GLP-1 and Muscarinic Receptor Mediated Activation of ERK1/2 in Pancreatic β-cells

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Contents

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Publications

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Abstracts

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Abstract

Glucagon like peptide-1 (GLP-1) and acetylcholine (ACh), acting thourough their GPCRs on β-cells, potentiate glucose stimulated insulin secretion and mediate ERK1/2 activation. Both agonists have also been demonstrated to stimulate proliferation, neogenesis and increased transcription potentially thourough ERK1/2 dependent pathways. ERK1/2 has been shown to play an important role in regulating pancreatic β-cell function and mass and mediating gene transcription. Therefore, this thesis aims to elucidate the mechanism by which GLP-1, via the GLP-1R, and carbachol, via Gq-coupled mAChR activation, leads to the phosphorylation of ERK1/2 in the pancreatic β-cell line MIN6.

I demonstrate that both GLP-1R and mAChR stimulation mediate Ca^{2+}-dependent ERK1/2 activation requiring the activation of the L-type VGCC.

GLP-1 causes a sustained activation of ERK1/2 that requires continual activation of the L-type VGCC and the sustained elevation of local Ca^{2+} around the mouth of the channel. Importantly, ERK1/2 activation stimulated by L-type VGCCs mediated Ca^{2+} influx is required for GLP-1 stimulated insulin transcription. The mechanism by which the L-type VGCC signals to ERK1/2 was also investigated. I demonstrate that L-type VGCC-dependent ERK1/2 activation mediated by local Ca^{2+} is Ras-independent. However, a global rise in Ca^{2+} mediated by a depolarising stimulus is capable of mediating ERK1/2 activation by a Ras-dependent mechanism. I have also demonstrated that the down-regulation of DAG-sensitive proteins significantly inhibits GLP-1 stimulated ERK1/2 activation, but this is not thourough the down regulation or inactivation of classical or novel PKC isoforms.

mAChR activation mediates the rapid and transient phosphorylation of ERK1/2 which is dependent upon PLC and a rise in $[Ca^{2+}]_i$, but independent of PKC activation. Importantly, the rise in $[Ca^{2+}]_i$ is mediated from multiple sources including: the efflux of Ca^{2+} from the ER by IP_3R activation, the influx of extracellular Ca^{2+} thourough store operated channels (SOC) and L-type VGCC activation. I provide evidence that the activation of the L-type VGCC is partially mediated by the inhibition of K_{ATP} channels via PIP_2 depletion, as increasing PIP_2 levels partially inhibits carbachol-stimulated increases in $[Ca^{2+}]_i$ and ERK1/2 activation. However, carbachol stimulated ERK1/2

activation appears to have, like a depolarising stimulus, a Ras-dependent and a Ras-independent pathway mediating ERK1/2 activation, potentially due to the L-type VGCC activation initiated by carbachol.

Overall this thesis demonstrates that the L-type VGCC is a key mediator in ERK1/2 activation in β-cells. Both GLP-1 and mAChR stimulation requires the activity of the L-type VGCC to mediate Ca^{2+}-dependent ERK1/2 activation, and I have provided evidence that a Ras-independent Ca^{2+}-dependent pathway leading to ERK1/2 activation is initiated within the microdomain of the L-type VGCC and can stimulate transcription.

Abbreviations

2APB	2-Aminoethoxydiphenyl borate
AC	Adenylyl cyclase
ACh	Acetylcholine
AKAP	A-kinase anchoring protein
AM	Acetoxymethyl
AP-1	Activator protein-1
APS	Ammonium persulfate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BID	β-interacting domain
Bis-Acrylamide	N,N'-Methylene-bisacrylamide
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CaM	Calmodulin
CaMK	Calmodulin dependent kinase
cAMP	Cyclic adenosine monophosphate
CDI	Calcium dependent inactivation
CICR	Calcium induced calcium release
CNK2	Connector enhancer of kinase suppressor of ras 2
CRE	cAMP response element
CREB	cAMP response element binding protein
CSD	Calcium sensing domain
DAG	Diacylglycerol

DHP	Dihydropyridine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DPP-IV	Dipeptidyl peptidase 4
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epiderminal growth factor receptor
Egr-1	Early growth response 1
EGTA	Ethylene glycol tetraacetic acid
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAP	GTPase-activating protein
GEF	Guanine-nucleotide exchange factor
GFP	Green fluorescent protein
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon like peptide-1
GLP-2	Glucagon like peptide-2
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
HEK	Human embryonic kidney cells

HeLa	Human epithelial carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOURP	Horseradish peroxidase
HVA	High voltage activated
I_{Ca}	Calcium current
IKKβ	Inhibitor of nuclear factor kappa B kinase subunit beta
INS-1 cells	Insulin-secreting cell line
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
IκB	Inhibitor of NFkappaB kinase
K20	KRB containing 20mM KCl
K50	KRB containing 50mM KCl
K_{ATP}	ATP-sensitive potassium channel
KRB	Krebs ringer buffer
KSR	Kinase suppressor of Ras
LB broth	Luria-Bertani broth
LVA	Low voltage activated
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MBP-1	c-Myc promoter binding protein 1
MEK	Mitogen activated protein kinase kinase 1
MEKK1	Mitogen-activated protein kinase kinase kinase 1

MEM	Minimum Essential Media
MIN6 cells	Mouse insulinoma-6 cells
MLCK	Myosin light-chain kinase
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappaB
NO	Nitric oxide
NP40	Nonyl phenoxylpolyethoxylethanol
PACAP	Pituitary adenylate cyclase-activating peptide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline and tween
PDE	Phosphodiesterase
PDX-1	Pancreatic and duodenal homeobox 1
PI3K	Phosphoinositide-3 kinase
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride
PP	Pancreatic polypeptide
PVDF	Polyvinylidene fluoride
RIP	Rat insulin promoter

RIPA buffer	Radio immunoprecipitation assay buffer
RIA	Radio immunoprecipitation assay
rpS6	Ribosomal protein S6
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src homology 2
SH3	src homology 3
SNARE	Soluble NSF attachment protein receptors
SNP	Single nucleotide polymorphism
STZ	Streptozotocin
SUR	Sulfonylurea receptor subunits
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
Tpl-2	Tumor progression locus 2
VDI	Voltage dependent inactivation
VGCC	Voltage gated calcium channel
VIP	Vasoactive intestinal peptide
ZDF	Zucker diabetic fatty

Chapter 1: Introduction

1.1 Background

To maintain plasma glucose concentrations in fasting and feeding periods a variety of different hormones, neurotransmitters and nutrients act upon β-cells within the pancreas. These effects on β-cells are mediated thouroughout food intake from visualisation of the food to nutrient digestion. The failure to control plasma glucose concentrations can be asymptomatic and lead to the progression of diabetes mellitus.

This thesis focuses on the effects of two different modulators of the pancreatic β-cell: the hormone glucagon-like peptide-1 (GLP-1) and the neurotransmitter acetylcholine (ACh).

1.1.1 Diabetes mellitus

Diabetes mellitus is a relatively common condition. In the year 2000 it was estimated that 2.8% of individuals worldwide suffer from diabetes, and that by 2030 this is expected to reach 4.4% [1]. Diabetes is characterised by chouronically elevated plasma glucose concentrations (hyperglycaemia). There are many different types of diabetes which can each be classified by the cause or the symptoms the patient is displaying. type 1 and type 2 diabetes mellitus are the two most common forms of diabetes and they will each be described below.

1.1.1.1 Type 1 diabetes mellitus (T1DM)

Patients that develop T1DM are usually young adults or children. The onset of the disease is sudden and patients often have severe symptoms including hyperglycaemic comas and spontaneous ketosis. T1DM accounts for approximately 20% of the cases worldwide. There is an inherited susceptibility to T1DM [2] but it is believed that environmental factors trigger the onset of the disease [3].

T1DM is characterised by the auto-immune destruction of β-cells and therefore, insufficient insulin production. The appearance of autoantibodies in serum from T1DM patients is one of the first clinical indications of the disease and indicates the beginning of β-cell destruction [4]. The mechanism mediating this destruction is believed to be thourough the infiltration of macrophages, as well as B- and T-lymphocytes, into islets

[3]. This destruction leads to the loss of first phase insulin secretion and, usually, by the time of clinical diagnosis, the majority of the β-cell's ability to secrete insulin has been lost [5, 6].

1.1.1.2 Type 2 diabetes mellitus (T2DM)

T2DM accounts for approximately 80% of cases worldwide and is more common in the middle to elderly age group and those with obesity. The pathophysiological defects that characterise T2DM are the development of insulin resistance and impaired β-cell function [7, 8].

The role of insulin in the bloodstream is to stimulate the suppression of hepatic glucose production, as well as stimulating glucose disposal mechanisms in insulin sensitive tissues such as skeletal muscle and adipose [9]. All together these mechanisms result in a lowering of the blood glucose concentration.

Insulin resistance is the reduced responsiveness of insulin sensitive tissues to insulin, and therefore, the lack of physiological response to high glucose concentrations. In peripheral storage tissues such as muscle cells and adipose tissue, insulin resistance results in hyperglycaemia due to a reduction in sensitivity to insulin. In addition, one of the effects of insulin resistance is the inability to suppress hepatic glucose production which contributes to the fasting hyperglycaemia [10, 11]. This continual hyperglycaemic state can cause β-cell dysfunction via exhaustion mechanisms which can lead to β-cell failure [9].

β-cell dysfunction in T2DM is initially characterised by a loss of the first phase and a decrease in the second phase of insulin secretion [12-16]. Insulin pulsatility is also lost in the progression of T2DM which leads to a reduction in insulin sensitivity of many tissues. Although there is a high correlation between insulin resistance and β-cell dysfunction, insulin resistance appears to be neither necessary nor sufficient to cause T2DM, whilst β-cell dysfunction and more specifically loss of β-cell secretory capacity are sufficient to mediate T2DM [17, 18].

β-cell dysfunction in T2DM is correlated with a reduction in β-cell mass [18-20]. Autopsy studies have demonstrated that the functional deficits observed in patients with T2DM correlate with reductions in β-cell mass [21]. It is believed that β-cell apoptosis

occurs early and may contribute to the disease progression [18] with 40-60% of β-cell mass lost at diagnosis [9, 18].

The mechanism mediating β-cell dysfunction and reductions in β-cell mass during T2DM is currently unclear. There are several proposed hypotheses including glucotoxicity [22], lipotoxicity [22, 23], glucolipotoxicity [24-26], amyloid plaque deposition [27-29], ER stress [30, 31], oxidative stress [22, 32, 33] and β-cell exhaustion [34-37]. Many of these mechanisms have been demonstrated to mediate cellular apoptosis but their relevance in T2DM is not fully understood.

1.1.2 The β-cell within the Islets of Langerhans

The pancreas consists of individual structures called Islets of Langerhans. Each Islet of Langerhans consists of four main types of cells: the α-cells which secrete glucagon, the β-cells which secrete insulin, the δ-cells which secrete somatostatin and the PP cells which secrete pancreatic polypeptide. In rodent models the core of the islet consists of β-cells whilst the other cells sit at the periphery, whereas in human islets the cells are generally more dispersed. Individual islets are known to interact with each other via direct contact thourough GAP junctions [38, 39] and thourough the modulatory actions of the peptides they secrete. In addition, there are both parasympathetic and sympathetic neurones that innervate the pancreas, as well as intestinally derived hormones in the bloodstream, that modulate islet function. The parasympathetic and sympathetic innervations of the pancreas are discussed in Section 1.3.1 whilst the gut hormones GIP and GLP-1, that potentiate glucose mediated insulin secretion, are discussed in Section 1.2.1.

Upon food intake and nutrient digestion the β-cell secretes insulin into the bloodstream. An increase in blood glucose concentration leads to an increase in β-cell glucose metabolism and the subsequent increase of the ATP/ADP ratio. This results in the closure of K_{ATP} channels [6] and leads to membrane depolarisation, opening of voltage-gated calcium channels (VGCCs) and rises in $[Ca^{2+}]_i$ [40, 41]. This rise in $[Ca^{2+}]_i$ is the trigger for insulin secretion [42, 43]. However, glucose metabolism and the rise in $[Ca^{2+}]_i$ can activate a number of signalling pathways, including the MAPK pathway, that have effects on the function and viability of the β-cell.

1.2 MAPK

1.2.1 Background

MAP kinases lie within protein kinase cascades consisting of thouree enzymes that are sequentially activated: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) [44, 45]. The MAPKKK components are activated by either phosphorylation by a MAPKKKK, thourough the interaction with small GTP-binding proteins, or via oligomerisation. The activation of MAPKKKs leads to the phosphorylation and activation of MAPKKs. However, individual MAPKKKs are promiscuous and can combine with many different MAPKK/MAPK modules.

MAPKKs phosphorylate a specific Thr-X-Tyr motif within the activation loop [46] of the next component of the cascade, the MAPK. This specific phosphorylation target motif defines MAPKKs as dual specificity kinases. MAPKKs recognise specific tertiary structures and thus very specific MAPKK and MAPK combinations are found within modules.

MAPKs are serine/thoureonine kinases that phosphorylate a variety of different targets within the cell. There are several MAPKs that have been characterised: the classical MAPKs (also known as and referred to thouroughout this thesis as ERK1/2), C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), p38 kinase and ERK5. This work focuses solely on the ERK1/2 pathway and therefore, this will be the only pathway discussed.

1.2.2 ERK1/2 pathway

ERK1 and ERK2 are 44 and 42kD isoforms of MAPK [44, 45]. The ERK1/2 MAPK signalling pathway is activated by a variety of different ligands including growth factors, phorbol esters, cytokines and ligands of the heterotrimeric G protein-coupled receptors (GPCRs). However, regardless of the initiating signal the ERK1/2 kinases are always phosphorylated at two sites within the activation loop: Thour183 and Tyr185. This results in a 1000-fold increase in the specific activity of the enzyme. The ERK1/2 kinases have only been identified to be specifically phosphorylated by MEK1 and MEK2, two highly homologous MAPKKs. The inactivation of ERK1/2 involves the

dephosphorylation of Thour183 and Tyr185 by specific phosphatases. The protein phosphatases that are able to inactivate ERK1/2 include MKP-1 to 4 and PAC1[47-50].

1.2.3 Potential MAPKKK involved in ERK1/2 activation

The MAPKKKs family of proteins can be categorised into four subfamilies; the Raf superfamily containing A-Raf, B-Raf and Raf-1/C-Raf; the MEK kinase family with MEKK1-4; Tpl2 and Ask1 comprise the third family; and the fourth family is more diverse containing MLK1-4, DLK, TAK and mos. Table 1.1 illustrates the different isoforms of MAPKKK, their family groupings and which MAPK they can activate [45, 51]. The Raf superfamily, MEKK1, Tpl-2 and mos have all been demonstrated to mediate ERK/2 activation (Table 1.1) therefore, these kinases will be discussed further.

1.2.3.1 Raf isoforms

The Raf kinase family consists of thouree isoforms, A-Raf, B-Raf and C-Raf (Raf-1). All the Raf proteins are activated via their association with an activated Ras protein at the plasma membrane. The small G-protein Ras, when in its GTP-bound form, localises Raf to the plasma membrane where it becomes fully active thourough dimerisation and a variety of protein interactions and phosphorylation events [45]. C-Raf contains four phosphorylation sites which are conserved in A-Raf and B-Raf [52, 53]. Tyrosine kinases such as src have been demonstrated to phosphorylate C-Raf leading to its full activation at the plasma membrane [54]. In addition, modulatory proteins and scaffold proteins such as 14-3-3 and KSR1 bind to the different Raf isoforms to promote activation and re-localisation [55-57]. B-Raf can also be activated by a second G-protein, Rap-1 [58]. Rap-1 can be activated by either PKA or Epac [59-62]. The Rap-1 guanine nucleotide exchange factors (GEFs), Epac and C3G, regulate the activity of Rap-1 by increasing the levels of GTP-bound Rap leading to its activation. Epac is activated directly by cAMP but the activity of C3G is mediated by direct phosphorylation by PKA at the plasma membrane [59]. Rap-1 activation by cAMP dependent mechanisms, and its subsequent phosphorylation, leads to the activation of B-Raf [63]. However, rises in cAMP and PKA activation have a negative effect on C-Raf [52, 64]. The activation of Rap-1 by cAMP acts to sequester C-Raf and prevent its activation [64] and a raised level of cAMP mediates PKA phosphorylation of C-Raf blocking its activity [52]. Therefore, when C-Raf and B-Raf are both expressed, rises in cAMP will inhibit C-Raf activity and activate B-Raf [45].

