been proposed that increased secretion of pancreatic glucagon during experimental diabetes, is responsible for the enhanced sodium-dependent glucose transport that occurs across the enterocyte BBM during this condition (Sharp & Debnam, 1994b; Thompson & Debnam, 1986). In light of these findings the effect of STZ-induced diabetes on renal glucagon receptor binding and mRNA expression levels was examined. Although there was a consistent increase in glucagon receptor mRNA expression in the PCT and cTAL, this difference failed to reach significance when expressed as arbitrary units. However, it is of interest that diabetes increases glucagon receptor mRNA expression in other cell types (Burcelin *et al.*, 1998; Tokuyama Y & Bell, 1993). Additionally, rat islet cells and hepatocytes cultured in high glucose concentrations display significantly increased levels of glucagon receptor mRNA (Abrahamsen & Nishimura, 1995; Burcelin *et al.*, 1998), therefore hyperglycaemia may be responsible for increased glucagon receptor mRNA expression during diabetes. Autoradiography performed on kidneys removed from control and STZ-induced diabetic animals revealed no apparent difference in specific ^{125}I-glucagon binding to proximal tubules. However, it has been proposed that even under conditions of reduced receptor expression, the ability of glucagon to activate signalling events may remain unchanged or even be elevated (Jiang & Zhang, 2003). Additionally, activation of this receptor has also been shown to be proportional to the extracellular concentration of free glucagon (Birnbaumer *et al.*, 1972). In agreement with previous reports (Jiang & Zhang, 2003; Unger & Orci, 1975), plasma glucagon levels were significantly increased during STZ-induced diabetes. Thus, even with no alteration in glucagon receptor expression, increased circulating levels of plasma glucagon may influence the diabetes-induced enhancement of renal GLUT-mediated glucose transport.

Although previous studies have shown the presence of GLP-1 receptor mRNA in the kidney (Bullock *et al.*, 1996; Dunphy *et al.*, 1998), there has been no information on its localisation along the nephron. In this present work, RT-PCR of defined nephron segments revealed that GLP-1 receptor mRNA is expressed exclusively in the proximal convoluted tubule. This finding suggests that GLP-1 may influence proximal tubular transport function, but also demonstrates the sensitivity of the technique and indicates that the low transcript levels of glucagon receptor in the proximal tubule is not a consequence of contamination with other nephron segments. Interestingly, the only documented effects of GLP-1 on renal transport function occurs in the PCT, where the peptide influences sodium reabsorption (Moreno *et al.*, 2002). Early studies into the binding properties of GLP-1 receptor demonstrated that superphysiological concentrations of glucagon could bind to and activate GLP-1 receptors (Graziano *et al.*, 1993; Gros *et al.*, 1993; Moens *et al.*, 1998), leading to an increase in both adenylate cyclase and phospholipase C activity (Dillon *et al.*, 1993; Wheeler *et al.*, 1993). Taken together these findings make this receptor a candidate for the diabetes-induced alterations in renal glucose transport. In contrast to the effects of STZ-induced diabetes on glucagon receptor expression, there was a trend for decreased GLP-1 receptor mRNA expression following induction of diabetes. This finding is in keeping with the effect of diabetes on the expression of GLP-1 receptor in pancreatic islet cells (Tokuyama Y & Bell, 1993) and the observation that culturing of this cell type in high glucose concentrations reduces GLP-1 receptor expression (Abrahamsen & Nishimura, 1995). Increased PKC activity has been documented following exposure of proximal tubule cells to high glucose concentrations (Park *et al.*, 2001; Phillips *et al.*, 1997) and in proximal tubule cells isolated from diabetic animals (Ha *et al.*, 2001). This is of relevance to the finding that increased cellular PKC activity desensitises GLP-1 receptor expression (Fehmann *et al.*, 1996; Widmann *et al.*, 1996), indicating the trend for reduced expression of GLP-1 receptor mRNA in the PCT of diabetic animals may be a consequence of hyperglycaemia-induced alterations in cellular PKC activity. From these findings it seems unlikely that the increased renal GLUT-

mediated glucose transport evident during diabetes occurs as a consequence of GLP-1 receptor activation.

To date there is no evidence for a role of GLP-2 in the regulation of renal function. Although it has been proposed that the kidney is involved in the clearance of this peptide from the circulation, there is no evidence for specific renal GLP-2 receptor binding sites (Thulesen *et al.*, 2000) or mRNA expression (Yusta *et al.*, 2000). However, a recent study has demonstrated that GLP-2 promotes the rapid insertion of GLUT2 protein at the enterocyte BBM (Au *et al.*, 2002). The observation that there are considerable similarities between the regulation of renal and intestinal glucose transport and the fact that glucagon receptor expression in the proximal tubule has previously been overlooked, raises the possibility that GLP-2 receptor expression and activation may play a role in the regulation of renal function.

5.4. Conclusion

In summary, it has been shown that glucagon receptor mRNA and specific glucagon binding sites can be detected in the renal proximal tubule and that glucagon promotes GLUT-mediated glucose transport across the proximal tubule BBM. This is in contrast to its effect on glucose uptake across the intestinal BBM, which is SGLT-mediated and cyclic AMP-dependent. Therefore, glucagon receptors present in the proximal tubule may display a distinct cellular signalling pathway from those expressed in more distal nephron segments, in that they are not coupled primarily to adenylate cyclase and thus cyclic AMP generation. Further studies are necessary to characterise the signal transduction pathway of these proximal tubule glucagon receptors. Diabetes has no affect on glucagon receptor mRNA expression or binding, however hyperglucagonemia may be capable of influencing renal glucose transport during diabetes even in the absence of changes in glucagon receptor expression.

Chapter 6.

Regulation of renal glucose transport by ATP and adenosine.

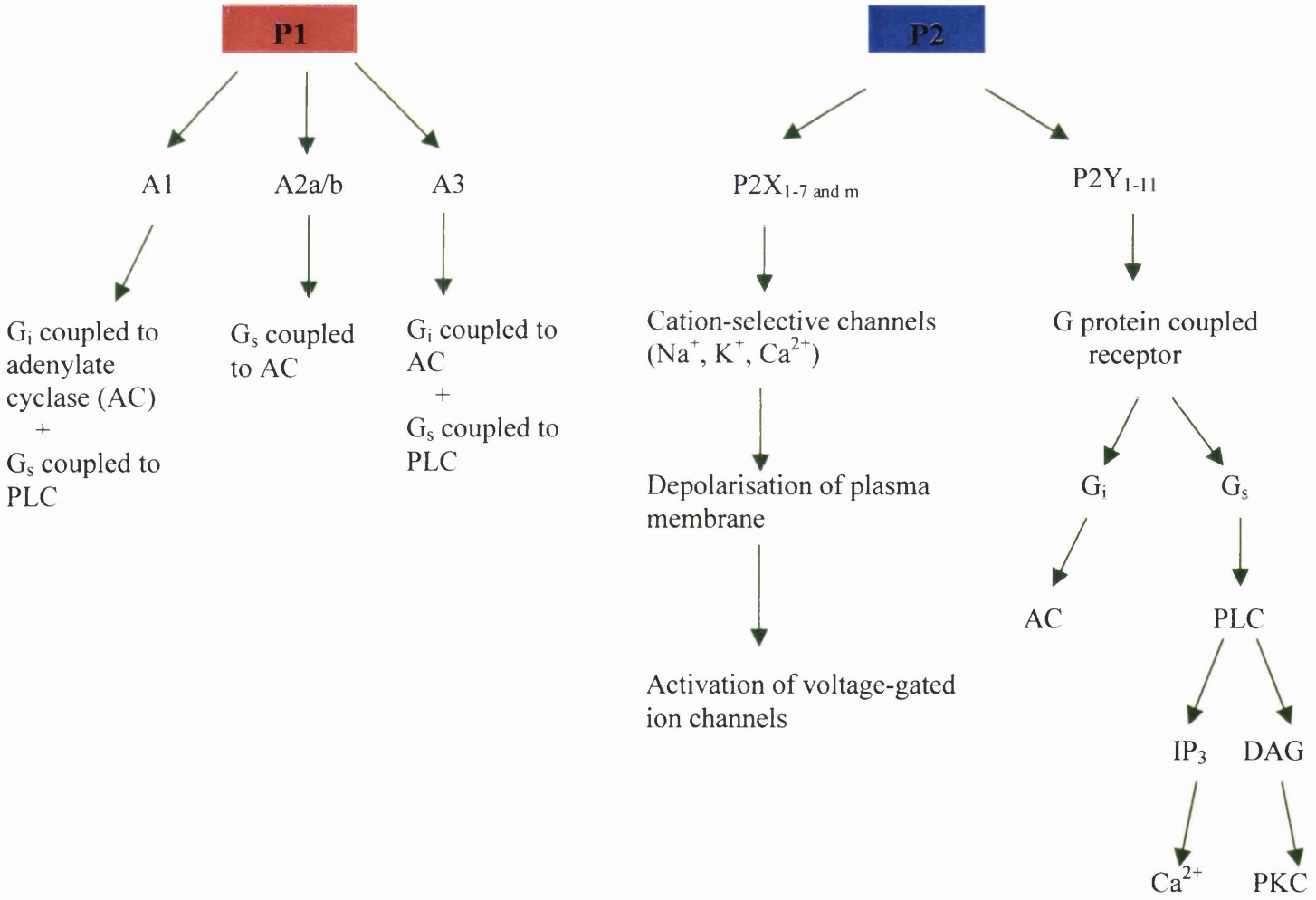
6.1. Introduction

Extracellular adenosine triphoshate (ATP) was first recognised to have important biological actions more than 70 years ago (Drury AN & Szent-Gyorgyi A, 1929). In the early 1970's it was proposed that ATP was the principle neurotransmitter of the autonomic nervous system (Burnstock, 1972). Following a large body of evidence for the role of ATP as a neurotransmitter and knowledge about specific extracellular receptors that mediate the affects of the nucleotide (reviewed by (Satchell, 2000)), Burnstock put forward a classification system for purine receptors, distinguishing between adenosine, P1, and ATP, P2 receptors (Burnstock, 1980a; Burnstock, 1980b).

Both P1 and P2 receptors are now subdivided based on their properties (Fig 6.1). P1 receptors have been subdivided into A1, A2a, A2b and A3. A1 receptors have a high affinity for adenosine, in the nM range; they can either inhibit adenylate cyclase or increase phospholipase C (PLC). A2 receptors have a lower affinity for adenosine, in the μM range. This group is subdivided into A2a and A2b. The A2a receptor subtype is positively coupled to protein kinase C (PKC), whilst the A2b subtype acts through Gs proteins to increase adenylate cyclase and is therefore positively coupled to protein kinase A (PKA). A3 receptor subtype has similar signalling properties to the A1 receptors but a lower affinity for adenosine (Zhou *et al.*, 1992).

P2 receptors have also been subdivided into different groups: P2X and P2Y on the basis of their receptor properties. At present there are 8 members of the P2X family, all of which are cation gated ion channels. Activation of these receptors results in rapid selective permeability to Na^+, K^+ and Ca^{2+}, through the receptors pore-like structure. The influx of cations causes membrane depolarisation and secondary activation of voltage-dependent Ca^{2+} channels. This transduction mechanism does not rely on the production of secondary messengers, so the response time is very rapid and occurs within 10ms (Ralevic & Burnstock, 1998).

Figure 6.1. Purinoceptor classification and intracellular signalling properties



These P2X receptor proteins have been named $P2X_1$-$P2X_7$ and the most recent isoform P2Xm, which has been cloned from skeletal muscle (Urano *et al.*, 1997). The different receptor subtypes vary in their ATP binding kinetics, with EC_{50} values for ATP ranging between 0.7 and 15μM for $P2X_1$-$P2X_6$, whilst $P2X_7$ requires 300μM ATP to elicit a current (Schwiebert & Kishore, 2001).

P2Y receptors are G-protein coupled receptors. 11 have been identified, although only $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ are accepted as valid members of the mammalian P2 receptor subfamily (Ralevic & Burnstock, 1998). P2 receptors have a broad natural ligand specificity, recognizing ATP, ADP, UTP and UDP; with the relative potency of these ligands being dependent on the P2Y receptor that is activated. Most P2Y receptors act via G-protein coupling to activate PLC, leading to the formation of inositol 1,4,5-triphosphate (IP_3) and mobilisation of intracellular calcium. Coupling to adenylate cyclase has also been described for some P2Y receptors. For example, activation of $P2Y_1$ and $P2Y_2$ receptors, causes stimulation of PLC and inhibition of adenylate cyclase. Whilst, the $P2Y_{11}$ receptor subtype is unique in the fact that it is positively coupled to both PLC and adenylate cyclase (Schwiebert & Kishore, 2001). Formation of IP_3 and mobilisation of intracellular Ca^{2+} as a result of P2Y receptor activation can elicit a variety of other intracellular responses, such as activation of PKC and phospholipase A_2 (PLA_2), calcium-dependent activation of K^+ channels and nitric oxide production. Furthermore, diacylglycerol (DAG) formation, as a result of PLC activation, can activate phospholipase D (PLD), the MAP kinase pathway and/or Ca^{2+} influx via voltage-operated Ca^{2+} channels. These downstream signalling events are dependent on the subtype of receptor that has been activated and the cell type in which the receptor is expressed. The response time for P2Y receptor activation is longer than that of the P2X receptor family, occurring within 100ms, due to the complexity of the second-messenger systems and/or ionic conductance's mediated by G-protein coupling (Ralevic & Burnstock, 1998).

Both P1 and P2 receptors are present in various tissues including the kidney (Ralevic & Burnstock, 1998). The exact distribution and role of these receptors in the kidney remains unclear, but is an active area of research. Most of the published findings on the distribution and function of the P2 receptors have used renal cell lines or primary cell cultures. However, recent reports have demonstrated their presence in native tissue. In brief, P2 receptors are expressed in the renal vasculature, in glomerular structures and throughout the nephron (Bailey *et al.*, 2000; Turner *et al.*, 2003). P1 purinoceptors are widely distributed in the renal vasculature, where the binding of adenosine to A1 and A2 receptors induces vasoconstriction and vasodilatation, respectively (Jackson & Dubey, 2001). Studies using various cell lines have also provided evidence for P1 receptors on renal epithelial cells (Coulson *et al.*, 1991; Kang *et al.*, 2001; Levier *et al.*, 1992; Prie *et al.*, 1995).

There are multiple pathways that result in the production of extracellular adenosine, to levels high enough to bring about P1 receptor activation. These include the sequential dephosphorylation of intracellular or extracellular ATP to adenosine. It is well established that intracellular adenosine can be transported out of the cell via bi-directional nucleoside transporters, that are dipyridamole sensitive, resulting in exposure of neighboring cells to raised adenosine concentrations (Cass *et al.*, 1998). The transmethylation pathway is another means by which adenosine is produced. This pathway involves the hydrolysis of *S*-adenosyl-L-homocysteine to L-homocysteine and adenosine and occurs at a relatively constant rate, therefore providing a basal level of adenosine production (Lloyd *et al.*, 1988). Production of adenosine by the cAMP-adenosine pathway is a relatively new concept (Jackson & Dubey, 2001). It is postulated that upon activation of hormone receptors coupled to adenylate cyclase, intracellular cAMP concentrations are increased. The resulting cAMP can be metabolised to AMP by cytosolic phosphodiesterases and the AMP broken down to adenosine by cytosolic 5'-nucleotidases. This intracellular adenosine may then exit the cell by the nucleoside transporters. Hormonally stimulated cAMP has also been shown to egress from the cell,

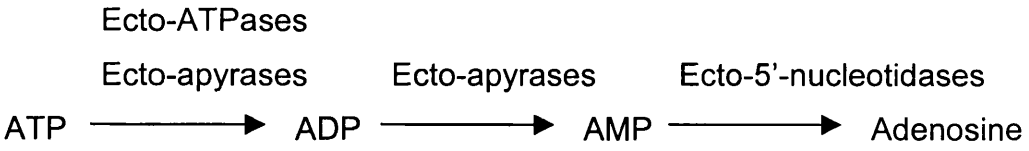
via the organic anion transporter (Davoren PR. *et al.*, 1963), where it is metabolised to AMP and adenosine by membrane bound phosphodiesterases and 5'-nucleotidases (Jackson & Dubey, 2001). This method of adenosine production is termed the extracellular cAMP-adenosine pathway. Adenosine can also be produced from exogenous cAMP (Jackson & Dubey, 2001); in fact cAMP produced from the liver has been demonstrated to affect renal function, either by the direct action of cAMP *per se* (Bankir *et al.*, 1997) or perhaps by its subsequent metabolism to adenosine.

Renal epithelial cells have been reported to contain ATP stores of up to 3-5 mM, whereas the extracellular concentration is very low (Gordon, 1986; Taylor *et al.*, 1998). This provides a large gradient for efflux of this nucleotide into the tubule lumen or renal interstitium. It has been hypothesized that ATP and its metabolites act in an autocrine/paracrine manner within the tissue microenvironment into which it has been released. It has been proposed that release of ATP from renal epithelial cells may occur by at least three different mechanisms: (i) conductive ATP transport through ATP release channels; (ii) facilitative diffusion through an ATP transporter or pore; and /or (iii) exocytosis of ATP-filled vesicles or granules (Schwiebert, 2001). Using various methods to measure ATP, it has been established that ATP liberated from a cell never exceeds 10μM. Therefore an individual cell is only releasing or sacrificing 0.1% or less of its total intracellular ATP pool (Schwiebert, 2001). Using cell lines and primary cell cultures, this group has shown that proximal tubule cells are the richest source of ATP in the kidney. Under basal conditions, this cell type releases ATP at high nanomolar concentrations, primarily across the brush border membrane. Whilst, in the presence of calcium agonist, or during pathological conditions such as hypoxia, hypertonicity, sheer stress or loss of cell viability, up to 5μM ATP can be released (Schwiebert & Kishore, 2001). The thick ascending limb and collecting duct are also a source of ATP, with low nanomolar concentrations being released (Schwiebert, 2001). From this information it has been hypothesized that ATP filtered by the glomerulus or

that released from the proximal tubule, travels down the nephron to regulate more distal nephron segments (Schwiebert, 2001).

ATP within the renal tubular lumen has several possible fates; (i) it could just simply diffuse or be swept away in the circulating environment; (ii) the ATP could be degraded by ecto-enzymes present on the cell surface to produce ATP metabolites (Fig 6.2). These metabolites may in turn act as agonists for the P2 receptors or in the case of adenosine act on P1 receptors; (iii) ATP *per se* can act on the P2 receptors that are expressed throughout the nephron (Schwiebert, 2001).

Fig 6.2 Degradation of extracellular ATP by membrane bound ecto-enzymes



ATP = adenosine triphosphate
ADP = adenosine diphosphate
AMP = adenosine monophosphate

In recent years, Unwin and coworkers have contributed to the scientific literature in the field of purinoceptors. The group has published data on the localisation of these receptors in the kidney (Bailey *et al.*, 2000; Turner C *et al.*, 2002; Turner *et al.*, 2003), and has started to delineate the role of ATP in regulating renal function (Bailey *et al.*, 2001b; Vonend O *et al.*, 2001). To combine the interests of the research group I work in and to expand the understanding of the role of purinoceptors in the regulation of renal glucose transport function, this project used BBM vesicles to establish if activation of purinoceptors alters renal BBM glucose transport.

6.2. Materials and methods

6.2.1. Incubation of cortical suspension

The protocol for the isolation and incubation of proximal tubule cells with ATP or adenosine was identical to that described in section 4.2.1. The tubule suspension was incubated for 30 minutes in the prescence of 100 µM and 1 mM ATP or 50 µM and 1 mM adenosine.