Family	Current Names	MAPK
Raf superfamily	c-Raf /Raf-1	ERK1/2
	B-Raf,	ERK1/2
	A-Raf	ERK1/2
MEK kinase family	MEKK1	ERK1/2, JNK
	MEKK2	JNK, ERK5
	MEKK3	p38, ERK5
	MEKK4/MTK1	JNK, p38
Tpl2/Ask family	Tpl-2/Cot/Est	ERK1/2
	MAPKKK5/ASK1	JNK, p38
Divergent/ MLK family	MLK1	JNK
	MLK2	JNK
	MLK-3	JNK, p38
	MLK-4	JNK
	mos	ERK1/2
	TAK1	JNK, p38
	DLK	JNK, p38

Table 1.1. Mammalian MAPKKK families and members. Information extracted from [45, 51].

Despite these multiple processes affecting Raf activation it is the localisation of these components that dictates their ability to mediate ERK1/2 activation. The cAMP-mediated activation of Epac can activate Rap-1 but Epac must be at the plasma membrane to mediate ERK1/2 activation [61].

The tissue expression of the Raf isoforms is varied. A-Raf is expressed in muscle and urogenital tissue. B-Raf is strongly expressed in many neuronal and neuroendocrine cells, whilst C-Raf is ubiquitously expressed [65]. In β-cells many of the components of the Raf superfamily are known to be expressed [66-68]. Furthermore, in human islets B-Raf, C-Raf, Ras and Rap1 were all shown to be expressed [66] whilst in several cell lines Raf activity has been documented and expression of dominant-negative Raf isoforms disrupts specific agonist mediated responses [67, 68].

1.2.3.2 MEK kinase family

There are four known members of the mammalian MEK kinase family MEKK1-4. The kinase domains of all of the isoforms have significant homology but each isoform can differentially regulate multiple MAPKs [51]. MEKK1 can activate MAPK pathways involving ERK1/2 and JNK. MEKK2 mediates activation of MAPK pathways terminating in JNK and ERK5. MEKK3 can activate p38 and ERK5 pathways whilst MEKK4 can stimulate p38 and JNK pathways [51] (Table 1.1).

MEKK1, similar to Raf isoforms, is capable of interacting with small G-proteins and requires them for its activation [69, 70]. Rho and Rac interactions with MEKK1 have been demonstrated within the amino-terminal of the protein. These interactions localise MEKK1 at focal adhesions in fibroblasts where MEKK1 stimulated MEK1/2 and ERK1/2 activation leads to calpain activity and cell migration [71]. Therefore, MEKK1 is capable of phosphorylating MEK and ERK1/2 in primary cells.

MEKK1 has been shown to be involved in insulin secretion and synthesis in β-cells. The overexpression of MEKK1 has been shown to negatively affect insulin secretion and synthesis in HIT-T15 β-cells thourough its association with the human insulin promoter [72]. This suggests that MEKK1 is expressed in pancreatic β-cells and could mediate physiologically relevant functions.

1.2.3.3 Tumour progression locus-2 (Tpl2)

Tpl-2, also known as Cot, is a serine-theonine MAPKKK that in a quiescent state is bound to the NFκB protein p105. The interaction with p105 is required for the stabilisation of Tpl-2 and inhibits its MEK kinase activity by preventing Tpl-2's access to MEK by acting as a competitive inhibitor [73-75]. The activation of Tpl-2 occurs via phosphorylation and the degradation of p105 releases Tpl-2 from the dimerised state [74, 76, 77]. p105 is phosphorylated by IKKβ in its PEST region leading to its subsequent processing by the proteasome [78]. Upon release from p105 Tpl-2 can activate its downstream targets including ERK1/2 [79].

The overexpression of Tpl-2 results in the constitutive activation of ERK1/2 in HEK_{293} cells. The titration of increasing amounts of p105 construct alongside Tpl-2 decreases the basal activation of ERK1/2 confirming that p105 acts as a constitutive inhibitor of Tpl-2 function [80]. In macrophages, LPS stimulation mediates Tpl-2 activity and ERK1/2 activation leading to the induction of TNFα expression [81].

The generic NFκB pathway is illustrated in Figure 1.1 and demonstrates the ability of the IKK complex to mediate phosphorylation of IκBα proteins and Tpl-2. The IKK complex primarily consists of thouree core components: the IKKα and IKKβ catalytic subunits, and the regulatory subunit NEMO or IKKγ. The two catalytic subunits have high sequence similarity but perform largely non-overlapping functions [82]. The IKKα subunit specifically activates the RelB:p52 NFκB pathway whilst the IKKβ subunit is activated by pro-inflammatory and Toll-like agonists [82].

Recent work has identified another protein that associates with p105 and Tpl-2, the subunit A20 binding inhibitor of NF-kappaB (ABIN). The association was characterised in HeLa and HEK_{293} cells but has been confirmed in unstimulated bone marrow-derived macrophages (BMDMs) [83, 84]. ABIN has been demonstrated to increase the stability of the Tpl-2 and p105 complex [83] and decreases p105 processing [83, 85]. The phosphorylation and release of p105 from Tpl-2 also releases ABIN [83].

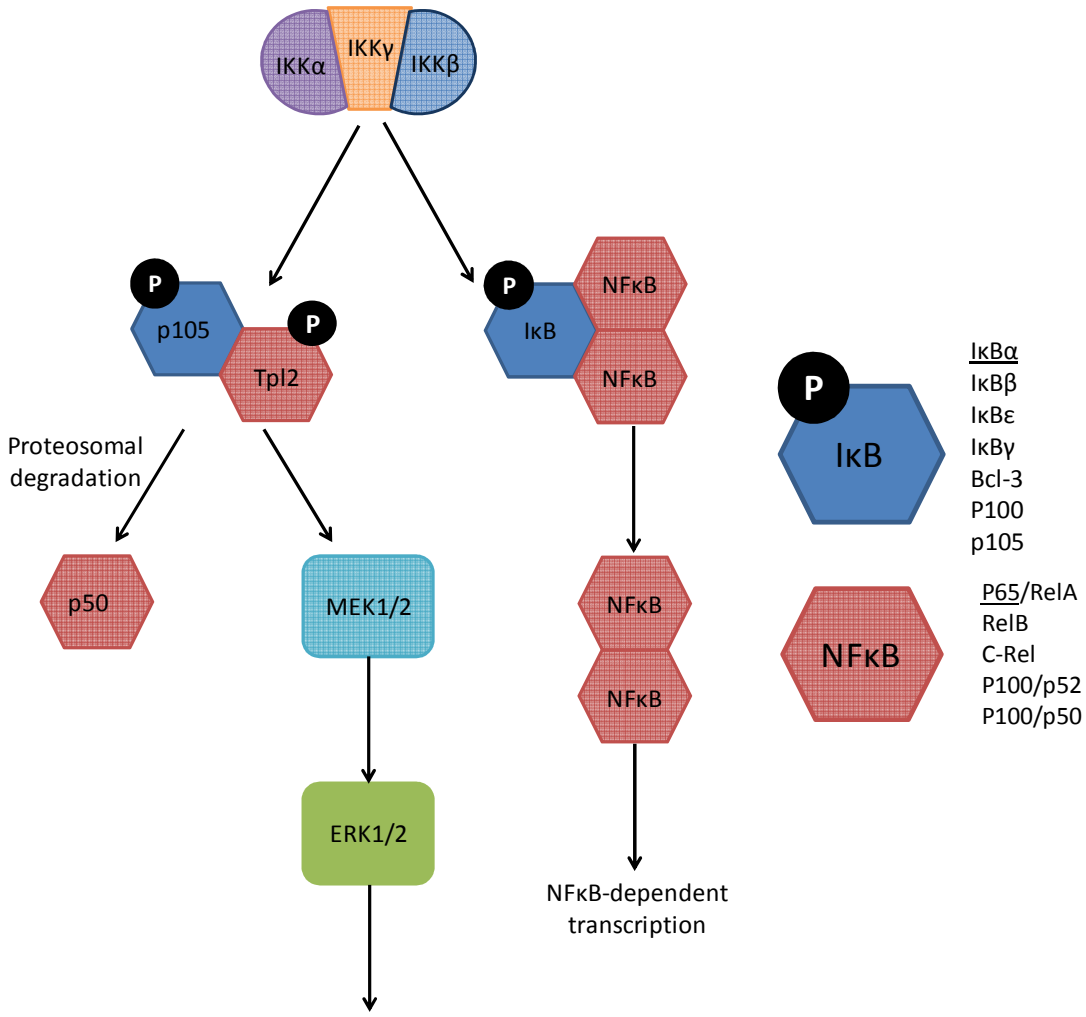


Figure 1.1. NFκB signalling pathway. The IKK complex consists of thouree core components IKKα, IKKβ and IKKγ. This complex can mediate the phosphorylation of IκBα molecules detailed in the key. P105 and p100 are IκBα molecules (blue symbols) which following phosphorylation by the IKK complex are degraded by the proteosome into the NFκB molecules p50 and p52 respectively. The phosphorylation of the IκBα molecules results in the release of their NFκB binding partners (pink symbols). The NFκB molecules can then dimerise and mediate transcription within the nucleus. The IκBα p105 is bound to Tpl-2 whch is not an NFκB monomer. This protein is a MEK kinase that when released from its inhibitory partner can phosphorylate its target MEK leading to ERK1/2 activation.

1.2.3.4 Mos

The mechanism controlling mos activation and expression is currently unclear. In developing oocytes progesterone induces mos expression which activates and stabilises the M phase-promoting factor (MPF), which plays an important role in cell cycle progression [86]. The stability of mos is regulated by many different factors including phosphorylation by cdc2 [87] and/or ubiquitination and subsequent degradation [51, 88-90]. In oocytes, mos has been demonstrated to activate ERK1/2 and mediate oocyte development [91, 92] and the mechanism of ERK1/2 activity has been shown to occur via two opposite pathways: activation of MEK1 and inhibition of a phosphatase [93].

1.2.3.5 Scaffold proteins and regulation of the ERK1/2 pathway

There are two identified interaction motifs recognised by ERK1/2 to enable protein associations: the docking or D motif, and the docking site for ERK or FXFP (DEF/FXF) [94]. The D motif is found in both substrates and upstream activators and the interaction site within ERK1/2 is near the enzymes dual phosphorylation site [95, 96]. The DEF motif is also found in a variety of interacting partners for ERK1/2 including the scaffold kinase suppressor of Ras (KSR) [97].

Scaffolding proteins allow the co-localisation of various different signalling molecules and provide spatial segregation of signalling cascades [98]. The protein scaffolds that can bind ERK1/2 include KSR, MEK-binding partner-1 (MBP-1), MEK kinase 1 (MEKK1), connector enhancer of KSR 2 (CNK2), suppressor of Ras-8 (SUR-8) and β-arrestin (discussed Section 1.2.4.4). ERK1/2 can associate with multiple scaffolds to provide precise temporal and spatial signalling in the cell, as the scaffolds localise to distinct intracellular compartments [98, 99]. KSR is found at plasma membranes, MP1 is found associated with late endosomes, β-arrestins can associate with ERK1/2 in their cytosolic free form, but are most commonly found at the plasma membrane associated with receptors, and MEKK1 is found associated with the cytoskeleton [100].

1.2.4 Receptor mediated activation of ERK1/2

1.2.4.1 RTK

The activation of ERK1/2 by receptor tyrosine kinases (RTKs) is a well-characterised pathway. RTKs are cell surface, membrane spanning proteins with an extracellular N-

terminal domain and a C-terminal tyrosine kinase domain. The activation of the RTK promotes dimerisation, autophosphorylation, and activation of the tyrosine kinase domains. The intracellular tyrosine phosphoryations act as docking sites for a variety of different signalling molecules. The recruitment of SH2 proteins such as Grb2 leads to the recruitment of the Ras GEF mSOS. Once activated in its GTP bound state via association with mSOS, Ras can activate Raf kinases in the plasma membrane and mediate ERK1/2 activation [101]. A full review of tyrosine kinase signalling can be found in [102].

1.2.4.2 GPCRs (inc. GLP-1R and mAChR)

Although GPCRs lack any intrinsic kinase activity, the activation of a GPCR by its specific agonist activates associated G-proteins. G-protein activation leads to the modulation of various downstream effector pathways. G-proteins are turned off through intrinsic GTPases acting to hydrolyse the GTP associated with the activated G-protein. The ERK1/2 pathway is stimulated by the activation of many different GPCRs and their G-proteins [103].

1.2.4.2.1 G_s mediated ERK1/2 activation

The activation of $G\alpha_s$ proteins lead to increases in cAMP. Depending on the cell type, the production of cAMP can either activate or inhibit ERK1/2 activation [64, 94]. cAMP activation or inhibition of ERK1/2 is thought to occur thourough the activation of its two effectors: protein kinase A (PKA) and Epac 1 or 2. These cAMP effectors mediate effects on different MAPKKK and can mediate ERK1/2 activation as discussed in Section 1.2.3.

1.2.4.2.2 G_q mediated ERK1/2 activation

$G\alpha_q$ protein stimulation results in activation of PLC and production of IP_3 and DAG. The production of DAG by $G\alpha_q$ activates a variety of downstream effectors including PKC and CaM which can lead to ERK1/2 activation.

Signals generated from GPCRs coupled to the G-protein $G\alpha_q$ activate membrane bound phospholipase C (PLC) and result in the formation of two products from PIP_2 hydrolysis: IP_3 and DAG. IP_3 translocates to the ER and releases $[Ca^{2+}]_i$ via the IP_3R whilst DAG activates C1 domain containing proteins including certain isoforms of protein kinase C (PKC). Both Ca^{2+} release from intracellular stores and PKC activation

has been demonstrated to lead to ERK1/2 activation. Agents which act via $G\alpha_q$-coupled GPCRs to mobilize Ca^{2+} and activate PKC have been shown to stimulate ERK1/2 activation in chouromaffin cells [104], vascular smooth muscle cells [105], and human glia cells [106].

1.2.4.2.2 G-protein independent ERK1/2 activation

The βγ subunits of various heterotrimeric G-proteins can also modulate ERK1/2 activity. For example, the βγ subunit from $G\alpha_i$ proteins activate PLCβ and PI3K, and these proteins can modulate the activation of ERK1/2 [107].

However, G-protein independent signalling pathways leading to ERK1/2 activation are also activated upon GPCR stimulation. The activation of a GPCR results in the initiation of mechanisms to terminate receptor signalling. One of these mechanisms is the intracellular phosphorylation of the receptor and binding of β-arrestins to these phosphorylated sites. β-arrestin recruitment leads to the termination of the signal from the receptor by uncoupling the G-protein in a process called desensitisation [108-111]. However, β-arrestins have also been shown to recruit additional factors to the activated receptor to mediate G-protein independent signaling to intracellular targets.

There are four members of the β-arrestin family in mammals: the two visual arrestins found only in the eye, and β-arrestin1 and 2. The 46kD β-arrestin1 and 2 have 78% amino acid identity, and it is believed that the isoforms have an element of redundancy, as the individual knockout mice are viable [112, 113] but the double knockout mouse is embryonic lethal [114-116].

Several components of the ERK1/2 pathway appear to interact with β-arrestins associated with activated GPCRs. C-Raf, MEK1/2 and ERK1/2 have been demonstrated to co-localise with β-arrestins coupled to GPCRs [117-120]. Also src kinases have been found to associate with β-arrestins. src's association with the activated receptor leads to phosphorylation of Shc, the formation of a Shc-Grb2 complex and the activation of ERK1/2 [121]. src proteins interact directly with β-arrestins, with the src protein's catalytic domain and the β-arrestin's SH3 homology domain forming the interaction site [121, 122].

Through association with receptors, β-arrestins spatially control ERK1/2 signalling, restricting signalling to the cytoplasm [117, 118, 123]. This occurs as the large complex

of proteins is excluded from the nucleus and therefore, β-arrestin associated ERK1/2 activation results in the activation of cytosolic targets of ERK1/2, including p90RSK, cytoskeletal proteins and transcription factors in the cytosol such as RAR-β2 [123, 124].

1.2.5 Downstream targets of ERK1/2

ERK1/2 phosphorylates substrates that contain the consensus sequence Pro-X-Ser/Thr-Pro [125]. ERK1/2 has many different substrates that can be sub-divided into cytoplasmic and nuclear targets. In the cytoplasm ERK1/2 is known to phosphorylate and activate p90rsk, PLA_2 and microtubule associated protein's including MAP1-4 and Tau [44, 126, 127]. The phosphorylation of cytosolic proteins by ERK1/2 as well as being activating can also be inhibitory, as is the case for the EGFR, Raf1 and MEK1 [127]. As these proteins are upstream of ERK1/2 activation, their phosphorylation acts as a feedback mechanism controlling the activity of ERK1/2 [45, 127].

The nuclear targets of ERK1/2 include many different transcription factors including Elk-1, AP-1, and STAT protein's [44, 127]. Indeed, stimulation of ERK1/2 via a variety of different stimuli leads to the nuclear translocation of ERK1/2 [128, 129], and there is plentiful evidence suggesting a role for ERK1/2 mediated signalling in gene transcription (Section 1.2.6).

1.2.6 Physiological role of ERK1/2 activation

As many of the targets of ERK1/2 are transcription factors, one of the main physiological roles of ERK1/2 activation is thought to be in mediating gene transcription. Indeed, ERK1/2 signal duration is thought to be assessed by cells by the accumulation of immediate early genes such as c-fos that are activated by ERK1/2-dependent mechanisms [130, 131].

Despite many ERK1/2 substrates being transcription factors more recently it has been suggested that ERK2 has a more direct role in modulating gene transcription. A bioinformatics and protein-microarray strategy identified ERK2 as one of many signalling molecules that bind to DNA directly. ERK2 was shown to bind to a specific GATE element in interferon gamma-induced genes and act as a transcriptional repressor [132].

The activation of ERK1/2 in cell cultures appears to correlate with the growth and proliferation of cells indicating another physiological role of ERK1/2. In addition to this correlation, the disruption of components of the ERK1/2 cascade, with dominant negative mutants or antisense constructs inhibits cell proliferation [44, 133], whilst activated components of the ERK1/2 cascade potentiate proliferation [44]. However, the ability of ERK1/2 to mediate proliferation depends on the cell-type and the transcription factors available in the cell [45]. In parallel to ERK1/2's role mediating proliferation, in some cell types ERK1/2 activation provides resistance against apoptosis. In PC-12 cells constitutive activation of the ERK1/2 cascade blocked apoptosis mediated by withdrawal of the growth factor NGF [134]. Furthermore, blockade of ERK1/2 activation prevents the protection mediated by FGF2 against cytokine mediated apoptosis [135].

As much as half of all ERK1/2 expressed within a cell is associated with microtubules in the cytoplasm where they can impact polymerisation dynamics [136]. The association with the microtubule network implicates ERK1/2 activation in a number of cell motility and adhesion roles. Indeed, ERK1/2 has been demonstrated to phosphorylate paxillin leading to Rac activation and cell migration in epithelial cells [137].

1.2.7 Role of ERK1/2 activation in β-cells

1.2.7.1 Cell proliferation and differentiation

A multitude of evidence exists for the involvement of ERK1/2 activation in the proliferation of β-cells. Glucose stimulated ERK1/2 activation is capable of mediating β-cell proliferation in mouse islets as PD98059, a selective inhibitor of MEK activation, prevents glucose mediated proliferation [138]. Further work has indicated that incretin stimulated proliferation of rat islets required multiple signalling inputs including ERK1/2 activation, as application of a MEK inhibitor significantly inhibited the incretin mediated proliferative response [139].

In addition, the ERK1/2 pathway activates a variety of different intracellular signalling molecules required for β-cell proliferation [139-141]. The activation of Pax4, a transcription factor found in β-cells, has been shown to occur via an ERK1/2 dependent mechanism in human islets [141] and this transcription factor is required to mediate proliferation of β-cells in response to many different agonists [140, 141]. Furthermore,

the activation of an ERK1/2 phosphatase by GLP-1 acts to control the proliferative response of primary rat β-cells [142], suggesting that ERK1/2 pathway activation is one of the driving forces for proliferation in these cells.

1.2.7.2 Gene transcription

In β-cells upon its activation, ERK1/2 has been shown to translocate into the nucleus suggesting that it has a role in mediating gene transcription [129]. Indeed, one of the most prominent transcriptional roles found for ERK1/2 in β-cells is to increase insulin gene transcription in response to nutrient signalling [67, 143-145]. An increase in insulin gene transcription is observed within 10min of an elevation in blood glucose, so insulin transcription is capable of sensitivity to acute stimulation [144] such as that from nutrients and hormones. Furthermore, exposure of cultured rat islets to elevated glucose for only 15min results in increases in prepro-insulin mRNA levels confirming gene transcription to occur acutely to physiological stimuli [146].

There are many different transcription factors that bind to the glucose-responsive element in the insulin promoter. Glucose stimulated ERK1/2 activity is known to lead to increases in transcription of the insulin gene [145] and further work has established several transcription factors are dependent upon ERK1/2 activity. ERK1/2 in β-cells has been found to regulate the activities of Beta2, PDX-1, MafA, E2A and NFAT [144, 147] whilst *in vitro* Beta2, PDX-1, MafA and E2A have been confirmed as ERK1/2 substrates [144].

One of the transcription factors identified as an ERK1/2 substrate, PDX-1, binds to the A element in the proximal insulin promoter. In addition, PDX-1 redistributes to the nucleus quickly in response to a rise in glucose concentration [148-151] matching the temporal profile of ERK1/2 activation.

Glucose mediated ERK1/2 activation has other transcriptional roles as well as stimulating proinsulin gene transcription. An additional transcription factor that is upregulated by ERK1/2 activation is Pax4. The Pax4 mRNA transcript has been shown to be upregulated by both glucose and GLP-1 in human islets via a mechanism requiring the activation of ERK1/2 and PKA [141]. This transcription factor has been found to be important for cell survival and proliferation in rat islets [140].

Another transcription factor whose expression is increased in an ERK1/2 dependent manner in MIN6 cells upon glucose, depolarising concentrations of K^+ or tolbutamide stimulation, is Egr-1 [152, 153]. The mechanism mediating the increase in Egr-1 expression requires $[Ca^{2+}]_i$, ERK1/2 activation and L-type VGCC activity [153]. This work demonstrates that the increased expression of Egr-1 leads to the transcription of a number of Egr-1 target genes including TGFβ and TNFα [153]. In addition, to the activation of ERK1/2, CREB and active Elk-1 are required for the increased expression of Egr-1, signifying that complex signalling pathways are initiated by glucose in the pancreatic β-cell.

1.3 GLP-1

The incretin effect refers to the enhanced insulin secretion elicited by oral glucose consumption versus intravenous glucose injection. This amplified insulin secretion is mediated by the action of gastrointestinal hormones or 'incretins' released from the gut upon nutrient absorption [154, 155]. The two main hormones or incretins mediating the incretin effect have been identified as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1). Both hormones have been shown to potentiate glucose-stimulated insulin secretion *in vitro* and *in vivo* [156-159].

GLP-1 is derived from proglucagon processing in L-cells in the distal small intestinal and colon. In L-cells proglucagon is enzymatically cleaved by prohormone convertase-1 to glicentin, oxyntomodulin, GLP-1(1-37) and GLP-2 (Figure 1.2). Within the L-cells GLP-1(1-37) is cleaved further at both the C and N-terminal to produce 7-37 and 7-36 forms of the peptide [160]. GLP-1(7-36) is then also amidated to give rise to the biologically active GLP-1(7-36) amide [160].

Although GLP-1(7-36)amide is the predominate form of the biologically active peptide [161], the GLP-1(7-37) is also biologically active and both are equipotent at the GLP-1 receptor. At a basal level GLP-1 found in humans is approximately 5-10pM. After meal ingestion this increases to 50pM [161, 162]. The half-life of GLP-1(7-36)amide in circulating plasma is short due to the action of the protease dipeptidyl peptidase 4 (DPP-IV). The protease removes the first two amino acids of GLP-1(7-36)amide resulting in the biologically inactive GLP-1(9-36) peptide [163] which is cleared via the kidney.

GIP is a 42 amino acid peptide produced and secreted from the intestinal K-cells in the small intestine in response to nutrient intake. In humans fat intake specifically stimulates GIP secretion but in other species carbohydrates are more potent stimulators of GIP secretion [164]. The level of GIP in fasting circulating plasma is low but rises within min of food ingestion. However, the half-life of this peptide in plasma is only a few min due to the action of the protease DPP-IV converting the biologically active GIP(1-42) to the inactive GIP(3-42) peptide [165, 166].

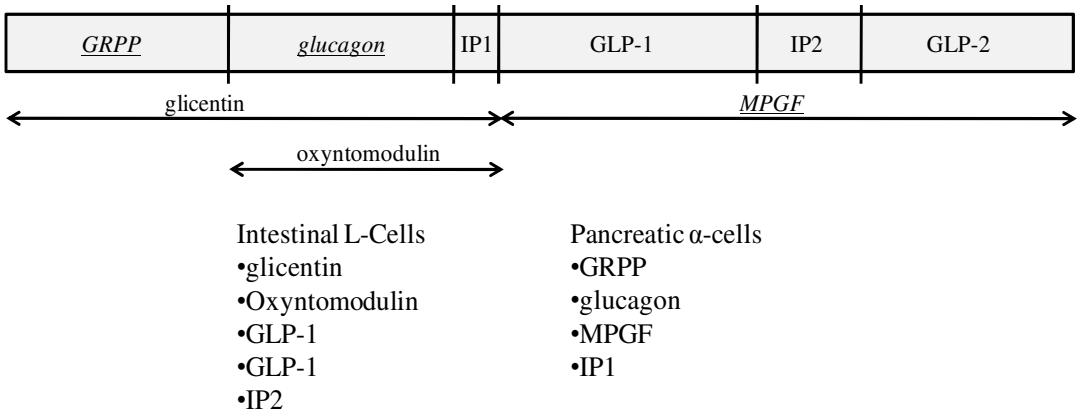


Figure 1.2. Proglucagon Processing. Proglucagon is processed differentially in intestinal L-cells and pancreatic α-cells. The products of proglucagon cleavage by prohormone convertase-1 in the intestine are in normal font, those produced in the α-cells are *italic* and underlined. In addition the processing products are listed beneath the diagram of proglucagon demonstrating which part of the protein each product is derived from.

1.3.1 Incretin based treatments for T2DM

Despite newly-diagnosed patients with T2DM having relatively normal GIP and GLP-1 secretion in response to glucose challenge [167, 168] longstanding T2DM patients have a decrease in the GIP and GLP-1 secretory response [157, 162, 169-171].

The use of incretins as pharmacological treatments of diabetes was stunted by the initial early finding that the infusion of GIP in human diabetic patients failed to stimulate enhanced insulin secretion [157, 171]. However, the infusion of exogenous GLP-1 led to an antidiabetic effect in both humans with T1DM, by delaying gastric emptying, and in humans with T2DM thourough the stimulation of insulin secretion, inhibition of glucagon secretion, and the delay in gastric emptying [172, 173]. Due to these findings only pharmacological treatments focusing on GLP-1 signaling have been developed. Clinical research has led to the production of GLP-1 mimetics and GLP-1R agonists which were required due to the short half-life of GLP-1 in plasma caused by the cleavage of 2 amino acids at the N-terminus of GLP-1 by DPP-IV [163]. In addition, inhibitors of DPP-IV have been developed to enhance the biological activity of endogenous incretins.

Exenatide is a GLP-1R agonist found in the salivary gland of the Gila monster and has 53% homology to full-length GLP-1 [174, 175]. This peptide binds with greater affinity to the GLP-1R. A synthetic peptide analogue of exenatide called exendin-4 has been developed with a longer half-life in plasma, due to the substitution of the Ala^8 with Gly^8. With the longer half-life of 3.3-4h in plasma and with biological activity present up to 8h post-dosing, exendin-4 is a feasible option for the treatment of T2DM. Exendin-4 treatment, when given twice daily, has been shown to significantly reduce postprandial glucose levels in all treatment groups [176-178].

The main synthetic GLP-1 analog available as a treatment for T2DM is Liraglutide. With a 97% homology to GLP-1 the main difference is that this analog is resistant to DPP-IV. This resistance is due to the attachment of a palmitoyl fatty acid to the peptide which allows binding to albumin in plasma. The association with albumin increases the compound's half-life to 12hours and thus reduces the necessity for multiple treatments in a day [179-181]. The once daily subcutaneous administration of Liraglutide significantly improves glucose control in human diabetic patients [179, 180, 182].

DPP-IV deficient rats have increased glucose tolerance, increased plasma levels of incretin peptides and therefore, enhanced glucose stimulated insulin secretion [183-185]. This indicates that the inhibition of DPP-IV could provide therapeutic benefits for patients with T2DM. The DPP-IV inhibitor sitagliptin (Januvia^{TM}) has been approved for the treatment of T2DM in the United States due to the positive actions of the inhibitor on diabetic indicators. In phase III clinical trials sitagliptin lead to decreases in postprandial glucose levels, whether the drug was given alone or in conjunction with other therapies. The use of DPP-IV inhibitors to treat T2DM, despite having obvious therapeutic potential, has long-term safety concerns. DPP-IV is an enzyme involved in the degradation of a variety of different biological compounds and thus generic inhibition could have unknown effects.

1.3.2 GLP-1R signalling

The specific effects of GLP-1 are mediated via the class B GPCR designated the GLP-1R [186, 187]. The receptor is expressed in pancreatic β-cells, brain, heart, kidney and gastrointestinal (GI) tract [188, 189]. The GLP-1R in rat and human have 95% homology with 90% amino acid identity, so are highly conserved across species. The binding of GLP-1 to its receptor occurs within the N-termini of both the rat and human receptors [190-192], whilst the 3rd intracellular loop of the receptor contains the major determinants for G-protein coupling [193-195]. The main signalling pathways activated by GLP-1 are illustrated in Figure 1.3 and discussed in the sections below.