6.2.2. ATP assay for measurement of ATP in cortical tubule suspension

The concentration of ATP in the cortical tubule suspension was measured prior to and following 30 minutes incubation at 37°C. An aliquot of tubule suspension was taken and the protein precipitated by the addition of 50% TCA, followed by centrifugation at 6000 x g for 5 minutes. The ATP content of the supernatant was measured using an ATP analysis kit (Assay kit No. 366-UV, Sigma, Dorset, UK) following the manufacturers instructions.

The principle of this assay relies on the conversion of ATP and 3-phosphoglycerate to ADP and 1,3-diphosphoglycerate by the enzyme phosphoglyceric phosphokinase (PGK). Followed by the conversion of 1,3-diphosphoglycerate and NADH to glyceraldehyde-3-phosphate, NAD and phosphate, by the enzyme glyceraldehyde phosphate dehydrogenase (GAPD), which is measured at 340nm, using a Beckman Du 650 spectrophotometer (Beckman-Coulter Bioresearch, Buckinghamshire, UK).

$$\text{ATP + 3-phosphoglycerate} \xrightarrow{\text{PGK}} \text{ADP + 1,3 diphosphoglycerate}$$

$$\text{1,3 diphosphoglycerate + NADH} \xrightarrow{\text{GAPH}} \text{NAD + Pi + glyceraldehyde-3-phosphate}$$

6.2.3. BBM vesicle preparation (as 2.2.3) and uptake studies (as 2.2.4.)

6.2.4. Western blotting (as 3.2.3.)

6.2.5. Statistics

Results are expressed as mean ± SEM of 6 vesicle preparations. Statistical comparisons were made using a Student's paired or unpaired *t* test. All analysis was performed using Graphpad Instat software with statistical significance taken as $P<0.05$.

6.3. Results

6.3.1. Concentration of ATP in cortical suspension

The data shown in Table 6.1 demonstrates that following 30 minutes of incubation of a cortical tubule suspension, where no ATP is added, there is an increase in ATP concentration within the incubation media. This finding is not unexpected considering the nature of the tubular suspension, where a proportion of the tubules may well be experiencing cellular damage, which would result in release of the intracellular ATP stores. Conversely, incubation of tubule cells with 100 μM or 1 mM ATP resulted in degradation of the nucleotide. This is most likely due to the presence of cytosolic and membrane bound phosphodiesterases and 5' -nucleotidases.

Table 6.1. ATP concentration in the cortical tubule suspension prior to (time zero) and following the 30 minute incubation period at 37°C after addition of 0, 100 μM or 1 mM ATP. Values are given in μM ± SEM and represent 6 preparations.

ATP concentration (μM)	Time zero	30 minutes
0	4.8 ± 2.1	28.2 ± 3.4
100	110.9 ± 6.8	63.5 ± 1.5
1000	1008 ± 15.9	718 ± 70

6.3.2. Effect of ATP on renal glucose transport

This series of experiments aimed to establish the effect of ATP on renal BBM glucose transport. A cortical tubule suspension was incubated with 100 μM or 1 mM ATP and BBM vesicles subsequently prepared. Glucose uptake experiments aimed to delineate the effect of the nucleotide on SGLT and GLUT-mediated glucose transport.

Glucose uptake experiments displayed a characteristic time-dependent overshoot, which was inhibited by phlorizin. Incubation with ATP or adenosine did not affect the fold-enrichment of alkaline phosphatase (control: 6.34 ± 0.43 *vs.* experimental: 5.9 ± 0.3, n=12, $P>0.5$ unpaired *t* test) or Na^+/K^+-ATPase (control: 0.75 ± 0.14 *vs.* experimental: 0.78 ± 0.15, n=12, $P>0.5$ unpaired *t* test). Vesicle-trapped space, as determined by incubation of BBM vesicles with 100 μM ^{3}H-glucose for 15 minutes, was also unaffected by treatment (control: 2.75 ± 0.23 *vs.* experimental: 2.47 ± 0.35 μl/mg protein, $n=12$, $P>0.5$ unpaired *t* test).

Incubation with 100 μM ATP had no significant effect on the V_{max} of SGLT-mediated transport compared with incubation with vehicle alone (Fig 6.3, A). Additionally there was no significant effect of the nucleotide on the K_m of SGLT-mediated transport (control: 489 ± 6.5 *vs.* 100 μM ATP: 488 ± 23 μM (P>0.9, paired t test). However, GLUT-mediated transport was increased by 103%, P<0.005 (Fig 6.3, B) following incubation with 100 μM ATP.

Incubation with 1 mM ATP resulted in the same pattern, with a significant increase in the rate of GLUT-mediated transport, by 42% (P<0.05, paired t test) (Fig 6.4, B) but no significant change in the V_{max} (Fig 6.4, A) or K_m (control: 457 ± 47 *vs.* 1 mM ATP 460 ± 39, P>0.9, paired t test) of SGLT-mediated transport.

Fig 6.3. Effect of 100μM ATP on **A** V_{max} of SGLT-mediated glucose uptake and **B** GLUT-mediated glucose uptake into renal BBM vesicles. $n = 6$, * $P<0.005$, paired t test.

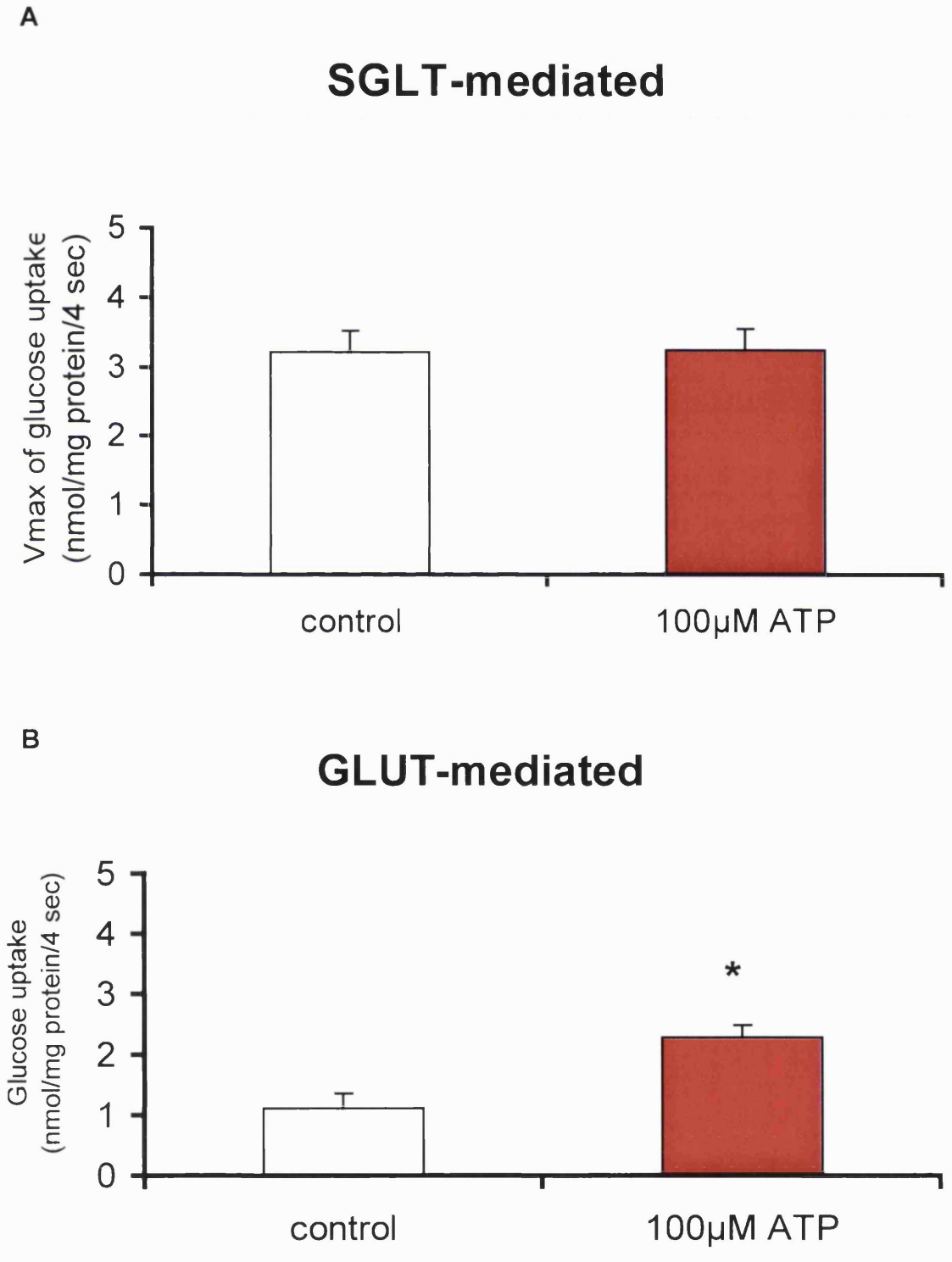
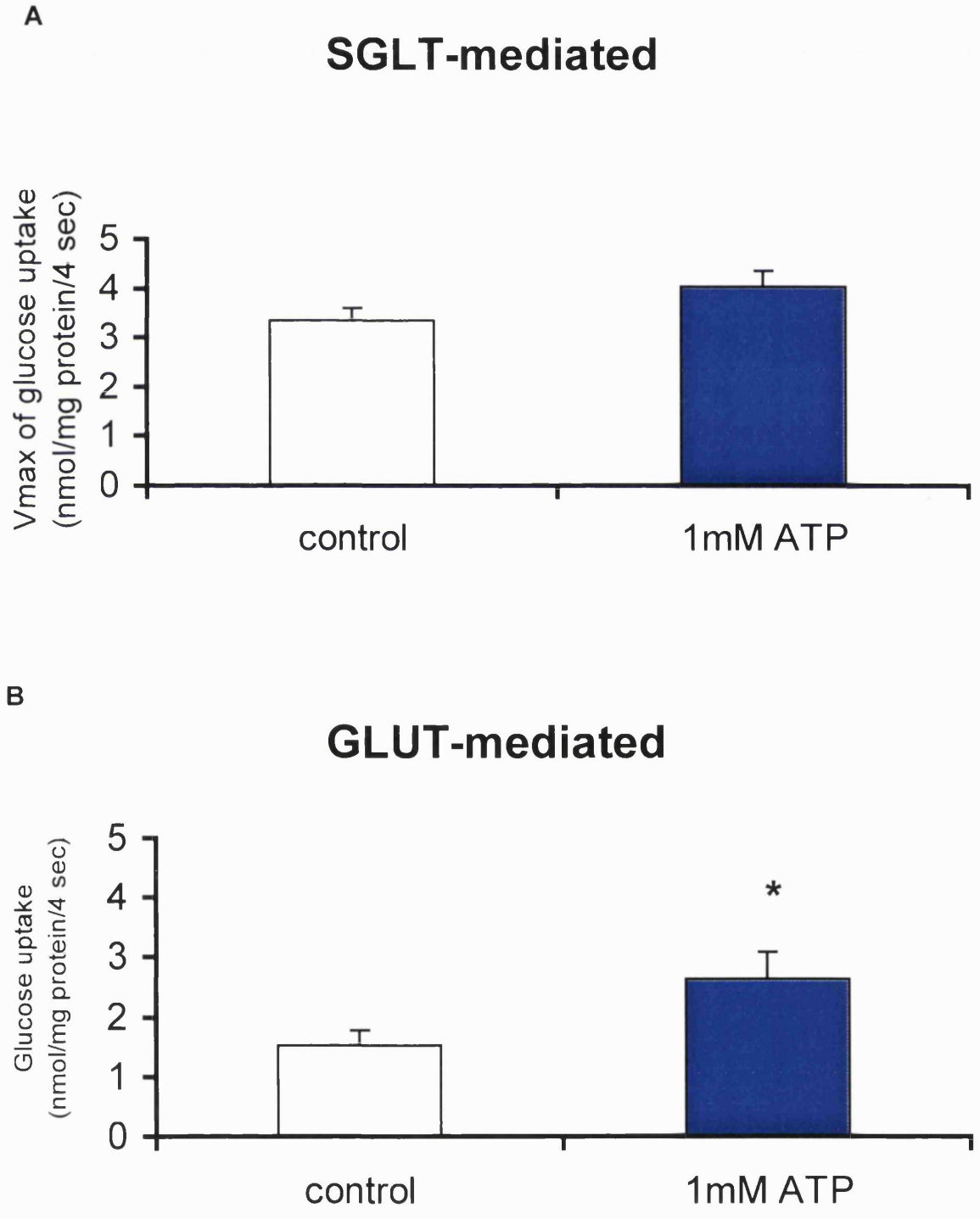