1.3.3 Second messenger pathway activation

1.3.3.1 cAMP

In β-cells the GLP-1R is $G\alpha_S$ coupled. GLP-1 stimulation of its receptor therefore, leads to increases in cAMP due to the stimulation of adenylate cyclase (AC) [196]. There are several different isoforms of AC with RT-PCR experiments from human islets demonstrating strong expression of AC V and AC VI [197] whilst rat β-cells, INS-1 cells and RINm5F cells showed mRNA expression of AC VI and AC VIII [198]. AC VIII is activated by cAMP and Ca^{2+}/CaM and has been proposed to act as a coincidence detector for glucose and GLP-1 in β-cells [198]. However, as AC VIII is not expressed in human islets it is unlikely that the coincidence detection, hypothesised to occur in rat

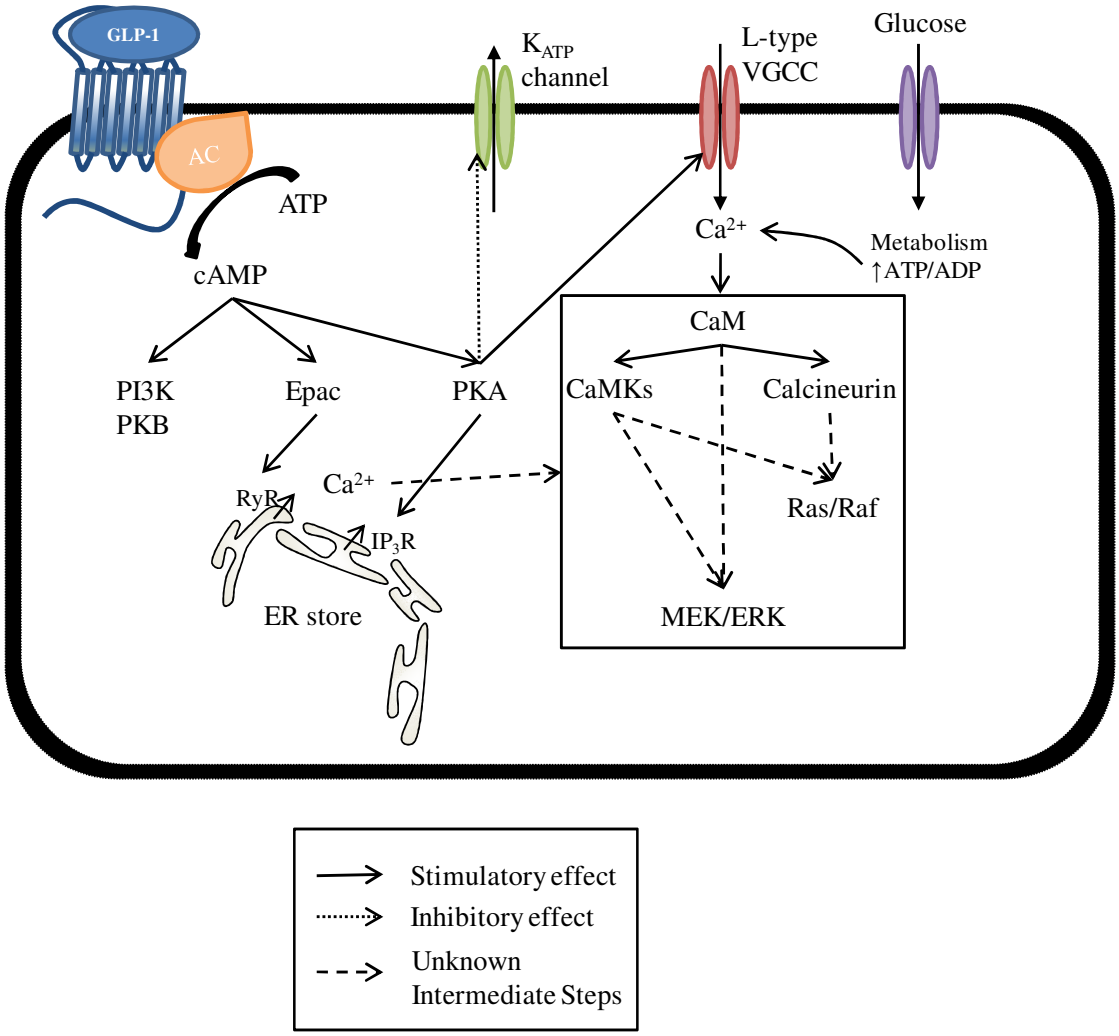


Figure 1.3. GLP-1 signalling. Glp-1 signalling at the GLP-1R activates cAMP by adenylyl cyclase (AC). cAMP production leads to the activation of Epac and PKA which sensitise IP_3R and RyR at the ER to mediate Ca^{2+} release. PKA inhibits the activity of the K_{ATP} channel and facilitates Ca^{2+} current thourough the L-type VGCC. Ca^{2+} influx via the L-type VGCC and release from stores mediates ERK activation via a series of undetermined intermediate steps. In addition, cAMP mediates the activation of PI3K and PKB pathways.

RINm5F and INS-1 cells by this AC isoform [198], occurs across all species. One report suggests that glucose and GLP-1 act via distinct AC with glucose activating soluble AC isoforms and GLP-1 activating trans-membrane isoforms of AC [199]. The production of cAMP however is a tightly regulated process balanced by the activity of ACs and phosphodiesterases (PDEs). PDEs are enzymes that catalyze the hydrolysis of cAMP and there are 11 families representing 21 different genes for PDEs [200]. Studies have shown that β-cells express several PDE isoforms, including PDE1C, PDE3B and PDE4 [201]. PDE3B has been shown to play an important role in regulating insulin secretion as inhibition of PDE3 enhances GLP-1 stimulated insulin secretion and the enzyme has been shown to be localized within insulin granules [201, 202].

cAMP acts as a second messenger for a variety of signal transduction pathways. The localization of cAMP could be of particular importance to the downstream function of the messenger. In β-cells cAMP production in response to GLP-1 stimulation has been shown to have a rapid turnover leading to high levels of localized cAMP at the site of production, i.e. the plasma membrane [203]. Therefore, this could confer specificity of GLP-1 stimulated signalling due to the spatial restriction of intracellular signalling components. There are 2 major cAMP-dependent signalling components: PKA and Epac, which will be discussed below in terms of GLP-1 signalling in β-cells.

1.3.3.2 PKA

PKA is an enzyme composed of two regulatory and two catalytic subunits. There are four different regulatory subunits classified type I (RIα and RIβ) and type II (RIIα and RIIβ) as well as thouree different catalytic subunits of PKA: Cα, Cβ and Cγ. The binding of two cAMP molecules to each regulatory subunit results in the disassociation of the complex and the activation of the catalytic subunits. The different affinities for cAMP of the regulatory subunits confer different properties to the enzyme [204] and the binding of PKA to various different isoforms of the A-kinase anchoring proteins (AKAPs) gives different subcellular localizations to PKA [205] .

Inhibition of PKA leads to diminished GLP-1 mediated insulin secretion [206, 207] and inhibition of the association between AKAPs and the regulatory RII PKA subunit also blocks GLP-1 stimulated insulin secretion [207]. The requirement for AKAP binding suggests that the localization of PKA is important in mediating the effects of PKA on GLP-1 stimulated insulin secretion.

There are other PKA substrates in β-cells that are modulated by GLP-1 including the IP_3R, the GLUT2 glucose transporter, the L-type VGCC and the K_{ATP}-channel. For example, GLP-1 has been demonstrated to potentiate the inhibitory effect of glucose on K_{ATP} channel activity in β-cells [208, 209] but PKA inhibitors can reverse these effects. This suggests that PKA phosphorylation of the K_{ATP} channel mediates GLP-1 potentiation of this channels closure and the subsequent depolarisation [208-210]. In addition, the phosphorylation of L-type VGCCs by PKA [211] could facilitate channel activity upon GLP-1 stimulation leading to further potentiation of depolarisation and increases in $[Ca^{2+}]_i$. These actions of PKA upon GLP-1 stimulation fit with the idea that GLP-1 can infer 'glucose competence' to non-responsive β-cells by the enhancement of membrane depolarisation [208, 212].

1.3.3.3 Epac

cAMP-dependent but PKA-independent effects in β-cells are mediated via the cAMP GEF cascade, also known as exchange proteins directly associated with cAMP (Epac) [213, 214]. These proteins are non-kinase effectors that mediate the activation of Ras superfamily GEF binding proteins including Rap1 and Rab3A [214, 215]. There are two isoforms of Epac that respond with high specificity to cAMP [213, 214]; they are referred to as Epac1 and Epac2 and are encoded by distinct genes [213]. The main difference between the two isoforms is the number of cAMP binding sites, with Epac1 containing 1 binding site whilst Epac2 has 2 [216]. Both isoforms of Epac have been identified in β-cells [217, 218] although Epac2 is the predominate isoform [219].

Epac2 activation has been shown to be required for insulin secretion as a reduction in Epac2 levels attenuates GLP-1 stimulated insulin secretion [220]. Also the interaction of Epac2 with Rim2, which is a Rab3A interacting protein, is believed to be involved in the priming of the insulin containing secretory vesicles in the β-cell [218]. In addition, Epac is thought to regulate insulin secretion via the modulation of K_{ATP} channels. The Epac selective agonist Sp-8-pCPT-2'-*O*-Me-cAMP was shown to inhibit the activity of K_{ATP} channels in β-cells [221]. As Epac has been demonstrated to interact with the SUR1 subunit of the K_{ATP} channel [218, 221, 222] it may be thourough this interaction that Epac modulates the channel's activity.

One further mechanism by which Epac can modulate insulin secretion is thourough the positive effect on intracellular Ca^{2+} levels. Epac has been demonstrated to interact with

the ryanodine receptor (RyR) in β-cells. In INS-1 cells incubation with ryanodine prevented the Epac analogue induced CICR [223] whilst overexpression of a dominant negative form of Epac blocked GLP-1 stimulated IP_3 dependent increases in $[Ca^{2+}]_i$ in MIN6 cells [224]. Furthermore, the association of Epac with the IP_3 receptor was shown to mediate increases in $[Ca^{2+}]_i$ subsequent to GLP-1 stimulation in INS-1 cells [225]. Epac could also play a role in signalling at the plasma membrane as it is interacts with Rim2 and piccolo which associate with insulin containing vesicles and L-type VGCCs [226]. These protein-protein interactions of Epac with the plasma membrane and intracellular Ca^{2+} channels promote the interaction of cAMP and Ca^{2+} signals to specialized domains. These domains may facilitate exocytosis and other Ca^{2+} dependent signalling β-cells.

1.3.3.4 PI3K and PKB

There are four classes of PI3 kinases (PI3K): I_A, I_B, II and III. Class I enzymes consist of an isoform of the p110 catalytic subunit (either α, β, δ, or γ) that interacts with a non-catalytic regulatory subunits. There are 5 non-catalytic subunits denoted p85α, p55α, p50α, p85β, p55γ and p101. Class I PI3Ks have been studied in pancreatic β-cells with the class I_B catalytic subunit p110γ [227], the class I_A p110α p110β subunits as well as the widely expressed p85α non-catalytic subunit demonstrated to be expressed [228]. A major downstream effector of PI3K is PKB. PKB is recruited to the cell membrane where the production of PIP_3 by PI3K causes the phosphorylation of PKB by PDK1 [229]. There are thouree isoforms of PKB, α, β and γ, all of which are expressed in pancreatic β-cells [228, 230].

PI3K activity has been reported in response to GLP-1 in several β-cell types including INS-1 cells, MIN6 cells and rat β-cells [231-235]. PI3K activation has been shown to be important in the insulin secretory response as mice lacking the p110γ isoform have secretory defects [227]. However, there appears to be compensation or redundancy between PI3K isoforms at least in terms of the proliferative response attributed to the kinase [227, 236]. The activation of PKB by phosphorylation on S473 and T308 in response to GLP-1 treatment [232] is thought to be instrumental for β-cell proliferation and survival. This is illustrated by the expression of a dominant negative form of PKB that completely reversed the protective effect of GLP-1 against apoptosis, whilst over-expression of a constitutively active PKB prevented agonist mediated apoptosis in

human islets [237, 238]. In addition, PKB activity leads to the activation of mTOR in β-cells. The activation of mTOR leads to the phosphorylation of rpS6 and this has been implicated in GLP-1R mediated increases in β-cell proliferation [239].

1.3.3.5 Ca^{2+}/CaM

Ca^{2+} activated calmodulin (CaM) regulates the activity of a number of intracellular substrates in β-cells including the CaMKII, and CaM-dependent enzymes such as PP2B or calcineurin.

CaMKII consists of multiple genes including α, β, δ and γ. The functional enzyme consists of multiple subunits and at high $[Ca^{2+}]_i$ the subunits undergo autophosphorylation and an increase in its affinity for CaM. Upon the reduction in $[Ca^{2+}]_i$ a large proportion of the enzyme remains active even in the absence of CaM association with resensitization requiring the action of a phosphatase [240]. In β-cells CaMKII is activated by insulin secretagogues in a manner that correlates with insulin secretion [240], suggesting that it may play an important global regulatory role in insulin exocytosis. In addition, GLP-1R activation activates CaMKII thourough increasing the influx of Ca^{2+} via the L-type VGCC and release of Ca^{2+} from the ER [241, 242]. CaMKII has also been shown to be involved in glucose and GLP-1 stimulated ERK1/2 activation after Ca^{2+} influx in β-cells [67, 68].

Calcineurin is a serine/thoureonine phosphatase that is activated by Ca^{2+}-bound CaM. The activation of calcineurin correlates with sustained rises in Ca^{2+} and removal of Ca^{2+} prevents the phosphatases activity [243]. Once activated by CaM calcineurin can dephosphorylate its target proteins. The most well characterised target of calcineurin is the transcription factor NFAT. Upon calcineurin mediated dephosphorylation, NFAT translocates into the nucleus and modulates gene transcription. Calcineurin activity has been demonstrated to have several roles in β-cells. Calcineurin inhibition has been shown to prevent GLP-1 stimulated insulin transcription via a decrease in NFAT binding within the insulin promoter [244]. In addition, the pharmacological inhibition of calcineurin prevents glucose and GLP-1 stimulated insulin secretion in β-cell lines [67, 239]. The activity of calcineurin is likely to be regulated in β-cells via the association of the phosphatase with AKAP 150. This AKAP in RINm5F cells was found to bind both PP2B and PKA with overexpression of AKAP150 lowering insulin secretion and PP2B

activity [244]. Thus the localization of calcineurin activity must be controlled to allow for its specific effects on insulin secretion and other signalling pathways in β-cells.

1.3.4.6 ERK1/2

ERK1/2 are MAPKs that are specifically activated by MEK1/2 and can be activated by a wide range of different mechanisms (reviewed in Section 1.2.4). In β-cells GLP-1 potentiates glucose stimulated ERK1/2 activation in several cell lines, including MIN6 cells and INS-1 cells, as well as human islets [66-68]. The activation of ERK1/2 by GLP-1, like glucose, has been shown to be dependent on a rise in intracellular free Ca^{2+} levels via Ca^{2+} influx thourough L-type VGCCs [68]. In addition, other factors such as β-arrestins, CaM, calcineurin and CaMKII have been shown to be required for glucose and GLP-1 mediated ERK activation [67, 68, 245].

1.3.4 GLP-1 and Ca^{2+} dependent signalling

1.3.4.1 GLP-1 evokes changes in $[Ca^{2+}]_i$

Glucose metabolism and subsequent membrane depolarisation leads to the activation of VGCCs. Ca^{2+} influx thourough L-type VGCCs is believed to mediate Ca^{2+} release from ER stores via calcium induced calcium release (CICR). GLP-1 is well documented to augment the increases in $[Ca^{2+}]_i$ mediated by glucose in β-cells [208, 224, 225, 246-250]. This has been shown to occur via Ca^{2+} influx thourough VGCCs and Ca^{2+} release from ER stores [6, 67, 68, 129, 224, 225, 250, 251] as the removal of extracellular Ca^{2+} and the inhibition of L-type VGCCs, prevents GLP-1 stimulated rises in $[Ca^{2+}]_i$ and Ca^{2+} dependent signalling [152, 225, 246, 249, 252]. In addition, GLP-1 can initiate a fast transient increase in $[Ca^{2+}]_i$ independently of glucose that occurs seconds after GPCR activation [246].

There are two proposed mechanisms by which GLP-1 stimulates the activity of the L-type VGCCs. Firstly, GLP-1 decreases the activity of the K_{ATP} channels thourough phosphorylation of the SUR1 subunit [208-210]. This leads to the potentiation of K_{ATP} channel closure in the presence of glucose thus resulting in increased VGCC activation and Ca^{2+} influx. The second mechanism by which GLP-1 mediates a rise in $[Ca^{2+}]_i$ via the L-type VGCC is thourough direct phosphorylation by PKA [211].

The release of Ca^{2+} from the ER by GLP-1 has been documented in several different β-cell lines but the extent of Ca^{2+} release from the stores varies amongst cell types. In HIT cells it was determined that although the initiation of Ca^{2+} oscillations stimulated by GLP-1 were within the vicinity of the plasma membrane the process required ryanodine receptor mediated CICR [253]. This is believed to be a generic mechanism for CICR; rises in $[Ca^{2+}]_i$ mediating activation of ryanodine receptors [212]. CICR has been demonstrated to occur in HIT-T15 cells [253], MIN6 cells [224], βTC3 cells [212] and INS-1 cells [225].

GLP-1 has also been documented to potentiate $[Ca^{2+}]_i$ levels via enhanced mobilisation of intracellular Ca^{2+} via a cAMP-dependent mechanism [224, 225, 250]. It has been reported that the cAMP effectors PKA and Epac sensitise ryanodine receptors [212, 224, 225, 246, 254] and/or IP_3 receptors [255, 256] to the effects of their agonists, Ca^{2+} and IP_3.

In addition, to the CICR mechanism described above which contributes to the overall and sustained $[Ca^{2+}]_i$ response to GLP-1, GLP-1 has been demonstrated to initiate a fast transient increase in $[Ca^{2+}]_i$ mediated by CICR. This fast response may or may not be superimposed on the sustained $[Ca^{2+}]_i$ response by GLP-1R activation [246]. This transient $[Ca^{2+}]_i$ response has been documented in rat and human β-cells and is sensitive to cAMP antagonists, L-type VGCC blockers, and antagonism of the ryanodine receptor [246]. This rise in $[Ca^{2+}]_i$ is thought to occur thourough the effects of immediate rises in cAMP, stimulated by the GLP-1R, on the L-type VGCC [246]. The physiological significance of this transient CICR is believed by the authors to contribute to the efficacy of the Ca^{2+}-dependent exocytotic trigger for insulin release [246].