Figure 6.4. Effect of 1 mM ATP on **A** V_{max} of SGLT-mediated glucose uptake and **B** GLUT-mediated glucose uptake into renal BBM vesicles. n = 6, * P<0.05, paired t test.



6.3.3. Effect of adenosine on renal glucose transport

Adenosine had similar effects on renal glucose transport as ATP. Incubation with 50 μM or 1 mM adenosine had no effect on the V_{max} (Fig. 6.5, A and 6.6, A) or the K_m of SGLT-mediated glucose transport (K_m of control: 468 ± 26 *vs.* adenosine 418 ± 53 μM, $P>0.3$, paired *t* test). 1 mM adenosine increased GLUT-mediated transport by 97%, $P<0.02$ (Fig 6.6, B) whereas 50 μM adenosine failed to evoke a significant response (Fig 6.5, B).

Since 50 μM adenosine was without effect on GLUT-mediated transport, it suggests that the response seen with ATP incubation is not due to metabolism of the nucleotide to its breakdown product adenosine. This suggestion is supported by the fact that measurement of the concentration of ATP in the cortical suspension following the 30 minute incubation was not significantly lower than the concentration added at the start of the incubation period. Therefore it could be hypothesised that ATP and adenosine evoke the same response via both P1 and P2 purinoceptor activation.

Figure 6.5. Effect of 50 μM adenosine on **A** Vmax of SGLT-mediated glucose uptake and **B** GLUT-mediated glucose uptake into renal BBM vesicles. *n* = 6

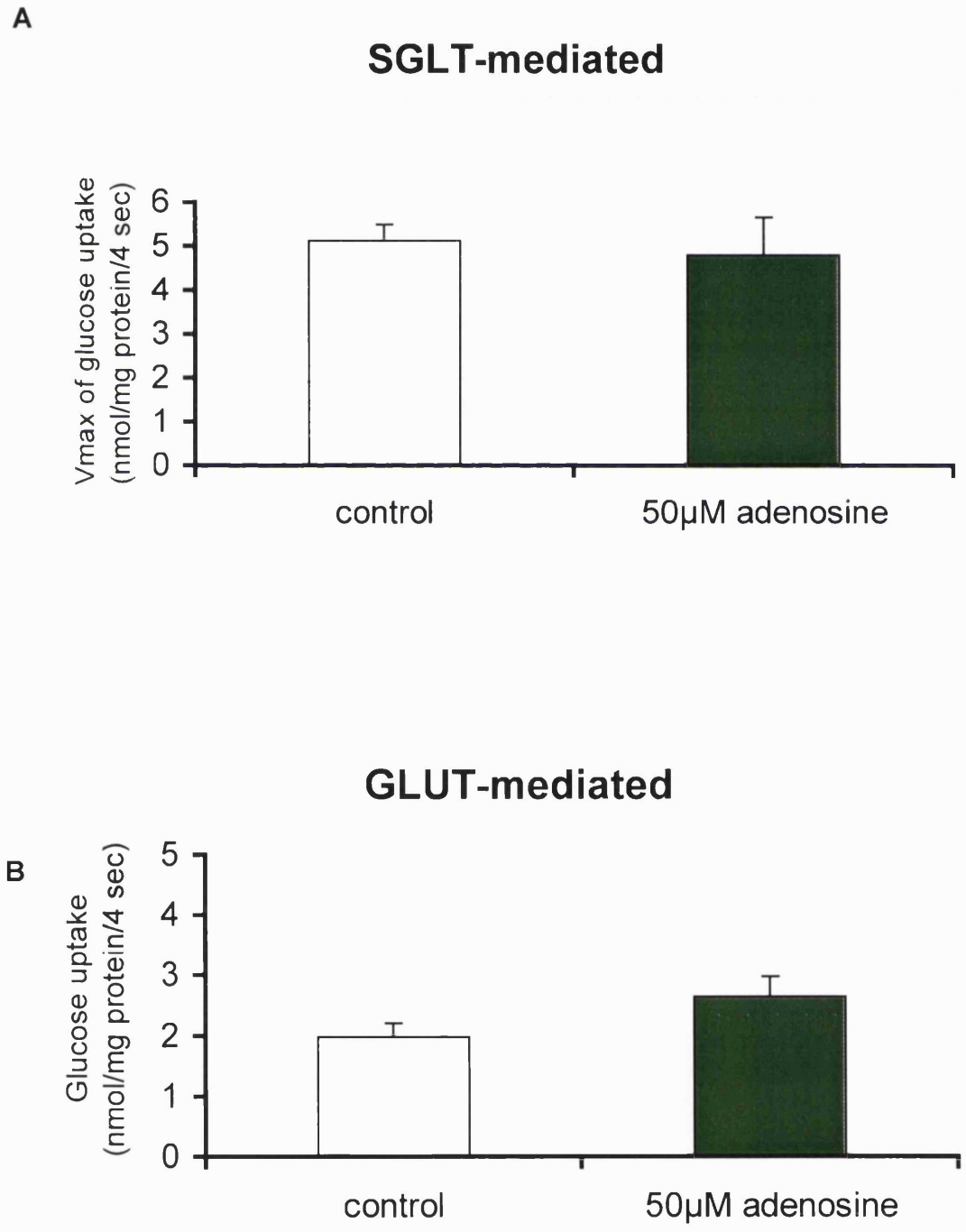
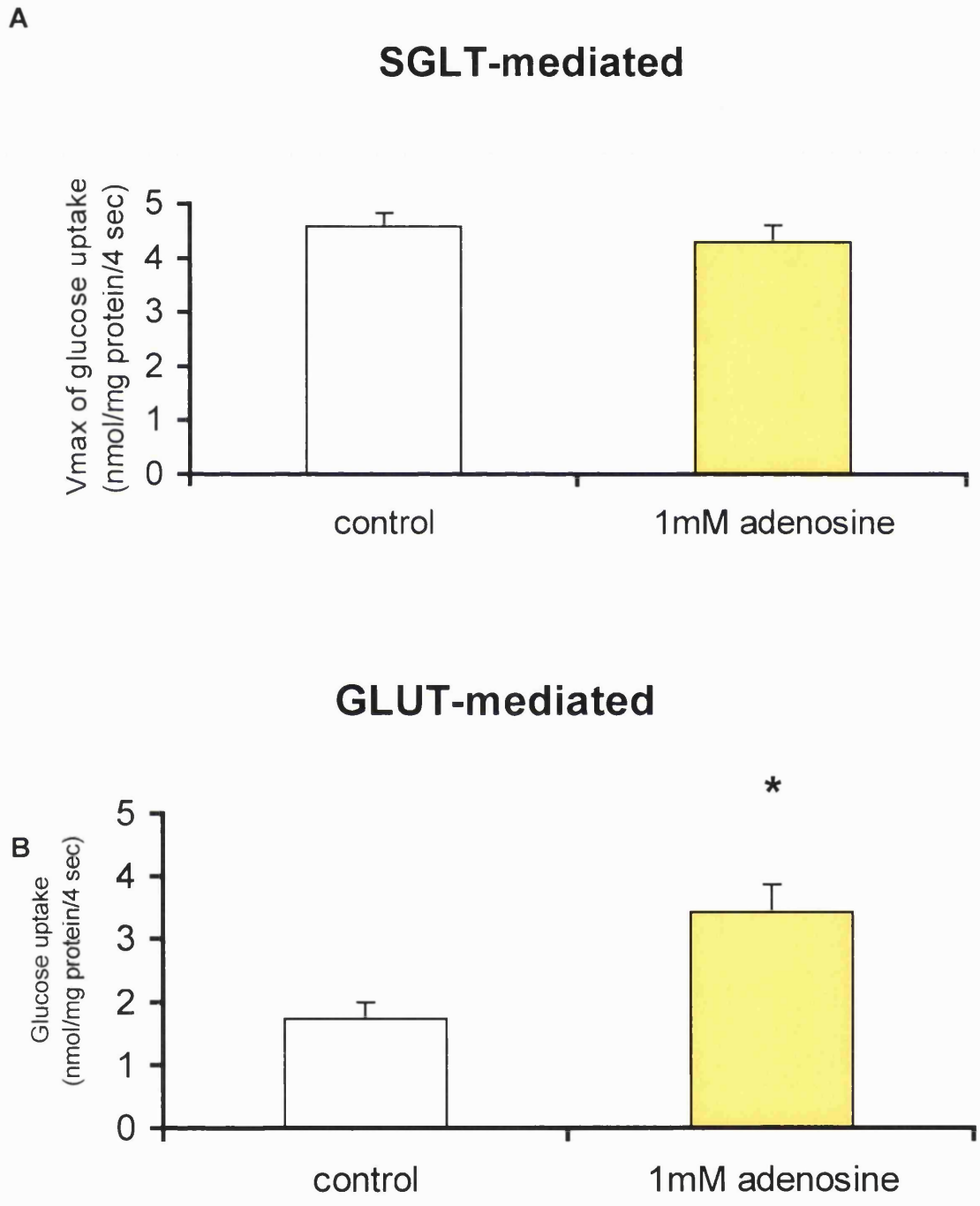


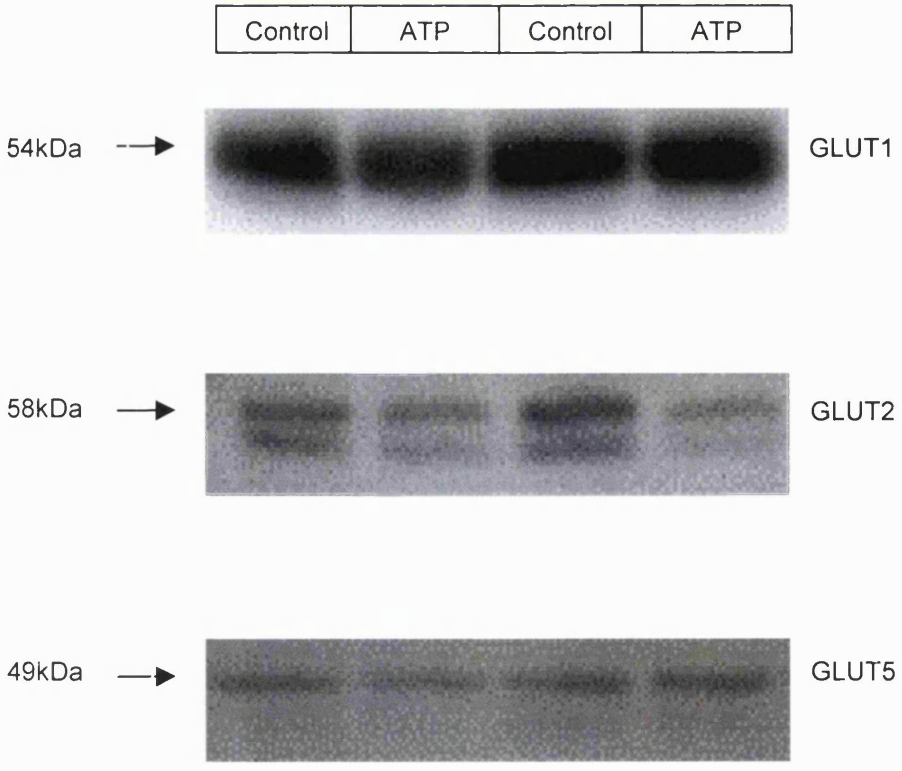
Figure 6.6. Effect of 1 mM adenosine on **A** V_{max} of SGLT-mediated glucose uptake and **B** GLUT-mediated glucose uptake into renal BBM vesicles. *n* = 6, * *P*<0.02, paired *t* test.



6.3.4. Western blots

Western blotting of BBM vesicles using antibodies raised against GLUT1, GLUT2 and GLUT5 revealed that the three transporters were detectable at the BBM at 54, 58 and 49 kDa respectively (Fig. 6.7). Exposure of 1 mM ATP to the cortical suspension resulted in no significant difference in the expression levels of the GLUT proteins (paired *t* test). GLUT2 protein levels were slightly decreased by 29% (density in arbitrary units, control: 1074 ± 753 *vs.* ATP: 762 ± 414), as were the levels of GLUT5 (control: 199 ± 51 *vs.* ATP: 137 ± 36). GLUT1 protein levels were elevated, by 32% (control: 4364 ± 935 vs. ATP: 5803 ± 1353) but none of these differences reached statistical significance.

Figure 6.7. Detection of GLUT1, GLUT2 and GLUT5 by western blotting using BBM vesicles prepared from renal cortex exposed to 1 mM ATP for 30 minutes at 37°C. Representative of $n = 6$.



6.4. Discussion

ATP has been demonstrated to have a variety of effects in the kidney. It has been shown to affect the renal microcirculation, through binding to P2X and P2Y receptors, which results in vasoconstriction or vasodilatation, respectively (Chan *et al.*, 1998). It also has a potential role in the regulation of tubuloglomerular feedback (Nishiyama *et al.*, 2001) and has been shown to stimulate release of renin from isolated renal cortical tissue (Churchill & Ellis, 1993). ATP can induce contraction of mesangial cells and therefore may have a role in the control of glomerular permeability and filtration. In this cell type, extracellular ATP can also promote cell growth and in the presence of other growth factors can affect cell proliferation (Chan *et al.*, 1998). ATP also has a role in the regulation of tubular function. In the cortical collecting duct, ATP can inhibit the hydrosmotic effect of arginine vasopressin (Edwards, 2002; Kishore *et al.*, 1995) and result in an increase in Cl^- secretion and a decrease in Na^+ reabsorption (McCoy *et al.*, 1999).

ATP has been reported to have several effects on proximal tubular function; it attenuates the parathyroid hormone-induced inhibition of phosphate transport (Lederer & McLeish, 1995), stimulates gluconeogenesis (Edgecombe *et al.*, 1997), activates basolateral chloride channels (Bouyer *et al.*, 1998) and inhibits the basolaterally located Na^+/K^+-ATPase, an enzyme that is involved in the maintenance of transepithelial Na^+ transport (Jin & Hopfer, 1997). However, these studies were unable to demonstrate the exact receptor subtype responsible for the physiological effects.

Adenosine has also been shown to have a wide range of effects on renal function, mainly through activation of the A1 and A2a receptor subtypes. Activation of A1 receptors has been shown to reduce renal blood flow and cause a reduction in GFR, thus adenosine has also been postulated to have a role in regulating tubuloglomerular feedback (McCoy *et al.*, 1993). Activation or inhibition of A1 receptors at the juxtaglomerular apparatus is known to be involved in the inhibition or stimulation of renin release, respectively. (Jackson & Dubey, 2001). A1 receptors have also been

demonstrated to be involved in the regulation of proximal tubular function. In cultured opossum kidney (OK) cells, which display a proximal tubule phenotype, the A1 specific agonist PIA (R-(-)-N^6-phenylisopropyladenosine) has been shown to increase sodium-dependent glucose and phosphate transport (Coulson *et al.*, 1991). In addition, microperfusion of proximal tubule cells with the highly specific A1 antagonist, FK-453, inhibits basolateral HCO_3 transport, in a cAMP-dependent manner (Takeda *et al.*, 1993). The role of A2 receptors in renal function is less well characterised, but activation of this P1 receptor subtype is involved in the enhancement of medullary blood flow and subsequent decrease in Na^+ reabsorption (Zou *et al.*, 1999). The A3 receptor protein has also been detected in the kidney by western blotting, but a role for this receptor in renal physiology has yet to be determined (Jackson & Dubey, 2001).

At present there are no studies into the role of ATP and adenosine in the control of renal glucose transport in native tissue. This study demonstrates that exposure of proximal tubules to varying concentrations of ATP or adenosine promotes GLUT-mediated glucose transport across the BBM. Western blotting of control and treated BBM revealed no change in transporter density. This finding suggests that ATP and adenosine promote GLUT-mediated transport by increasing the intrinsic activity of one or more of the GLUT transporters present at the BBM. The finding that adenosine promoted glucose transport at 1 mM but was without effect at 50 μM, suggests that the ATP induced response was not due to its degradation to adenosine, and subsequent stimulation of P1 receptors. Instead, it could be hypothesised that adenosine and ATP *per se* can evoke the same response through P1 and P2 receptor subtypes, respectively.

The principles of ATP release and its degradation, together with the multiple subtypes and subfamilies of the purinoceptors, each of which have complex signal transduction cascades, makes understanding the role of ATP in cell function complex. This is further complicated in the kidney due to its 10 or more distinct segments and multiple cell types within each segment, each of

which is in contact with the tubular fluid or interstitium that contains nucleotide or nucleoside agonists. The fact that multiple purinoceptors can be expressed on the same cell type and even on the same membrane domain (Schwiebert & Kishore, 2001) further complicates deciphering the exact receptor involved in a given response. The results from this study do not reveal the receptor subtype involved in the ATP or adenosine induced response. However, from the findings presented in chapter 4, where thapsigargin and PMA increase GLUT-mediated transport, whilst cAMP increases SGLT-mediated transport it could be presumed that ATP and adenosine work on the receptor subtypes that are positively coupled to PLC rather than adenylate cyclase. Since the majority of filtered glucose is reabsorbed in the proximal tubule (Tune & Burg, 1971), it could be postulated that the effects seen in this study are due to activation of one or more of the receptor subtypes known to be expressed in this nephron segment that are coupled to PLC.