1.3.4.2 Insulin secretion

Glucose stimulated insulin secretion has two distinct phases. The first phase is where global $[Ca^{2+}]_i$ changes occur in response to glucose metabolism, and this initiates insulin secretion which is maintained for approximately 10min. This initial phase of secretion mediates the fusion of large dense vesicles docked to L-type VGCCs in the readily releasable pool. The second phase of insulin secretion is characterised as a sustained plateau of insulin secretion occurring for an additional 25-30min [257]. The vesicles that are not docked to the channels are involved in the second phase of insulin secretion

and are primed for membrane fusion by intracellular signals such as Ca^{2+} and ATP during the second phase [42, 257, 258].

Insulin secretion is initiated by the inhibition of K_{ATP} channels via glucose metabolism. GLP-1's ability to potentiate glucose stimulated insulin secretion is mediated by the phosphorylation of the K_{ATP} channels SUR1 subunit. Inhibition of the K_{ATP} channel mediates depolarisation and initiates Ca^{2+} influx thourough VGCCs. The depolarisation of the β-cell allows Ca^{2+} to act on the exocytotic machinery fusing the insulin containing vesicles to the plasma membrane [42, 43]. Ca^{2+} dependent exocytosis involves syntaxin and SNAPS localised to the insulin containing vesicles that are responsive only to Ca^{2+} influx thourough L-type VGCCs. However, there is also a secondary pool of vesicles that are responsive to global rather than localised Ca^{2+} rises [259, 260] and these vesicles are likely to be involved in the second phase of insulin secretion. GLP-1 is able to potentiate Ca^{2+}-dependent glucose stimulated insulin secretion thourough increasing the $[Ca^{2+}]_i$ response of the β-cell. As both the first and second phase are Ca^{2+}-dependent GLP-1 will potentiate the effects of glucose on both phases of insulin secretion.

1.3.4.3 ERK1/2

GLP-1 potentiates glucose stimulated ERK1/2 activation in several insulinoma cell lines, including MIN6 cells, INS-1 cells, and human islets [66-68]. The activation of ERK1/2 by GLP-1, like glucose, has been shown to be dependent on a rise in $[Ca^{2+}]_i$ levels via Ca^{2+} influx thourough L-type VGCCs [68, 129, 261].

In MIN6 cells the removal of extracellular Ca^{2+} with EGTA prevents GLP-1 stimulated ERK1/2 activation demonstrating the requirement of extracellular Ca^{2+} for ERK1/2 activation [68]. The activation of the L-type VGCC is necessary for glucose and GLP-1 mediated ERK1/2 activation. Furthermore, L-type VGCC activation alone, using the L-type VGCC activator BayK-8644, is sufficient for ERK1/2 activation [68]. In addition to the role of extracellular Ca^{2+} there has been a report demonstrating the requirement of Ca^{2+} efflux from ER stores in GLP-1 mediated ERK1/2 activation. In INS-1 cells the application of SERCA pump inhibitors, such as thapsigargin, to deplete stores, as well as inhibitors of ryanodine and IP_3 receptors attenuate GLP-1 stimulated ERK1/2 activation [67].

Many Ca^{2+}-dependent signalling molecules have been implicated in glucose and GLP-1 mediated ERK1/2 activation such as β-arrestins, CaM, calcineurin and CaMKII [67, 68, 245, 262]. The requirement of CaM for glucose and GLP-1 stimulated ERK1/2 activation has been demonstrated in several β-cell lines using the calmodulin inhibitor W7 [67, 68]. Furthermore, several studies have implicated CaM indirectly in GLP-1 mediated ERK1/2 activation by identifying CaM-targets as potential mediators of GLP-1 stimulated ERK1/2 activation [67, 68, 239]. Pharmacological inhibition of calcineurin, a CaM-dependent phosphatase, has been demonstrated to significantly inhibit GLP-1 stimulated ERK1/2 activation in INS-1 cells [67] and MIN6 cells [239]. Furthermore, the use of KN62 and KN93 CaMKII inhibitors implicated CaMKII in GLP-1 stimulated ERK1/2 activation [67, 68].

β-arrestin has been shown to associate with agonist-stimulated GPCRs to induce desensitization and promote scaffolding of intracellular signalling proteins [108-111, 116]. In β-cells β-arrestin has been demonstrated to mediate signalling of some GPCR agonists, including GLP-1 and PACAP [245, 262]. The siRNA mediated knockdown of β-arrestin1 significantly attenuated GLP-1 mediated ERK1/2 activation at 5 and 10min in INS-1 cells in the absence of stimulatory glucose concentrations [262]. Furthermore, siRNA mediated knockdown of β-arrestin1 was shown to significantly affect the long-lasting ERK1/2 activation mediated by GLP-1 in INS-1 cells [263]. In addition, in mouse islets β-arrestin knockout does inhibit the modest GLP-1 stimulated ERK1/2 activation that occurs in the absence of glucose in these cells but the role of β-arrestin in the GLP-1 potentiation of ERK1/2 signalling has not been evaluated.

It has been suggested that two temporally distinct pathways mediate ERK1/2 activation in response to GLP-1, a G-protein dependent pathway and a β-arrestin dependent pathway. The β-arrestin knockout studies suggest that GLP-1 can signal to ERK1/2 independently of G-protein activation and in the absence of glucose via β-arrestin recruitment to the GLP-1R.

1.3.5 Physiological role of GLP-1 in β-cells

In β-cells GLP-1 regulates a number of physiological functions. Thourough the potentiation of glucose mediated signalling, GLP-1 can potentiate glucose stimulated insulin secretion and modulate the transcription of specific genes including preproinsulin. These short-term actions of GLP-1 enable the β-cell to respond and adapt

to physiological stimuli. Long-term effects of GLP-1 include the ability to regulate β-cell mass thourough changes in proliferation, apoptosis and differentiation.

1.3.5.1 Regulation of mass

The mechanism that GLP-1 utilizes to mediate increases in β-cell mass is thought to be a combination of several pathways: the stimulation of proliferation, the inhibition of apoptosis and the differentiation of stem cells [264-266].

In T2DM β-cell mass is reduced by up to 60% [18, 19]. The application of GLP-1R agonists or inhibitors of DPP-IV can increase β-cell mass in normal or diabetic mice [267-271]. The continuous infusion of GLP-1 can delay the age dependent decline of β-cell function in Wistar rats due to an increase in β-cell mass [271]. Furthermore, GLP-1R agonists decrease the severity of the diabetic phenotype in the db/db obese and T2DM mice. This is correlated with increases in β-cell mass [268-270].

One of the mechanisms that GLP-1 is thought to mediate increases in β-cell mass, is thourough the stimulation of β-cell proliferation [264-266]. The application of GLP-1R agonists *in vivo* or *in vitro* induces proliferation of β-cells to increase β-cell mass [272, 273]. GLP-1 treatment of human fetal islets caused maturation of the β-cells [274] whilst in rat islets GLP-1 treatment led to a 3-fold increase in the number of β-cells [272]. In β-cell lines GLP-1 is also able to promote proliferation. In several independent reports in INS-1 cells GLP-1 increased the incorporation of thymidine into replicating DNA indicative of proliferation [233, 236, 275, 276] and using the same methodology this was also shown in rat purified β-cells [142]. The proliferative action of GLP-1 has been shown to occur via a variety of signalling pathways. The overexpression of a dominant negative form of the novel PKCξ suppressed the proliferative effect of GLP-1 in the INS-1 cell line [275]. However, the overexpression of a dominant negative form of PKB similarly prevented GLP-1 stimulated proliferation [276]. In other work GLP-1R mediated proliferation has been shown to involve the EGFR and src kinase, with inhibitors of these signalling molecules preventing the GLP-1 response in INS-1 cells and rat islets [236]. With a variety of intracellular signalling molecules implicated in mediating proliferation, additional work has been carried out assessing the limiting factors for GLP-1 mediated proliferation. CREMα and DUSP14 have both been identified as proteins that limit GLP-1 mediated proliferation. shourNA mediated knockdown of these proteins increased the proliferative response of GLP-1 in primary

rat β-cells [142]. CREMα and DUSP14 correspond to proteins involved in the repression of CREB mediated transcription and ERK1/2 activation thus this work implicates CREB and ERK1/2 in the proliferative response of β-cells to GLP-1. Long-term effects on β-cell proliferation by GLP-1 could be mediated by the upregulation of IRS2 gene expression [277].

The anti-apoptotic effect of GLP-1 complements the action of the incretin on the proliferation of β-cells to increase β-cell mass [278]. The anti-apoptotic effect of GLP-1 has been demonstrated thourough the coapplication of various potent inducers of apoptosis and GLP-1 on β-cells. GLP-1 has been shown to inhibit the negative effects of cytokines, peroxide, fattyacids and streptozotocin in rodent β-cell lines [235, 238, 266, 279, 280] confirming its anti-apoptotic effects. GLP-1 has also been shown to mediate anti-apoptotic effects in human islets as application of GLP-1 led to down-regulation of active caspase-3 and bcl-2, two markers of apoptosis, at the mRNA and protein levels [281]. The signalling mechanisms that have been proposed to mediate the anti-apoptotic effects of GLP-1 include the PI3K and PKB pathways. The activation of PKB thourough CREB and IRS-2, and independently thourough the IGF-1R has been implicated in the anti-apoptotic effects of GLP-1. GLP-1 via an increase in CREB activity upregulates the expression of IRS-2 and increases PKB phosphorylation, resulting in inhibition of apoptosis [282]. GLP-1 stimulation of the IGF-1R, via an autocrine loop, activates PKB and inhibition of this signalling prevents the anti-apoptotic effect of GLP-1 in MIN6 and purified mouse β-cells [283].

In addition to the proliferative and anti-apoptotic effects of GLP-1 contributing to increases in β-cell mass, the incretin has been demonstrated to stimulate differentiation of cell types into cells with a β-cell phenotype. The suggestion that GLP-1 stimulates differentiation of stem cells into islet precursors follows the observations that the application of GLP-1 agonists leads to increased number of small (and therefore presumed to be newly formed) islets in rodents [270, 273, 284, 285]. The evidence for differentiation is the observation that GLP-1 application in rodents induces the expression of PDX-1 positive cells in small ducts [286], where islet neogenesis is believed to occur, and GLP-1 agonists initiate islet specific differentiation markers in human progenitor cells [274, 287, 288]. In addition, GLP-1 treatment leads to the differentiation of PANC-1 and ARIP-1, which are ductal cell lines, into insulin secreting cells [289].

1.3.5.2 Regulation of gene transcription

GLP-1, given that it is documented to stimulate a variety of different intracellular signalling pathways, is capable of promoting transcription of a wide range of proteins. However, in β-cells GLP-1s most characterised transcriptional target is insulin.

GLP-1 stimulation of β-cell lines increases insulin content and mRNA levels [196, 286, 290] and has been documented to dose-dependently increase insulin gene expression from rat insulin promoter (RIP) constructs expressed in INS-1 cells [291]. The CRE binding site within the 410bp rat insulin promoter has been documented as the main site for GLP-1 stimulation with both CREB and other bZIP transcription factors believed to be functional at this site [291].

GLP-1 has also been shown to stimulate insulin transcription at the human insulin promoter [281, 292], however studies on the human insulin promoter show subtle differences in GLP-1 stimulation. The human promoter contains 4 CRE binding sites as opposed to the single site in the rodent promoter [292] and GLP-1 appears to stimulate CRE-dependent transcription of insulin at these sites via a different mechanism. CRE1 is likely to be permanently occupied and thus does not participate in GLP-1 stimulated insulin transcription. CRE2 is activated by cAMP probably via the interactions of ATF2 and CREB-1. However CRE3 and CRE4 stimulation by GLP-1 occurs by pathways other than those involving PKA and cAMP and with a large variety of proteins observed to bind to CREs the candidate transcription factor remains elusive [292].

A microarray study using glucose and a cAMP agonist to mimic the action of GLP-1 has been shown to upregulate the transcription of 1278 immediate early genes (IEG) after 4 hours treatment in MIN6 cells [293]. One of the key findings of the microarray study is the large proportion of target genes transcribed in MIN6 cells, in response to glucose and cAMP co-stimulation, requires the action of the AP-1 transcription factor [293]. One particular IEG documented to be transcribed in response to GLP-1 stimulation is the ERK1/2-dependent IEG c-Fos. In MIN6 cells c-Fos accumulation in response to GLP-1 stimulation is biphasic with the initial peak requiring ERK1/2-dependent transcription and the plateau phase quiring the stabilization of the protein via ERK1/2 [294].

GLP-1 is also documented to increase the transcription of several factors that are important in the proliferative response of the hormone. The Pax4 mRNA transcript, shown to be important for cell survival and proliferation [140] has been shown to be upregulated by both glucose and GLP-1 in human islets via a mechanism requiring the activation of ERK1/2 and PKA [141]. Furthermore, the transcription of the cyclin D1 protein is time-dependently induced by GLP-1 in INS-1E cells and positively correlates with GLP-1 stimulated β-cell proliferation [139]. The ability of GLP-1 to stimulate cyclin D1 transcription was confirmed by transiently transfecting a cyclin D1 luciferase reporter construct into rat islets and INS-1 cells [139].

1.4 Acetylcholine

1.4.1 Autonomic stimulation

The parasympathetic stimulation of islets originates from sensory inputs from the oral, visual and olfactory systems, combining with neuronal signals from the gut and the liver, in the hypothalamus [295]. The combination of these signals leads to the activation of the vagal nerve which descends to the exocrine tissue thourough the parasympathetic ganglion. In the endocrine tissue the vagal nerve separates into vagal fibres which target the Islets of Langerhans in the pancreas. A variety of different transmitters including acetylcholine (ACh), vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) are released from these vagal fibres [296-308] to act upon individual cells within the centre and periphery of the islet [307, 309-312].

The activation of vagal nerves has been shown to be essential for normal pancreatic β-cell function. The application of nicotinic receptor and muscarinic receptor antagonists prevents insulin secretion [297] and severing the vagal nerve prevents full insulin responsiveness.

The sympathetic fibres innervating the pancreas originate from the thoracic and upper lumbar sections of the spinal cord [313]. The activation of the sympathetic fibres leads to noradrenaline stimulation and activation of intraganglionic neurones. These intraganglionic neurones descend to the pancreas resulting in the stimulation of islets with norephinephourine, galanin and NPY [296, 308, 314-316]. Activation of sympathetic neurones therefore inhibits insulin secretion and stimulates glucagon secretion [296, 317]. Therefore, the parasympathetic and sympathetic neurones of the autonomic nervous system act in conjunction to manage fluctuating plasma glucose concentrations thourough neuronal innervations of the β-cell.

1.4.2 Muscarinic receptor activation during the pre-absorptive and absorptive phases of feeding

Muscarinic receptor activation of β-cells has been proposed to occur upon food intake via release from intrapancreatic vagal nerve endings during both the preabsorptive (cephalic phase) and absorptive phase of feeding [296, 318, 319], illustrated in Figure

1.4. ACh signalling has been demonstrated to be important for β-cells to respond to plasma glucose concentrations as atropine administration or severing of the vagus nerve delays rises of plasma insulin [320-326].

The preabsorptive phase of food intake occurs before any post-absorptive glyceamic elevation [323, 325, 327-336] and as small rises in circulating insulin are apparent within 3-4min of ingestion [337], a mechanism independent of absorption must affect insulin secretion from the β-cells. The preabsorptive phase of feeding is stimulated by visual, oral and olfactory neurons [330, 338, 339] and is responsible for up to 20% of the rise in insulin levels thouroughout food intake [325, 327]. Both cholinergic and non-cholinergic mechanisms are thought to contribute to the preabsorptive phase of insulin secretion [296, 335] as removal of the vagal nerves prevents rises in insulin prior to nutrient absorption [296, 326, 327, 340, 341]. The presence of the preabsorptive phase of insulin release in humans has been shown thourough imaginary food ingestion thourough hypnosis and sensory mechanisms [342]. These studies demonstrate the modulation of insulin secretion in the absence of nutrient intake signifying a role for sensory mechanisms in mediating insulin secretion in humans. During the preabsorptive phase of feeding there is a small but physiologically crucial stimulation of insulin secretion which potentiates subsequent glucose stimulated insulin secretion from the β-cell [335, 343-346]. The preabsorptive rise in plasma insulin allows a finer control of plasma glucose levels upon glucose absorption. This is illustrated by work in rats with deinnervated islets whereby the rise in plasma glucose levels is not adjusted as efficiently as innervated rats unless insulin is injected prior to nutrient absorption [347]. The effect of mAChR activation prior to rises to glucose has been illustrated *in vitro* with prior exposure to carbachol resulting in dose-dependent increases in the subsequent glucose stimulated insulin secretion in isolated islets [348, 349].