The distribution of P2 receptors appears to be species dependent (Bailey *et al.*, 2000) and the possibility of strain differences also exists. Therefore to postulate which receptor subtype is responsible for the increase in GLUT-mediated transport, it would be important to know which receptors are present in the proximal tubule of the strain of rat used in the present study. The distribution of both P2X and P2Y receptors in the kidney of Sprague-Dawley rats has been studied using immunohistochemistry, RT-PCR and pharmacological studies. It has been established that the mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ is present in microdissected proximal tubule segments (Bailey *et al.*, 2000; Bailey *et al.*, 2001a). The pharmacological profile is indicative of expression of $P2Y_1$, $P2Y_2$ or $P2Y_4$ (as they have a similar agonist profile) and $P2Y_6$ (Bailey *et al.*, 2000; Bailey *et al.*, 2001a). Immunohistochemistry confirmed the presence of $P2Y_4$ on the basolateral membrane of the proximal convoluted tubule (PCT), whilst $P2Y_1$ was present at the apical membrane of the straight proximal tubule (PST) (Turner *et al.*, 2003). $P2Y_6$ immunoreactivity was also detected in the PCT and PST, with the cell cytoplasm displaying positive staining rather than a specific membrane. $P2X_4$ was also detected in cortical nephron segments, although

at lower levels than $P2Y_4$, whilst, $P2X_6$ was detected at low levels throughout the entire nephron (Turner C *et al.*, 2002; Turner *et al.*, 2003). Although the mRNA for $P2Y_2$ was detected in the proximal tubule, its presence was unable to be confirmed using subtype specific antibodies. This finding suggests that this receptor subtype is either expressed at such a low level it isn't detectable using immunohistochemistry, or that it is only expressed under certain conditions (Turner *et al.*, 2003).

The first step in furthering our knowledge on the role of ATP in the regulation of renal glucose transport would be the use of nonselective agonists that activate both P2X and P2Y purinoceptors, for example ATPγS (adenosine 5'O-(3-thiotriphosphate) or 2MeSATP (2-methylthio ATP) (Ralevic & Burnstock, 1998). To establish an effect of P2X receptors on renal glucose transport, the agonists α,β-meATP (α,β-Methylene adenosine 5'-triphosphate), β,γ-meATP (β,γ-Methylene adenosine 5'-triphosphate) or Bz-ATP (2',3'-O-(4-Benzoyl-benzoyl)-ATP) could be used as these have been shown to activate P2X receptors without any effect on the P2Y subtypes (Schwiebert, 2001). Broad range agonists for P2Y receptors include ADP (adenosine 5'diphosphate), UDP (uridine 5' diphosphate), UTPγ*S* (uridine 5'-O-(3'thiotriphosphate) and 2Me*S*ADP (2-methylthioadenosine diphosphate) (Ralevic & Burnstock, 1998). Although it has been demonstrated that $P2Y_1$, $P2Y_4$ and $P2Y_6$ are present in the proximal tubule of the species of rat used in this study (Turner *et al.*, 2003): it would be extremely difficult to establish the exact receptor subtype responsible for the increase in GLUT-mediated glucose transport as agonists that are specific for just one subtype of P2Y receptor have yet to be synthesised.

LLC-PK cells, which have a proximal tubule phenotype, have been shown to express mainly A2 receptors at the BBM and A1 receptors at the BLM (McCoy *et al.*, 1993). Selective agonists for A1 receptors, such as CPA (N^6-cyclopentyladenosine) or CCPA (2-chloro-N^6- cyclopentyladenosine) and CGS-21680 (2-[*p*-(carboxyethyl)phenylethylamino]-5'-*N*-ethyl-carboxamidoadenosine), or DPMA (*N*-[2-(3,5-dimethoxyphenyl)-2-(2-

methylphenyl)ethyl]adenosine) for A2 receptors, could be used to determine the receptor subtype involved in the adenosine induced increase in GLUT-mediated glucose transport. In addition, adenosine may be promoting GLUT-mediated transport by uptake of the nucleoside into the cells rather than activation of the specific receptors; this hypothesis could be tested by the addition of dipyridamole (an inhibitor of the nucleoside transporter) to the cortical tubule suspension.

It is of interest that streptozotocin-induced diabetes has been shown to increase glomerular expression of $P2X_7$ (Vonend *et al.*, 2001). This receptor forms a large non-selective ion pore following stimulation by ATP, which has been linked to cell death by apoptosis. Vonend *et al.* proposed that increased cell surface expression of $P2X_7$ and its subsequent stimulation, by ATP that is being filtered or released from adjacent injured cells, may be an important factor in the apoptotic cell death and glomerular damage that is known to occur in diabetes. Such a phenomenon may also occur in the proximal tubule, perhaps by $P2X_7$ or one of the other P2X receptors expressed in this nephron segment.

6.5. Conclusion

This study demonstrates that ATP and adenosine specifically increase renal GLUT-mediated glucose transport, but does not influence SGLT-mediated transport. The receptor subtypes involved in the response are unknown at present. As described in chapter 4, the regulation of GLUT-mediated glucose transport appears to involve activation of PKC and/or increased intracellular calcium levels rather than an increase in intracellular cAMP. It could therefore be hypothesised that the receptors involved in the adenosine and ATP-induced response would activate PLC rather than adenylate cyclase.

Studies have shown that $P2Y_1$, $P2Y_4$ and $P2Y_6$ are expressed in the proximal tubule, each of which is positively coupled to PLC; therefore these subtypes may be candidates for the ATP-induced increase in glucose transport. Both

A1 and A2 receptors are expressed in the proximal tubule, although only A1 receptors are linked to PLC, suggesting this subtype may be responsible for the adenosine-induced increase in GLUT-mediated glucose transport

Chapter 7.

General discussion

7.1. Aims of experiments described in this thesis

In the past decade substantial advances have been made into establishing the cellular mechanisms involved in diabetes-induced renal cellular damage. Data from clinical trials and experimental models of diabetes have shown a strong correlation between hyperglycaemia and the progression of diabetic nephropathy (Larkins & Dunlop, 1992; Nishikawa *et al.*, 2000). Most studies have concentrated on the effect of high glucose levels on mesangial cells, as changes in glomerular function and glomerulosclerosis are hallmarks of the disease. Studies have provided convincing evidence that overexpression of GLUT1 is a key factor that predisposes this cell type to glucose-induced damage (Heilig *et al.*, 1995b; Heilig *et al.*, 2001). However, diabetes is also reported to stimulate proximal tubular hypertrophy and thickening of the tubular basement membrane (Phillips *et al.*, 1999b), culminating in alterations in cellular function of this nephron segment, yet the underlying mechanisms involved have not been studied in detail. The aim of the experiments described in this thesis was to investigate the effect of streptozotocin-induced diabetes on proximal tubular glucose transport and to establish the cellular mechanisms involved in its regulation.

7.2. Effect of streptozotocin-induced diabetes on proximal tubular glucose transport.

The most striking finding from the experiments described in this thesis is that STZ-induced diabetes promotes the expression of GLUT2 at the proximal tubule BBM. Measurement of glucose transport, using BBM vesicles, revealed that facilitative glucose transport was increased during diabetes, and could be abolished by overnight fasting. Western blotting and immunohistochemistry demonstrated that BBM expression of GLUT2 correlated with the changes in transport rate. Thus, expression of this transporter at the BBM appears to occur in response to changes in the glycaemic status of the animal, and may provide a low affinity/high capacity route for reabsorption during hyperglycaemia (Fig. 7.1).