Due to the quick degradation of acetylcholine in plasma, to measure the continued effect of parasympathetic stimulation during the absorptive phase of nutrient digestion, the presence of pancreatic-polypeptide (PP) in the plasma is measured. PP secretion is abolished by application of atropine or vagotomy [350, 351], so provides a direct readout of parasympathetic activity. PP secretion follows a biphasic pattern with the

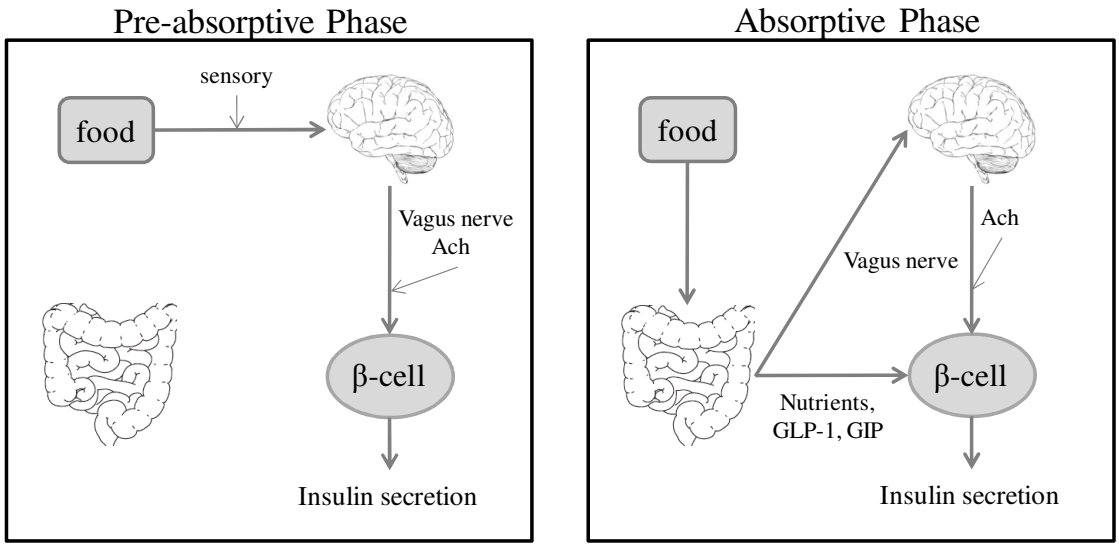


Figure 1.4. Effects on β-cells during the two physiological phases of food intake. There are 2 phases of food intake: the pre-absorptive and the absorptive. During the pre-absorptive phase of feeding sensory stimulations mediate vagal nerve stimulation of the β-cells leading to insulin secretion. During the absorptive phase of feeding the presence of nutrients in the intestine leads to vagal nerve stimulation of the brain to continue neuronal stimulation of the β-cell but in addition nutrient absorption leads to incretin release from the intestine. Nutrients and incretins stimulate β-cells to mediate insulin secretion.

rapid first phase correlating to the pre-absorptive insulin response whilst the sustained secretory phase of PP gives evidence of a sustained activation of parasympathetic neurones during the absorptive insulin response [318, 352]. During this absorptive phase of feeding, activation of the mAChR acts to potentiate glucose stimulated insulin secretion [318]. The existence of parasympathetic innervations in both the pre-absorptive and absorptive phases of digestion highlights multiple glucose-dependent and independent functions for mAChR stimulation of β-cells.

1.4.3 mAChRs in the β-cell

Neurotransmitters, such as ACh, are found to stimulate a variety of different cell types both centrally and peripherally. Cholinergic stimulation of peripheral tissues is mediated by ACh acting via both nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR) [353-355] [355]. nAChRs form an ionotropic family of cholinergic receptors that function as cation channels, that upon activation allow the influx of mono- and divalent cations such as Na^+ and Ca^{2+}. mAChRs are metabotropic, functioning as GPCRs to mediate specific second messenger and cell signalling cascades. These metabotropic mAChRs have 5 distinct subtypes; denoted $M_{1\text{-}5}$ [356, 357]. The 5 subtypes are encoded by 5 individual genes and their presence in different systems can be identified by the receptor's binding affinities for specific agonists and antagonists [358]. The effectors that the 5 different GPCR muscarinic subtypes regulate depend on the coupling of their G-proteins. M_2 and M_4 preferentially couple to $G\alpha_{i/o}$ leading to inhibition of AC and a reduction in cAMP levels. However, M_1, M_3, and M_5 mAChR subtypes preferentially couple to $G\alpha_{q/11}$ leading to the activation of phospholipase Cβ, and the generation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) from PIP_2 [358]. IP_3 and DAG act as second messengers and stimulate Ca^{2+} mobilisation and PKC activation respectively, which in turn can activate Ca^{2+}-dependent ACs and ion channels [359-361].

Multiple mAChR subtypes have been identified in β-cells [319, 362-365]. mAChR activation in β-cells does not lead to the inhibition of adenylyl cylases [366, 367] but muscarinic signalling is insensitive to pertussis toxin [368, 369], and there is evidence of PIP_2 hydrolysis [368, 370-372]. Therefore, the $G_{q/11}$ coupled M_1, M_3 and M_5 subtypes are thought to be the main candidates for mediating muscarinic agonist effects.

The selective knockout of M_3 receptors in β-cells showed that this subtype is primarily responsible for cholinergic action [319]. These mice have significant glucose intolerance and lowered plasma insulin levels. Conversely, mice selectively overexpressing the M_3 mAChR in β-cells have increased glucose tolerance and enhanced plasma insulin levels. Furthermore, these mice are resistant to diet induced glucose intolerance and hyperglycaemia [319].

1.4.4 Muscarinic Gq-receptor signalling

1.4.4.1 PLC

The catalysis of PIP_2 hydrolysis is controlled by the ubiquitously expressed enzyme phospholipase C (PLC). This reaction generates IP_3 and DAG. There are fourteen mammalian isoforms of PLC but members of the PLCβ family are activated by heterotrimeric G_q proteins in response to GPCR activation [373, 374]. To activate PLC isoforms a Ca^{2+} ion is required to bind the active site of the enzyme [375] and other Ca^{2+} ions can bind to several different domains within the structure of PLC enzymes. These domains that have Ca^{2+}-binding properties include C2-domains and EF-hands [375, 376].

mAChR activation in β-cells leads to the stimulation of PLCβ activity via the activation of the pertussis and cholera-toxin insensitive G_q G-protein [373, 377]. The activation of mAChRs causes DAG and IP_3 accumulation in both primary β-cells and insulin-secreting cell lines [378, 379] and Ca^{2+} potentiates this signalling response [378, 380-384].

1.4.3.2 DAG and PKC

DAG comprises of a glycerol backbone with two of the hydroxyl groups esterified with long-chain fatty acids. This second messenger is liposoluble and remains at the plasma membrane to activate DAG dependent kinases such as PKC. The stimulation of mAChRs in β-cells leads to the production of two species of DAG enriched in either palmitate or arachidonate fatty acids. These species have differential time courses of accumulation in β-cells which may effect the activation of DAG-dependent kinases such as PKC [385, 386]. Indeed, muscarinic agonists have been shown to induce the translocation of various PKC isoforms to the plasma membrane in β-cells [387, 388].

The most well-documented DAG-kinases are PKCs. The PKC family consists of several groups of kinases classified by their sensitivity to Ca^{2+} and DAG. Conventional PKC isoforms (cPKC) including α, βI, βII and γ require both by Ca^{2+} and DAG for activation. Novel PKC isoforms (nPKC) include δ, ε, η, and θ and are activated by DAG alone. Atypical PKC isoforms include ξ and τ/λ and are activated independently of both Ca^{2+} and DAG. In β-cells a variety of different PKC isoforms have been reported to be expressed including α, β, δ, ε, ξ and τ [389-399].

One of the major functional effects of mAChR activation of PKC in β-cells is its positive role in stimulating insulin secretion. PKC is thought to sensitise the secretory machinery in β-cells to Ca^{2+} [400-404]. Research using both generic inhibitors and pseudo-substrates of PKC has suggested that the major PKC isoform expressed in β-cells, PKCα, is the likely mediator of PKC-dependent effects of mAChR stimulated insulin secretion [381, 388, 405-408].

1.4.3.3 ERK1/2

Stimulation of mAChRs in a variety of excitable and non-excitable cells has been shown to activate the ERK1/2 pathway. M_3 receptors mediate signalling to ERK1/2 but the mechanism of this is poorly understood. In a human neuroblastoma SK-N-BE2(C) cell line M_3 mediated ERK1/2 activation requires PKCε, Ras, Raf and MEK activity but not PI 3-kinase activation. It is also Ca^{2+}-independent [409, 410]. However, in intestinal epithelial cells, thyroid epithelial cells and primary cortical cultures, mAChR mediated ERK1/2 activation requires Ca^{2+} mobilisation and is independent of PKC [411-413]. Thus tissue and cell specific differences influence the mechanism of ERK1/2 activation upon mAChR stimulation [414-418].

In isolated cultured rat islets brief exposure to carbachol has been shown to mediate ERK1/2 phosphorylation [419], but the mechanism underlying this phosphorylation was not determined. ERK1/2 activation in β-cells has a wide variety of physiological functions. The activation of ERK1/2 has been shown to be important in the stimulation of β-cell proliferation, differentiation, survival and gene transcription [261, 420, 421]. In the β-cell line MIN6, glucose-stimulated ERK1/2 activation has been shown to play an important role in enhancing insulin exocytosis via the phosphorylation of synapsin I [422]. Interestingly, mAChR activation has been shown to prime β-cells for subsequent glucose stimulated insulin secretion *in vitro*, with glucose stimulated insulin secretion

increased after preincubation with muscarinic agonists [348, 349]. Therefore, it is conceivable ERK1/2 activation observed upon mAChR activation plays a role in preparation of β-cells for insulin secretion. Furthermore, the expression of both M1 and M3 receptors is increased during proliferation [423] suggesting a specific role for the cholinergic pathway in proliferation. ERK1/2 activation in β-cells has also been shown to stimulate proliferation as glucose and incretin stimulated ERK1/2 activation leads to an increase in β-cell number in rat and mouse islets [138, 139]. In other cell types the stimulation with muscarinic agonists is also believed to mediate Ca^{2+} and/or ERK1/2-dependent cell growth and proliferation. For example, the proliferation of human lung fibroblasts is mediated by an ERK1/2-dependent mechanism thourough the activation of mAChRs [424]. Furthermore, the rise in $[Ca^{2+}]_i$ subsequent to mAChR activation in human breast cancer cells leads to ERK1/2 activation as well as increases in protein synthesis and cell proliferation [416].

1.4.3.4 Transcription

mAChR activation in a variety of cells leads to gene transcription of several important transcripts. In αT3M3 gonadotrophs and neuroblastoma cells, mAChR stimulation increases the expression of the early growth response factor 1 (Egr-1) transcription factor, and this requires ERK1/2 dependent phosphorylation of CREB [425-427]. Another important transcriptional target for mAChR activation is the matrix metalloproteinase (MMP) genes in human colon cells, whose expression by an ERK1/2 dependent mechanism provides a novel feed-forward mechanism for cell proliferation [428, 429]. Currently, mAChR activation in β-cells has not been shown to activate transcription although it is known to activate ERK1/2 [419]. Therefore, it is likely that mAChR activation of ERK1/2 regulates transcription.

1.4.3.5 Ca^{2+} response

ACh stimulation of β-cells leads to concentration-dependent rises in $[Ca^{2+}]_i$ consisting of an initial peak followed by a small sustained elevation [430]. The amplitude of the transient peak of $[Ca^{2+}]_i$ is dependent on the glucose concentration present before and during ACh stimulation [380, 406, 431-435]. In lower glucose concentrations there is a smaller peak Ca^{2+} response, presumably due to the lack of refilling of the IP_3 sensitive intracellular stores after IP_3 depletion [434, 436, 437].

The rise in $[Ca^{2+}]_i$ in β-cells upon muscarinic stimulation is thought to comprise of several different mechanisms. The thouree main mechanisms known to contribute to the rise in $[Ca^{2+}]_i$ include the activation of IP_3-stimulated Ca^{2+} efflux from the ER, opening of VGCCs and capactitive Ca^{2+} entry [430, 438-440].

The production of IP_3 upon mAChR stimulation of PLCβ leads to the mobilisation of Ca^{2+} from IP_3-sensitive intracellular stores. In MIN6 and INS-1 β-cell lines as well as mouse and rat islets this mobilisation can be suppressed by thapsigargin and CPA, two SERCA pump inhibitors [430, 441-445], and a drop in $[Ca^{2+}]_{ER}$ can be visualised upon carbachol stimulation [438, 439], confirming that the IP_3-mediated rises in $[Ca^{2+}]_i$ originate from the ER. However, a relatively high concentration of ACh is required for the mobilisation of intracellular stored Ca^{2+} [380, 430] whereas the stimulation of Ca^{2+} influx is much more sensitive to mAChR activation [430].

In the absence of glucose, ACh is able to stimulate increases in $[Ca^{2+}]_i$ which is inhibited by diazoxide (a K_{ATP} channel activator) or by blocking VGCCs [430]. ACh in primary β-cells is generally thought not to be able to mediate depolarisation sufficiently to activate VGCCs in non-depolarising conditions. However, some evidence exists that high concentrations of ACh can mediate a small depolarisation, sufficient to stimulate insulin secretion [380, 446-448].

ACh also stimulates capacitative Ca^{2+} entry: the stimulation of voltage-independent Ca^{2+} influx channels via the emptying of intracellular stores [449, 450]. This Ca^{2+} response affects membrane potential and can therefore influence the activity of the VGCCs in the plasma membrane [451-453]. The capacitative entry has been shown in β-cells to be small and, contradictory to other systems, is not dependent on the energy status of the cell, PKC activation, phosphatases or tyrosine kinases [440]. Similar to the requirement for intracellular Ca^{2+} mobilisation, relatively high concentrations of muscarinic agonists are required to stimulate capacitative Ca^{2+} entry in β-cells [440].

In general therefore, in non-depolarising conditions a high concentration of muscarinic agonist is required to stimulate the activation of VGCCs, IP_3-mediated Ca^{2+} release and the subsequent capacititative Ca^{2+} entry.

1.4.3.6 Insulin secretion

One of the main roles of cholinergic stimulation of β-cells is to modulate glucose-dependent insulin secretion. ACh targets β-cells via its release, along with other transmitters, from peripheral autonomic nerves. Food intake is known to trigger an increase in parasympathetic nerve activity and ACh is released from intrapancreatic vagal nerve endings during the preabsorptive (cephalic phase) and absorptive phase of feeding [296, 318, 319].

The effects of ACh on insulin secretion are generally thought to be dependent on glucose [380, 388, 449, 454]. At low glucose concentrations the ACh only produces a small depolarisation mediated by Na^+ influx which has been shown to be insufficient in mouse islets to stimulate VGCC-dependent Ca^{2+} influx [446]. However, in some cell line models a high concentration of ACh mediates nifedipine sensitive insulin secretion [447, 448]. In addition, electrophysiological studies have revealed that ACh causes depolarisation of rat β-cells in a concentration dependent-manner, leading to bursts of action potentials which are potentiated by glucose [380, 446, 448, 455-458]. The ACh mediated depolarisation is likely via L-type VGCC opening as the increases in $[Ca^{2+}]_i$ elicited by ACh in islets was abolished by nifedipine. Nifedipine application completely inhibited the glucose-dependent effects of ACh on insulin secretion [455]. In addition, in the absence of extracellular Ca^{2+}, only high concentrations of ACh mobilized enough $[Ca^{2+}]_i$ to trigger insulin release [380], suggesting that extracellular Ca^{2+} influx is the main signal mediating ACh action on insulin secretion in β-cells.

1.4.4 Muscarinic receptor dysfunction in T2DM

The importance of the mAChR signalling at the β-cell has been demonstrated using a β-cell specific M_3 knock out mouse. The specific knockout of the M_3 mAChR leads to impaired glucose tolerance and insulin secretion [319]. However, there are few documented reports of mAChR signalling dysfunction in diabetic patients. Due to the dramatic physiological effects of the β-cell specific M_3 receptor knockout, it is likely that polymorphisms and mutations of the channel exist and participate in the aetiology of diabetes. One report suggests a link between the M_3 receptor and T2DM. In a study evaluating Pima Indians, the authors located a single nucleotide polymorphism (SNP) within the M_3 receptor gene associated with T2DM. Furthermore, the transcript

containing the SNP within the 5'UTR was expressed in human islets but the functional properties of the receptor were not investigated [459].

1.5 Aims of the thesis

The overall aims of this thesis are to investigate the molecular mechanisms by which agonists of either the GLP-1R or muscarinic receptors mediate ERK1/2 activation in the pancreatic β-cell. The thesis will also aim to investigate the role of Ca^{2+} influx through the L-type VGCC in signalling to ERK1/2. Furthermore, the physiological significance of ERK1/2 activation by these agonists will be investigated.

Chapter 2: Materials and methods

2.1 General reagents and materials

Unless stated, all chemicals were of analytical grade and were purchased from Sigma or Fisher. Bacterial cell culture reagents were from Melford. Disposable plastics (1.5ml microfuge tubes, non-filtered pipette tips) were obtained from Sarstedt. Filtered tips were purchased from Axygen. PD184532, BIM I, Ro320432, Gö6976, BAPTA-AM and EGTA-AM were all purchased from Calbiochem. Nifedipine, diazoxide, phorbol 12-myristate 13-acetate (PMA), thapsigargin, ryanodine, dantrolene, xestospongin C, 2APB, carbachol and GLP-1(7-36 amide) were all purchased from Sigma. Molecular probes supplied Fluo-4 AM, Fura-Red, Fura-2 free acid and Fura-2-AM. Ionomycin was purchased from Tocris. The luciferase assay system (Cat No E1500) and the dual-luciferase reporter assay system (Cat No E1910) were purchased from Promega. Tissue culture plates and flasks were from NUNC or TPP. Tissue culture pipettes were from Greiner or Corning.

2.2 Buffers

2X HEPES buffered saline (HBS)

8g NaCl

0.2g Na_2HPO_4-$7H_2O$

6.5g HEPES

pH to 7.0 and make up to 500ml with distilled H_2O. Re-pH, filter-sterilise, aliquot and store at -20°C

Krebs ringer bicarbonate buffer (KRB)

115mM NaCl

5mM KCl

10mM $NaHCO_3$

2.5mM $MgCl_2$

2.5mM $CaCl_2$

20mM HEPES pH 7.4

Luria-bertani media (LB)

1% w/v Tryptone

0.5% w/v Yeast Extract

1% w/v NaCl

1.5% w/v Bacteriological Agar (for solid media only)

Ampicillin (Filter sterilised)

Used at a final concentration of 100µg/ml

Kanamycin (Filter sterilised)

Used at a final concentration of 50µg/ml

1X TAE (Tris-Acetate EDTA)

40mM tris-acetate

1mM EDTA pH 8

6X DNA loading buffer

50% v/v glycerol

0.1M EDTA pH 8

1% w/v SDS

1mg/ml bromophenol blue

1mg/ml xylene cyanol

10X Tris-Glycine buffer (for 1l)

30g Tris base

144g Glycine

SDS-PAGE running buffer

1X Tris-Glycine buffer

0.1% w/v SDS

Semi-dry transfer buffer

1X Tris-Glycine buffer

0.01% w/v SDS

20% v/v Methanol

10X PBS (Phosphate Buffered Saline; 1l)

3g KCl

100g NaCl

14g Na_2HPO_4

3g KH_2PO_4

pH to 7.4 and make up to 1l

PBS-tween (PBST)

1X PBS

0.1% v/v Tween-20

Laemmli sample buffer (4X)

0.25M Tris pH 6.8

4% w/v SDS

40% v/v Glycerol

10% v/v β-mercaptoethanol

20μg/ml Bromophenol blue

MIN6 lysis buffer

1% v/v Triton X-100

10mM β-glycerophosphate pH 7.4

50mM Tris-HCl pH 7.5

1mM EDTA pH 8

1mM EGTA

1mM Sodium orthovanadate

1mM Benzamidine

0.2mM PMSF

1µg/ml Leupeptin

1µg/ml Pepstatin A

0.1% v/v β-mercaptoethanol

50mM NaF

RIPA buffer

150mM NaCl

50mM Tris-HCl pH 8.0

10.5% DOC

0.5% SDS

1% NP40

1mM Sodium orthovanadate

10mM β-glycerophosphate pH 7.4

0.1% v/v β-mercaptoethanol

1mM Benzamidine

0.2mM PMSF

1µg/ml Leupeptin

1µg/ml Pepstatin A

2.3 Plasmid constructs

Protein Expressed	*Tag/Vector*	*Provided by*	*Reference*
His-Cav1.2-DHPi	His/pEGFPN1	Dr Ricardo Dolmetsch, Stanford School of Medicine	[460]
Cav1.2-DHPi-GFP	GFP/pcDNA3	Dr Greg Hockerman, Purdue University	[461]
His-Cav1.3-DHPi	His/pcDNA3	Dr Ricardo Dolmetsch, Stanford School of Medicine	[460]
Cav1.3-DHPi-GFP	GFP/pcDNA3	Dr Greg Hockerman, Purdue University	[461]
β_1 auxiliary subunit	pcDNA3	Dr Ricardo Dolmetsch, Stanford School of Medicine	[460]
β_3 auxiliary subunit	pcDNA3	Dr Greg Hockerman, Purdue University	[461]
$\alpha_2\delta$ auxiliary subunit	pcDNA3	Dr Greg Hockerman, Purdue University	[461]
His-Cav1.2-DHPi-IA	His/pcDNA3	Dr Ricardo Dolmetsch, Stanford School of Medicine	[460]
His-Cav1.2-DHPi-IV	His/pcDNA3	Dr Ricardo Dolmetsch, Stanford School of Medicine	[460]

HA-Tpl2	HA/	Dr Shao-Cong Sun, Pennsylvania State University	[74]
HA-Tpl2	HA/	Dr Philip Tsichlis, Tufts-New England Medical Centre, Boston	[80]
HA-Tpl2-K167M-DN	HA/	Dr Philip Tsichlis, Tufts-New England Medical Centre, Boston	[80]
PIP5K KA		Prof RAJ Challiss, University of Leicester	n/a
PIP5K KD		Prof RAJ Challiss, University of Leicester	n/a
eGFP-$PH_{PLC\delta1}$	GFP	Prof RAJ Challiss, University of Leicester	[462]
D1ER-FRET cameleon	GFP	Prof R Tsien, Department of Pharmacology and Howard Hughes Medical Institute, University of California	[463]
pFOXLUC410 preproinsulin promoter	pFOXLUC vector under control of -410 to +1 of the rat insulin promoter	Dr Kevin Docherty, University of Aberdeen Dr M. German, University of California at San Francisco Medical School	[292]
IKKα WT	FLAG	Dr Raj Patel, University of Leicester	[464]
IKKα K44A	FLAG	Dr Raj Patel, University of	[464]

		Leicester	
IKKβ WT	FLAG	Dr Raj Patel, University of Leicester	[464]
IKKβ K44AA	FLAG	Dr Raj Patel, University of Leicester	[464]
IKKα WT	HA	Dr Michael Karin, University of California	[465]
IKKα AA	HA	Dr Michael Karin, University of California	[465]
IKKβ WT	HA	Dr Michael Karin, University of California	[465]
IKKβ AA	HA	Dr Michael Karin, University of California	[465]
TPI intronless Renilla		Prof Melissa Moore, Brandeis University	[466]
eGFP (Clontech)	GFP	Clontech, Mountain View, CA	n/a
eGFP-ERK	GFP	Dr Yoshimura, Kyusyu University, Japan	n/a

2.4 Adenoviruses

Protein Expressed	*Tag*	*Provided by*
Cav1.2-DHPi adenovirus	GFP	Made by Dr Edith Gomez

Cav1.3-DHPi adenovirus	GFP	Made by Dr Edith Gomez
CaMKIIδ DN adenovirus	GFP	Made by Dr Claire Moore
RasN17 adenovirus	HA	Prof. Barbara Kahn, Beth Israel Deaconess Medical Center
RapGap adenovirus	GFP	Made by Dr Claire Moore
CaM WT adenovirus	GFP on an independent promoter	Dr Liz Seward, University of Sheffield, originally from David Yue, John Hopkins University School of Medicine, Baltimore
$CaM_{1,2,3,4}$ adenovirus	GFP on an independent promoter	Dr Liz Seward, University of Sheffield, originally from David Yue, John Hopkins University School of Medicine, Baltimore
WT PKCε adenovirus	GFP	Made by Dr Edith Gomez
PIP5K WT adenovirus	None	Dr Show-Ling Shyng, Oregon Health & Science University
PIP5K DN adenovirus	None	Dr Show-Ling Shyng, Oregon Health & Science University

2.5 Antibodies

Primary Antibody	*Supplier/Cat No*	*Dilution*	*Buffer*	*Secondary*	*Application*
Phospho-ERK1/2	CST/4695	1/1000	5% BSA in PBS-T	Rabbit	WB, IF

ERK2	CST/9108	1/1000	5% BSA in PBS-T	Rabbit	WB
Phospho-MEK	CST/9154	1/1000	5% BSA in PBS-T	Rabbit	WB
Phospho-CREB S133	CST/9198	1/1000	5% BSA in PBS-T	Rabbit	WB
Tpl-2 (M20)	Santa Cruz	1/500	5% milk in PBS-T	Rabbit	IP, WB
GAPDH-HOURP	CST/3683	1/10,000	5% BSA in PBS-T	N/A	WB
IκBα	CST/4814	1/1000	5% BSA in PBS-T	Mouse	WB
Phospho-p65	CST/3033	1/1000	5% BSA in PBS-T	Rabbit	WB
IKKβ	CST/2678	1/1000	5% BSA in PBS-T	Rabbit	WB, IP
pIKKαβ	CST/2697	1/1000	5% BSA in PBS-T	Rabbit	WB
αGFP	AbCam/ab6556	1/1000	5% BSA in PBS-T	Rabbit	WB
αHA	Roche/ 11583816001	1/1000	5% milk in PBS-T	Mouse	WB, IP
αHis	Sigma/H1029	1/1000	5% milk in PBS-T	Mouse	WB, IP
αMyc (9E10)	Sigma/M4439	1/1000	5% milk in PBS-T	Mouse	WB

WB = Western Blotting, IP = Immunoprecipitation, IF = Immunoflourescence

2.6 siRNA oligos

Name	*Sequence*
Tpl-2 siRNA (40) MAP3K8MSS218540	CCUAGCAACAUUGUAUUCAUGUCUA UAGACAUGAAUACAAUGUUGCUAGG

(Invitrogen)	
Tpl-2 siRNA (41) MAP3K8MSS218541 (Invitrogen)	GGCCCAUGAGAGAAUUUGAAAUUAU AUAAUUUCAAAUUCUCUCUCAUGGGCC
Tpl-2 siRNA (42) MAP3K8MSS218542 (Invitrogen)	CCAAGAAAGUGAUCCACCAUGAUAU AUAUCAUGGUGGAUCACUUUCUUGG
Tpl-2 siRNA Designed (Invitrogen)	AAUCGAUUCGGAUGUUCUCCUUGUU AACAAGGAGAACAUCCGAAUCGAU

2.7 Cell culture techniques

2.7.1 Mammalian cell culture

Cell lines used in this study were Human Embryonic Kidney 293 (HEK_{293}), RAW264.7 macrophages (provided by Dr Bernard Burke, University of Leicester), Mouse Insulinoma 6 cells (MIN6), which respond to physiological changes in glucose concentrations [467] were kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University Medical School, Japan and rat insulinoma clone 1e (INS-1e) cells were kindly supplied by Prof. Claes B. Wollheim, MD, Department of Cell Physiology and Metabolism, University Medical Center, Geneva.

2.7.2 Maintenance of cell lines

HEK_{293} cells were used at approximately 80% confluence and were grown in full Dulbecco's Modified Eagle's Medium containing 25mM glucose supplemented with 10% heat-inactivated foetal calf serum, 100µg/ml streptomycin, 100units/ml neomycin and 100units/ml penicillin sulphate, equilibrated with 5% CO_2, 95% air at 37°C. Cells were split every 2-3 days to prevent over confluence.

RAW264.7 cells were used at ~80% confluence and split every 3-5 days. RAW264.7 cells were maintained in RPMI 1640 medium containing 11mM glucose supplemented with 10% heat-inactivated FCS, 100µg/ml streptomycin, 100units/ml neomycin and 100units/ml penicillin sulphate, equilibrated with 5% CO_2, 95% air at 37°C.

MIN6 cells were used at approximately 80% confluence between passages 16 to 40. MIN6 cells were grown in DMEM containing 25mM glucose supplemented with 15% heat-inactivated FCS, 100µg/ml streptomycin, 100units/ml neomycin 100units/ml penicillin sulphate, 40mM $NaHCO_3$ and 75µM β-mercaptoethanol, equilibrated with 5% CO_2, 95% air at 37°C. The medium was changed every 2-3 days.

INS-1E cells were cultured in RPMI-1640 supplemented with 5% FCS, 100µg/ml streptomycin, 100units/ml penicillin sulphate, 100units/ml neomycin, 1mM sodium pyruvate, 10mM HEPES and 50µM β-mercaptoethanol. The media was changed every 2-3 days.

2.7.3 Cell splitting

When ~80% confluence was reached, cells were washed once in 1X for 2-5min at 37°C. Cells were resuspended in DMEM as soon as they started to detach from the plate. Resuspended MIN6 and INS1-E cells were split 1:3 to 1:4 for maintenance, or as required for experiments; HEK_{293} cells were split 1:5 to 1:10 for maintenance, or as required for experiments; and RAW264.7 were split 1:3 for maintenance or as required for experiments.

2.8 Gene transduction and manipulation techniques

2.8.1 Calcium phosphate mediated DNA transfection

Cells were split to 20-30% confluence 6-10 hours prior to transfection. For a 6cm diameter plate, a solution containing 5µg of DNA (GFP tagged constructs were used, or if plasmid did not contain GFP, they were co-transfected with plasmids encoding pEGFP) and 244mM $CaCl_2$ with a final volume of 180µl was prepared. This solution was added drop-wise to 180µl 2XHBS, mixing gently upon addition. Air was bubbled thourough the DNA/$CaCl_2$/HBS mixture to break up any precipitate. The DNA/$CaCl_2$/HBS mixture was then added directly to the cells by dropping slowly and evenly into the medium. After 16-24hours of incubation at 37°C/5% CO_2, the medium

was removed and fresh medium was added to the cells. Incubation was resumed for a further 24hours prior to experimentation. Transfection efficiency was assessed by monitoring the levels of GFP expression, using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transfection was typically between 70-90% at the time of experimentation.

2.8.2 Lipofectamine mediated DNA transfection

DNA was added to opti-MEM media in the absence of serum. Simultaneously, the appropriate volume of lipofectamine was added to the same volume of Opti-MEM media in the absence of serum. These solutions were incubated for 5min at room temperature and then they were combined by adding the DNA to the lipofectamine solution. The solution was mixed gently by flicking then left at room temperature for 20min. The media was changed on the cells to opti-MEM and the complexes added to the cells. The complexes were left on the cells overnight then the media changed to complete media for another 24hours before experimentation.