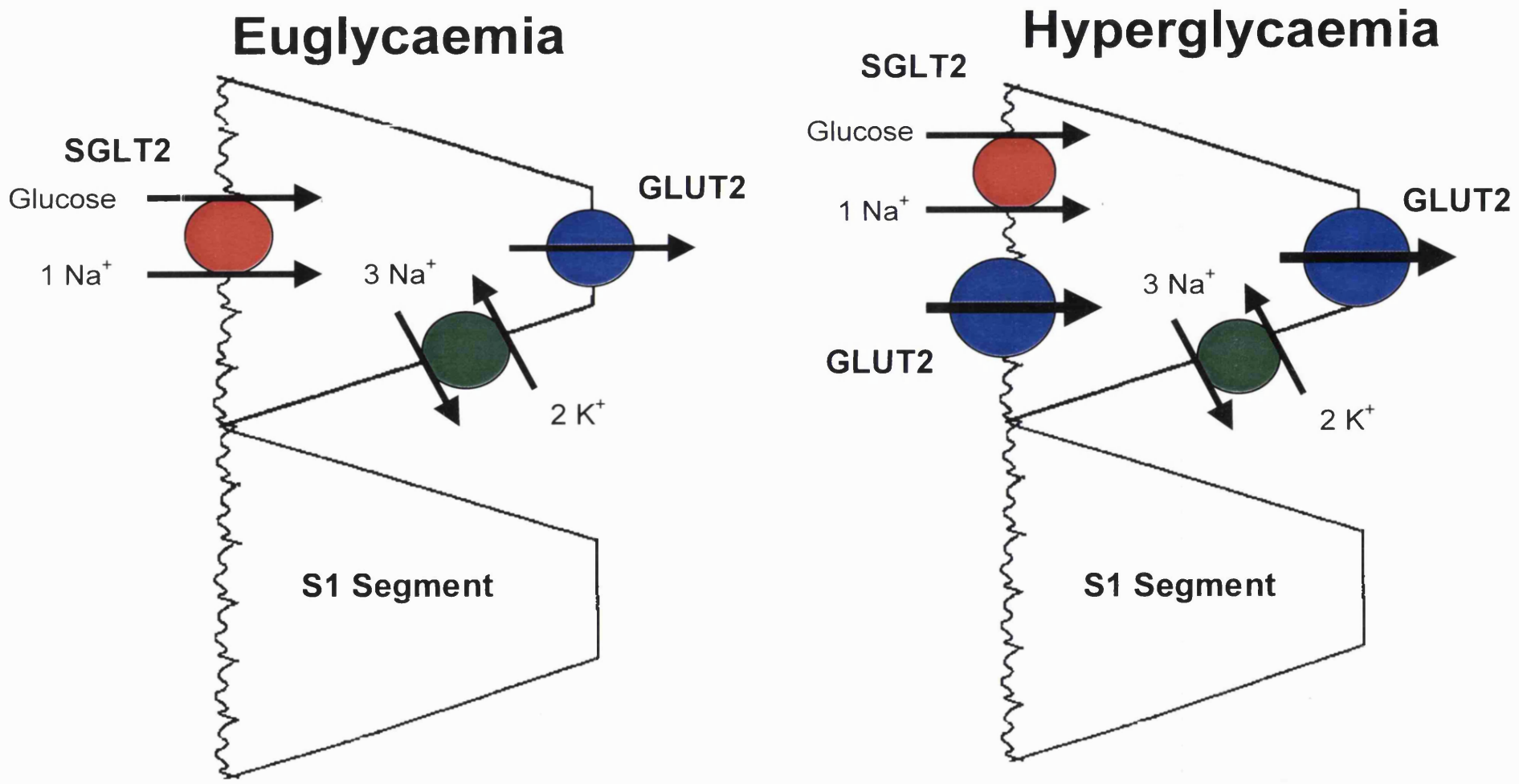
Although this is the first study demonstrating a role for facilitative glucose transport across the renal BBM, in intestinal enterocytes, where the transport process is similar to the kidney, STZ-induced diabetes increases BBM levels of GLUT1, GLUT2 and GLUT5 (Boyer *et al.*, 1996; Corpe *et al.*, 1996). Additionally, facilitative transport across the intestinal BBM has also now been implicated in the absorption of sugars following the assimilation of a meal (Kellett, 2001). It has been proposed that adaptation of GLUT2 and GLUT5 at the enterocyte BBM provides rapid and precise regulation of the absorptive capacity to match dietary intake in situations of sugar abundance (Gouyon *et al.*, 2003a). Involvement of GLUT2 at the renal BBM during physiological conditions has yet to be established, but adaptation in response to postprandial fluctuations in plasma glucose levels may occur based on the similarities between the renal and intestinal glucose transport mechanisms.

Although there are remarkable similarities between the enterocyte and proximal tubule in terms of glucose transport, there appears to be one major inconsistency. There is considerable evidence that GLUT2 is expressed at the enterocyte BBM even under conditions of euglycaemia (Affleck *et al.*, 2003; Au *et al.*, 2002; Helliwell *et al.*, 2000b). However, from the experiments described in this thesis, GLUT2 could not be detected at the proximal tubule BBM of control animals using western blotting or immunohistochemistry. Helliwell *et al.* have proposed that a role for GLUT2 in intestinal glucose absorption has been overlooked due to the rapid dynamics of the transport system, which is dependent on the composition of the diet on which the animal is maintained and the type of experimental procedure used for investigation (Helliwell *et al.*, 2000b). Although rapid shuttling out of the BBM may be a reason for our failure to detect GLUT2 at the BBM of control animals by western blotting, it seems unlikely to be of consequence following the *in vivo* fixation protocol used for immunohistochemistry and confocal microscopy. However, in this context, it has recently been proposed that antibodies raised against the C-terminal of GLUT2 are inappropriate for the detection of this protein at the enterocyte BBM in sections of whole tissue, even though it can be successfully used to

detect the protein by western blotting of BBM vesicles (Affleck *et al.*, 2003). The authors of this study went on to demonstrate that antiserum raised to the extracellular loop of GLUT2 enabled the detection of the protein at the enterocyte BBM of whole tissue sections, which could be enhanced by digestion of the glycosylation sugars with N-endoglycosidase. The use of a similar experimental procedure may reveal that GLUT2 is infact present at the proximal tubule BBM of control animals; however, this seems unlikely as in contrast to the intestine, GLUT2 can not be detected in renal BBM vesicles prepared from control animals.

Kellett and colleagues have demonstrated that activation of PKC-βII is required for the insertion of GLUT2 into the enterocyte BBM (Kellett, 2001) and that the presence of luminal sugars activates this PKC isoform via cleavage of the native enzyme to its active form (Helliwell *et al.*, 2003). Interestingly, the presence of mannitol in the luminal perfusate evoked changes in PKC-βII cleavage, but insertion of GLUT2 into the BBM was favored by glucose and fructose over mannitol (Helliwell *et al.*, 2003). Thus it is possible that even the presence of non-transported sugars evokes changes in the distribution of GLUT2. The authors of this study have proposed that the effect of mannitol is not a consequence of changes in osmolarity but occurs as a result of a sugar sensing mechanism. In this context, preliminary data investigating the short-term regulation of GLUT2 at the proximal tubule BBM has shown that 2 hour vascular infusion of 20% glucose and mannitol are both capable of inducing expression of GLUT2 at the BBM, although expression at this site is favored by infusion of glucose (personal observations).

Figure 7.1. Adaptation of proximal tubule glucose reabsorption during hyperglycaemia



7.3. Differential regulation of renal glucose transport

The experiments described in chapter 4 provided important information on the cellular mechanisms involved in the regulation of renal glucose transport. The results clearly demonstrate that sodium-dependent and independent glucose transport is regulated by different cellular signalling events. Regulation of sodium-dependent glucose transport occurs via cAMP-induced insertion of SGLT1 protein into the proximal tubule BBM. This finding is in keeping with the now widely recognised role of the PKA pathway in the regulation of SGLT-mediated glucose transport (Wright *et al.*, 1997). In contrast, regulation of facilitative glucose transport occurs via a pathway involving both PKC and calcium signalling. Interestingly, perfusion of the small intestine *in vitro* with PMA promotes facilitative transport, via the rapid insertion of GLUT2 at the enterocyte BBM (Helliwell *et al.*, 2000b). Further studies revealed that activation of PKC-βII plays a key role in the PMA and sugar-induced insertion of GLUT2 into the enterocyte BBM (Helliwell *et al.*, 2003). In slight contrast to this finding, the effect of PMA on renal facilitative glucose transport appears to result from changes in intrinsic activity of one or more of the facilitative transporters already present in the membrane. However, such changes have also been documented in response to activation of the p38 MAP kinase and PI 3-kinase signalling pathways (Helliwell *et al.*, 2000a).

The numerous papers published by Helliwell *et al.* describing the dynamic regulation of GLUT2 at the enterocyte BBM have been extremely well designed and have been possible due to the relative simplicity of *in vivo* experiments in the small intestine. In the kidney, comparable experiments would involve the more elaborate technique of *in vivo* microperfusion. Such experiments would allow the effects of STZ-induced diabetes, diet or infused compounds on glucose transport in individual proximal tubules to be examined. The addition of specific transport inhibitors to the tubule perfusate would enable the contribution of the individual glucose transporter isoforms to be defined. In the small intestine, Helliwell *et al.* used an *ex-vivo* model to

evaluate the role of different signalling pathways in the regulation of BBM glucose transporters. These experiments required the pre-perfusion of pathway inhibitors and activators for periods of up to 45 minutes *in vivo,* followed by perfusion of the jejunum *in vitro*. This approach was adopted because the compounds take time to evoke a response and the jejunum has limited viability *in vitro*. Such studies in the kidney would have to be carried out exclusively *in vivo* and would require long periods of luminal perfusion to ensure maximal inhibition by the compounds. Due to the high flow rate of proximal tubules, long-term perfusion would be technically challenging. To overcome this problem microperfusion experiments could be used in conjunction with perfusion of the renal artery with the specific inhibitors. However, this approach would target both the BLM and BBM facilitative transporters and it would not be possible to correlate changes in transport function with changes in the expression of the glucose transporters. The use of isolated proximal tubule cells to perform such studies would be inappropriate, as the pathways that are likely to be involved in the regulation of facilitative transport are stress related and perhaps most importantly PKC isoforms are inactivated upon removal of tissue from its *in vivo* setting (Helliwell *et al.*, 2000b). Consequently, the findings of such experiments may prove inconclusive or ambiguous. Therefore the use of a proximal tubule cell line, such as LLC-PK cells, derived from porcine kidney, may be more appropriate. The use of signalling pathway activators and transporter inhibitors would be possible in this experimental setting, as the cells could be pre-incubated with the relevant compounds prior to transport measurements. Additionally, changes in transport rates could be correlated with changes in glucose transporter expression, PKC activation and accumulation of advanced glycation endproducts.

7.4. Consequences of increased glucose transport during diabetes

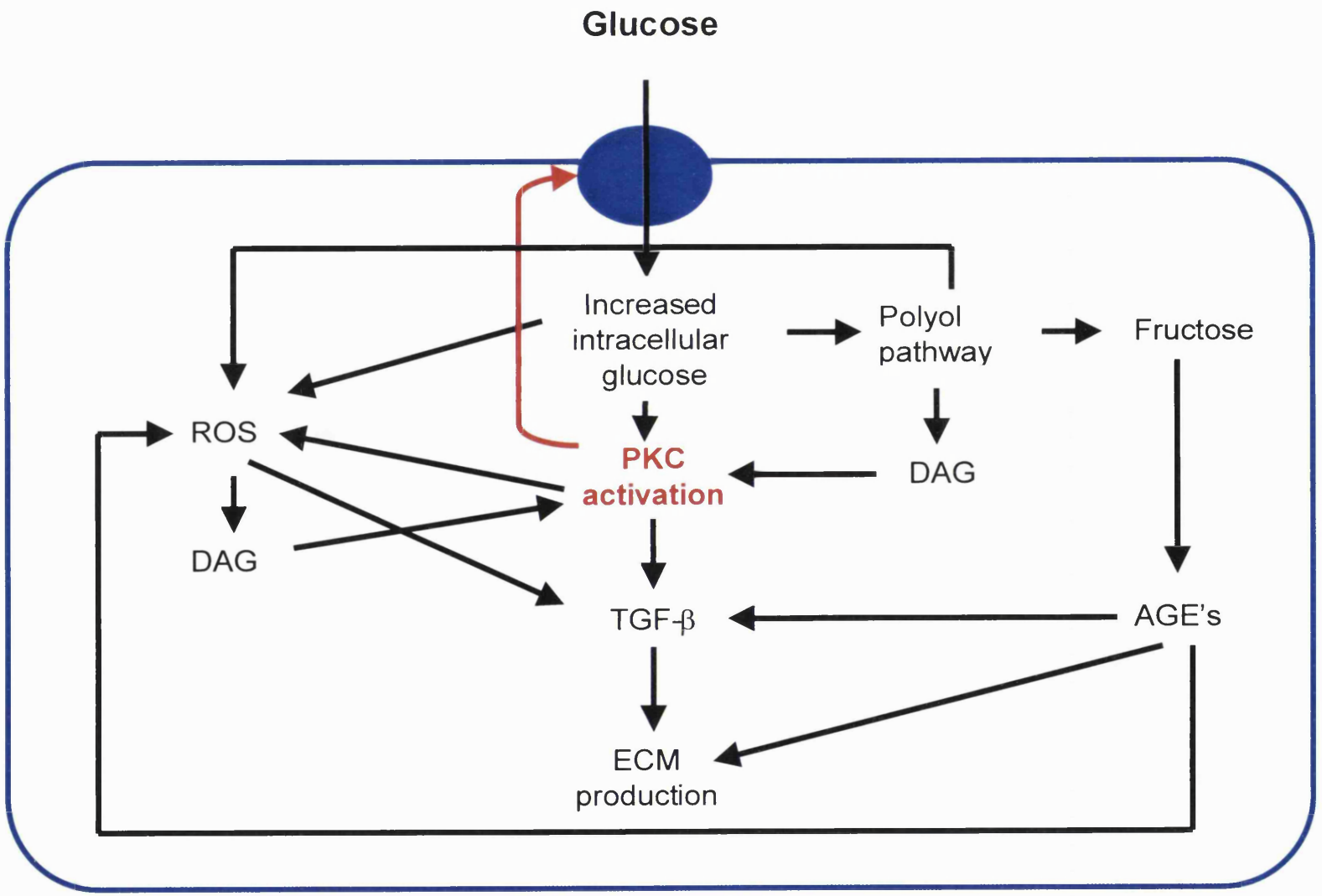
In mesangial cells, it has been proposed that increased GLUT1 expression occurs as a result of glucose induced PKC activation, leading to a variety of cellular events that culminate in cellular damage characteristic of diabetic nephropathy (Heilig *et al.*, 2001). A number of PKC isoforms have been

implicated in this cellular damage, however, activation of PKC-β appears to induce the most dramatic pathological changes and inhibition of this isoform ameliorates the cellular damage associated with the disease (Haneda *et al.*, 2001; Koya *et al.*, 2000).

As described above, activation of the PKC signalling pathway by PMA increases GLUT-mediated glucose uptake in proximal tubule cells and the small intestine. In enterocytes, increased glucose uptake correlates to the PKC-βII induced insertion of GLUT2 into the BBM. Interestingly, proximal tubule cells isolated from the kidneys of STZ-diabetic animals display increased activation of a variety of PKC isoforms (Ha *et al.*, 2001; Whiteside & Dlugosz, 2002), which can be mimicked by incubation of proximal tubule cells in high glucose concentrations (Park *et al.*, 2001; Symonian *et al.*, 1998). Thus, it could be envisaged that glucose induced activation of PKC regulates the expression of GLUT2 at the proximal tubule BBM, resulting in an increase in the intracellular glucose concentration, which may ultimately lead to cellular damage in this nephron region (Fig. 7.2).

In this context, it has been shown that mutations in the GLUT2 gene, leading to the translation of functionally inactive proteins, is the key factor in the pathogenesis of Fanconi-Bickle syndrome (Santer *et al.*, 1997). In the proximal tubule, the lack of a functional GLUT2 protein at the BLM results in reduced glucose efflux, and consequently, raised intracellular glucose concentrations. Alterations in glucose metabolism subsequently occur, leading to glycogen accumulation and loss of proximal tubule function (Santer *et al.*, 1998). Therefore, insights into this rare disease have demonstrated a role for abnormal proximal tubule glucose handling in the development of diabetic-like nephropathy.

Figure 7.2. Potential mechanisms resulting in over-expression of GLUT2 at the BBM of proximal tubules and GLUT1 in mesangial cells and the subsequent cellular pathways documented to influence glucose-induced cellular damage.



7.5. Regulation of renal glucose transport by glucagon

Studies using intestinal enterocytes have demonstrated that glucagon promotes sodium-dependent glucose transport at the BBM (Debnam & Sharp, 1993; Thompson & Debnam, 1986), a response that is likely to be mediated by increased intracellular cAMP levels (Sharp & Debnam, 1994b). In addition, increased secretion of pancreatic glucagon during experimental diabetes has been proposed to be responsible for the enhanced sodium-dependent glucose transport that occurs across the enterocyte BBM (Debnam & Ebrahim, 1989). These studies raised the question of whether glucagon might also be involved in the regulation of renal glucose transport during diabetes. In contrast to the small intestine, incubation of proximal tubule cells with glucagon promoted GLUT-mediated glucose transport but failed to evoke a significant effect on SGLT-mediated transport.

Although glucagon has been shown to influence proximal tubule function (Ahloulay *et al.*, 1995; de Rouffignac *et al.*, 1991), a direct role for the hormone on this nephron segment is generally not considered to occur, as early glucagon binding and functional studies failed to localise the receptor to this nephron region (Bailly *et al.*, 1980; Butlen & Morel, 1985). The results presented in chapter 5 demonstrated that glucagon receptor mRNA is detectable in microdissected proximal tubules, whilst autoradiography revealed the presence of specific glucagon binding sites in the renal cortex. Interestingly, using cultured baby hamster kidney cells, it has been demonstrated that glucagon receptors can stimulate a second intracellular signalling pathway, involving the activation of phospholipase C (PLC) (Hansen *et al.*, 1998). Activation of this alternative pathway has also been reported in hepatocytes (Wakelam *et al.*, 1986). Stimulation of the PLC signalling pathway involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 subsequently stimulates the release of calcium from intracellular stores and DAG activates PKC. The finding that renal GLUT-mediated glucose transport is stimulated by both PKC and raised intracellular calcium concentrations makes it tempting to speculate that the

glucagon-induced increase in facilitative glucose transport occurs as a result of stimulation of receptors expressed in the proximal tubule which are coupled to this alternative signalling pathway. Indeed, preliminary (unpublished) data has shown that incubation of isolated proximal tubule cells with glucagon stimulates a 2-fold increase in IP_3 production. Although in STZ-induced diabetes there was no increase in glucagon receptor mRNA expression or receptor binding sites, the hyperglucagonaemia associated with the disease may influence glucose transport in the proximal tubule.

7.6. Conclusion

In conclusion, the experiments described in this thesis have provided evidence that the facilitative glucose transporter, GLUT2, plays an important role in the reabsorption of glucose at the proximal tubule BBM during hyperglycaemia. Regulation of renal sodium-dependent and independent glucose transport occurs via distinct signalling pathways, which display striking similarities to the regulatory mechanisms involved in the control of intestinal glucose transport.

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Publications

Full Papers

1) J. Marks, E.S. Debnam, M.R. Dashwood, S.K. Srai and R.J. Unwin. (2003) Detection of glucagon receptor mRNA in the rat proximal tubule: potential role for glucagon in the control of renal glucose transport. *Clinical Science* **104 (3),** 253-258

2) Joanne Marks, Nicolas J.C. Carvou, Edward S. Debnam, Surjit K. Srai and Robert J. Unwin. (2003) Diabetes increases facilitative glucose uptake and GLUT2 expression at the proximal tubule brush border membrane. *J. Physiology* **553 (1)**, 137-145

Abstracts

1. Debnam ES, Unwin RJ, Marks J, Oliva C. (1997) Diabetes Mellitus promotes phlorizin-insentitive but not SGLT-mediated glucose transport across isolated rat renal brush border membrane. *J. Physiol.* **504P**, 141P

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4. J Marks, ES Debnam, RJ Unwin. (1998) Rapid stimulation by pancreatic glucagon of facilitated glucose transport across luminal membrane prepared from isolated rat renal cortex. *J. Physiol.* **513P**, 45P.

5. J Marks, ES Debnam, R Dong, SK Srai, RJ Unwin. (1998) Differential effect of glucagon and cAMP on glucose transport across rat renal brush border. *J. Am. Soc. Nephrol.* **9**, 403A, A2057.

6. J Marks, ES Debnam, RJ Unwin. (1999) Protein kinase C (PKC) activation is involved in acute regluation of phlorizin-insensitive glucose transport across isolated renal brush border membrane. *J.Physiol.* **518P**, 26P.

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8. J Marks, ES Debnam, RJ Unwin, SK Srai. (2000) Potential role for purinoceptors in the regulation of glucose transport across isolated rat renal BBM. *J. Physiol.* **527P**, 15P.

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11. J Marks, ES Debnam, L. Churchill, SK Srai, RJ Unwin. (2002) Diabetic hyperglycaemia causes the expression of GLUT2 at the proximal tubule brush border membrane. *J. Am. Soc. Nephrol.* **13**, A0277.

12. J. Marks, ES Debnam, L. Churchill, SK Srai, RJ Unwin. (2003) GLUT2 protein is translocated to the rat proximal tubule BBM in response to diabetic hyperglycaemia. *J. Physiol.* **547P**, PC65