Plate type	*Volume of media on cells (µl)*	*Lipofectamine volume (µl)*	*DNA (µg)*	*Media volume for incubation (µl)*
24 well	300	2	0.8	50
4cm	1000	10	4	225

2.8.3 Transduction of cell lines with recombinant adenoviruses

Adenoviral mediated transduction of cell lines was performed as follows. Growth medium was removed from cells and replaced with DMEM supplemented with antibiotics as appropriate (see below), minus FCS. High titre viral stock was added to the cells (for details see Figure legends) and incubated at 37°C/5% CO_2. The plates were rocked gently every 15min and after one hour complete medium (containing FCS) as appropriate (see below) was added to the cells, and the incubation continued for a further 24-48hours prior to experimentation. Adenoviral infection efficiency was assessed by monitoring the levels of GFP expression using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transduction was typically between 80-95% at the time of experimentation.

Plate/Flask type	*Volume of –FCS media on cells*	*Volume of +FCS media added to cells*
4cm	400µl	1.2ml
T75	3.5ml	9ml
T175	10ml	30ml

2.8.4 Electroporation mediated siRNA oligo tranfection

MIN6 cells were trypsinised as normal for cell culture, and after resuspension in standard media, spun down at 170g for 5min at room temperature. Cells were resuspended in 40ml PBS and counted using a haemocytomter. To use the haemocytomteter pipette 10ul of cells at the top of the coverslip, this should fill the space between the coverslip and the haemocytomter. The required amount of cells were respun and resuspend in 10µl solution R (MP-100 Electroporation Kit) for each well required. The resuspended cells were added to the oligo in a sterile eppendorf. For each well 20ng of siRNA oligo was required. All stocks of the specific and control siRNAs are 20µM. A 10µl electroporation gold tip was taken from the kit and 10µl of the cell/siRNA mix was placed into the apparatus ensuring no bubbles were present and electroporated at 900V for 30msec with 2 pulses. Electroporated cells were then placed in media without antibiotics overnight and an equal volume of 2x antibiotic media added the following morning. Cells were generally transfected with stealth siRNA constructs purchased from Invitrogen alongside a scrambled siRNA as a negative control, a FITC-conjugated siRNA as a marker of transfection efficiency and 2 mock conditions, mock and mock with electric current. At 72hours post-transfection the efficiency was assessed by measuring FITC signal using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transduction was typically between 85-95% at the time of experimentation.

2.9 Treatment of cell lines

Prior to treatment, the medium was removed and the cells washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (KRB buffer). The cells were then incubated for 1hour at 37°C/5% CO_2 in KRB buffer supplemented with low glucose concentrations (see Figure legends for details) subsequent to incubation in KRB buffer,

cells were further incubated in test substances for the times (details of treatments are provided in the Figure legends). When cells were treated with elevated extracellular K^+ concentration, the Na^+ concentration was decreased to maintain isotonicity. In the calcium-free experiments, after pre-incubation, the cells were incubated for 10min in a nominal calcium KRB buffer (buffered with EGTA and calculated using a fluorimeter) and stimulated in the same buffer. Treatments were stopped by either the addition of ice-cold MIN6 lysis buffer (1% Triton, 10mM β-glycerophosphate, 50mM Tris-HCl, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 1mM benzamidine HCl, 0.2mM phenylmethylsulfonyl fluoride, 1µg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol, and 50mM sodium fluoride); RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 8.0, 0.5% DOC, 0.5% SDS, 1% NP40, 1mM sodium orthovanadate, 10mM β-glycerophosphate pH 7.4, 1mM benzamidine HCl, 0.2mM phenylmethylsulfonyl fluoride, 1µg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol) or by addition of 1x Lamelli sample buffer. The cells were scraped off of 24-well, 4cm or 6cm diameter plates into a clean microfuge tube. The lysate was vortexed for 10sec if in MIN6 lysis buffer or RIPA buffer and centrifuged at 16000 x *g* at 4°C for 10min and the post nuclear supernatant was removed and transferred to a fresh microfuge tube. With samples lysed in 1x Lamelli sample buffer, lysates where sonciated for 10sec and left to settle before centrifuged at 16000 x *g* at 4°C for 10min. All cell lysates were then stored at -80°C until required.

2.10 Bradford assay

The Bradford protein assay was used to determine protein content of cell lysates. Bradford reagent (Bio-Rad) was diluted with distilled water 1/5 for use. 2µl of protein samples of unknown protein content was mixed with 1ml of diluted Bradford reagent and incubated for 5min at room temperature. The A_{595} of the reaction mixture was measured on a WPA spectrophotometer and the protein content of unknowns was determined by linear regression against a standard curve of BSA protein standards (0 to 20µg). Protein contents of individual lysates were adjusted to the sample with the lowest protein content using the appropriate lysis buffer used in the experiment.

2.11 Bacterial cell techniques

2.11.1 Bacterial strains

Strain	*Resistance*	*Supplier*
Chemically competent DH5α (Subcloning Efficiency)	N/A	Invitrogen
DH5α	N/A	NEB

2.11.2 Chemically competent cells

Competent DH5α cells were purchased from Invitrogen. 50-100µl of competent cells were thawed on ice for 5 min, and then added gently to ~ 100ng of DNA in a pre-chilled tube. The cells and DNA were mixed by gently pipetting once up and down. Cells were then incubated on ice for 30min. Cells were heat shocked at 42°C for 25-30sec, then left on ice for a further 2min. Cells which had been transformed with plasmids conferring ampicillin resistance were plated directly on to LB-agar containing ampicillin. Cells which had been transformed with plasmids allowing resistance to kanamycin were recovered by adding a single colony to 500µl of warm LB for 1hour at 37°C with constant shaking at 225rpm. Cells were then centrifuged for 1min at 3,000rpm in an Eppendorf bench top microfuge and 400µl of the LB was discarded. The cells were resuspended gently in the remaining LB and plated on to LB-agar plates containing kanamycin. Plates were incubated overnight, upside down at 37°C.

2.11.3 TAE-agarose gel electrophoresis

DNA samples were prepared by the addition of DNA loading buffer. Agarose (Melford) (0.7%-1.5%) was dissolved in 1XTAE by heating in a microwave for ~2min. Once cooled to ~60°C, 0.1µg/ml ethidium bromide was added and the gel allowed to set. DNA samples were loaded alongside either 100bp or 1kb DNA ladders (New England Biolabs) as appropriate. Agarose gels were run horizontally immersed in 1X TAE at 90 volts for ~45min or as required for resolution of bands.

2.12 DNA purification techniques

2.12.1 Plasmid DNA purification

Plasmid DNA was prepared from overnight bacterial cultures (containing appropriate antibiotics) following manufacturer's instructions. Plasmid minipreps were prepared from 5ml of overnight culture using the Genelute Plasmid Miniprep kit. Miniprep DNA was eluted in 100µl of sterile distilled H_2O. DNA maxipreps were prepared from 100ml of overnight culture using the Ultraclean Endotoxin-Free Plamid Maxi prep Kit (Qiagen) or PurElute IEX Plasmid Maxiprep kit (EdgeBio) and the DNA eluted in 1X TE. Maxiprep DNA was stored in 1X TE. All DNA minipreps/maxipreps were stored at -20°C until required.

2.12.2 Plasmid DNA purification by caesium chloride density gradients

Plasmid DNA was also purified by centrifugation thourough a caesium chloride (CsCl) density gradient. Plasmid DNA was prepared from 500ml of overnight bacterial cultures (containing appropriate antibiotics). The bacteria were then pelleted by centrifugation for 10min at 7700 x *g* at 4˚C. The pellet was then resuspended in 10ml buffer P1 (10mM EDTA, 50mM Tris-HCl pH8.0). The cells were then lysed by the addition of 20ml freshly prepared buffer P2 (0.2M NaOH, 1% SDS) followed by the addition of 15ml of buffer P3 (3M potassium acetate pH5.5). The lysate was then cleared by centrifugation for 15min at 12,000 x *g* at 4˚C. The DNA was precipitated by the addition of an equal volume of ice-cold isopropanol, followed by incubation at -20°C for 30min. The precipitated DNA was pelleted by centrifugation at 12,000 x *g* for 10min at 4°C. The DNA pellet was resuspended in a 5.5ml of TE buffer containing 6g of caesium chloride and 5mg/ml ethidium bromide, which was loaded into polyallomer tubes and subjected to ultracentrifugation at 100,000 *x g* in a TLA 100.3 rotor for 16-20hours. The ethidium bromide stained plasmid DNA bands, equilibrated within the caesium chloride density gradient after ultracentrifugation, were visualized under long wave UV light and the lower banding was removed with a 21 gauge needle on a 1ml syringe. The DNA band was collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol by adding 2.5 volumes of cold 95% ethanol. The DNA was centrifuged at 10,000 *x g* for 45min in the Sorvall GSA rotor to recover the DNA then the supernatant was gently

decanted. 80% ethanol was added then centrifuged as before, decanted, and the DNA pellet was air dried. The DNA was resuspended in 1X TE buffer and stored at -20$^{\circ C}$.

2.13 Recombinant adenoviral techniques

2.13.1 Re-amplification of high titre adenovirus stocks

90% of the adenoviral supernatant was used to re-infect 5 T-75 flasks of HEK_{293} cells. When GFP was present, viral productions were monitored by GFP expression on a Nikon fluorescence microscope fitted with a mercury lamp. Approximately 2 days post-infection, when 70-90% of the cells were floating, the cells were harvested and subjected to four cycles of freeze/thaw/vortex as described above. The virus supernatant became the high titre virus stock.

2.13.2 Harvesting virus from HEK_{293} cells

Cells were washed off the flasks, transferred to 50ml conical tubes and pelleted at 1800 x *g* for 5min at 4˚C in an Eppendorf 5810R centrifuge. The pellets were resuspended in 1ml PBS, frozen in a dry ice/ethanol bath then thawed in a 37°C water bath and vortexed. This freeze/thaw/vortex cycle was repeated 3 more times to fully lyse the cells. The samples were spun again at 3200 x *g* for 10min at 4˚C to pellet cell debris. The pellet was discarded and the resulting viral supernatant was stored at -80°C.

2.13.3 Adenovirus purification by CsCl centrifugation

The viral supernatants of selected viruses where purified using discontinuous CsCl centrifugation. When being purified by this method, the HEK_{293} cells were harvested and resuspended in 20ml DMEM containing 5% FCS before the freeze/thaw cycles. The supernatants were re-spun at centrifuged at 16,000 × *g* for 5min at 4°C to pellet any remaining cell debris.

CsCl gradients were prepared in Sorvall PA36ml tubes. 8ml of CsCl 1.4 (53g+87ml of Tris-HCl pH7.9) was slowly pipetted and then very gently overlaid with 6ml of CsCl1.2 (26.8g+92ml of Tris-HCl pH7.9). The virus supernatant was then layered on top of the gradient and the tube filled with 10mM Tris-HCl 7.9 to complete the volume if required. After ensuring the tubes were balanced they were centrifuged in a Sorvall AH629 rotor at 100,000 x g for 90min at 4°C ensuring the deceleration rate was 0. Upon removing the tubes from the buckets two bands were visible within the gradient.

The bottom band (live virus) was removed using an 18G needle and syringe carefully from the tube, not disturbing the defective particles above.

The infective particles were dialysed for 20hours at 4°C in a 3.5K slide-a-lyzer cassette into1L dialysis buffer (10mM Tris-HCl pH8, 25% glycerol, 50mM NaCl and 0.05% BSA), changing the buffer once during the incubation. Dialysed material is the infective, purified virus.

2.14 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.13.1 Gel formulation

The running gel was poured between glass plates and then immediately overlaid with distilled water. When the running gel had set (~ 30min) the water was removed and the stacking gel poured on top. The comb was inserted immediately after and the gel left to set for ~30min.

2.14.2 Running of gels

SDS-PAGE gels were immersed in SDS-PAGE running buffer in ATTO system gel tanks. Protein samples prepared were added to Laemmli sample buffer (4X) to give a 1X final. Samples were then vortexed and centrifuged at 16000 x *g* at 4˚C for 1min. Protein samples were boiled for 3min, then centrifuged briefly at full-speed and loaded alongside prestained protein markers (broad range; NEB). SDS-PAGE gels were run vertically at 180 volts for ~ 90min until the bromophenol blue just ran off the edge of the gel.

2.14.3 SDS-PAGE

SOLUTION	*7.5%*	*12.5%*	*STACKING*
40% Acrylamide (BDH)	2.925ml	4.725ml	1.24ml
2% Bis-Acrylamide[1]	1.56ml	2.52ml	0.65ml
1.5M Tris-HCl, pH 8.8	3.75ml	3.75ml	-

1M Tris-HCl, pH 6.8	-	-	1.25ml
H_2O	6.54ml	3.78ml	6.7ml
10% SDS	0.15ml	0.15ml	0.1ml
TEMED[2]	7.5µl	7.5µl	10µl
10%APS[3] (Add just before pouring)	110µl	110µl	75µl

Acrylamide: Bis acrylamide ratio of 37.5:1

1: N,N'-methylene bis-acrylamide (BDH/Bio-Rad)

2: NNN'N' tetramethylethylenediamine

3: Ammonium persulphate

2.15 Western blotting

Protein samples were run on SDS-PAGE gels as described in section 2.7.5. Proteins were transferred on to Immobilon-P PVDF filters (Millipore) using a semi-dry transfer cell. Briefly, membranes were soaked in 100% methanol to wet them. 6 Whatman 3MM papers per gel, the membrane and the gel were then equlibrated in semi-dry transfer buffer for 5min. 3 Whatman papers were placed on the cell and air bubbles removed. The membrane was then layered onto these papers. The gel was placed on top of the membrane and 3 more Whatman papers placed on top. Any air bubbles were removed and excess transfer buffer removed. The transfer was then performed at 15 volts for 38min. Transfer efficiency was assessed by transfer of the prestained protein markers.

Following transfer, the membrane was blocked in 5% milk PBST/TBST (depending on which buffer the primary antibody was diluted in) for 1hour at room temperature. Where antibodies were diluted in 5% PBST/TBST milk, the blocking step was followed by an overnight incubation at 4°C in the specific antibody. If primary antibodies were diluted in 5% BSA PBST/TBST, the membrane was washed 3 times for 5min in PBST/TBST prior to incubation. Antibody concentrations were adjusted according to manufacturer's instructions. The following day, the membrane was washed thouree

times for 5min in PBST/TBST. The membrane was then incubated in the appropriate secondary antibody (secondary antibodies from NEB was diluted 1:3000 and made in 5% milk PBST/TBST) for 1hour at room temperature. The membrane was then washed thouree times for 10min with PBST/TBST. Detection of proteins was performed by enhanced chemiluminescence. Membranes were exposed to X-ray film (GRI) for 1min to 1hour, depending on signal. Proteins of interest were identified by comparison of their size to broad range protein markers.

2.16 Ca^{2+} imaging techniques

2.16.1 Epifluorescence intracellular Ca^{2+} imaging

MIN6 cell were loaded with 2µM Fura-2-AM in dye loading buffer (KRB + 1.87µl 20% pluronic acid/ml) for 1hour at 37°C. Coverslips were mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope and left in 200µl KRB buffer as described in individual figure legends. To measure $[Ca^{2+}]_i$ the cells were excited at 340 and 380nm using a SpectraMASTER II monochouromator (PerkinElmer Life Sciences) at 1 second intervals and emissions collected at wavelengths above 520nm. All epifluorescence Ca^{2+} imaging experiments were carried out at room temperature.

2.16.2 Single cell confocal Ca^{2+} imaging

MIN6 cells were spilt 1:3 onto 25mm diameter glass coverslips and left for a minimum of 3 days before experimentation. Before imaging, the wells were washed with KRB supplemented with 2mM glucose and left for 30min at room temperature. After 30min, the KRB was replaced with dye loading solution (KRB +1mg/ml BSA +1.87µl/ml 20% pluronic acid) containing 2µM of Fluo-4 for another 30min. The cells were washed twice with KRB supplemented with 2mM glucose. All Ca^{2+} imaging experiments were carried out at room temperature.

A PERKinElmer UltraVIEW confocal microscope was used to measure the intracellular Ca^{2+} concentration. Cells were excited using the 488nm laser line and 485nm excitation filter, and the emitted fluorescence was captured at wavelengths >520nm, with images collected at approximately 2sec intervals. Data was analysed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and reported as the average fluorescence of all cells in the field of view +S.E.M.

2.16.3 NOVOstar Ca^{2+} imaging

MIN6 cells were split 1:2 into 96 well plates (200µl total volume) and left to culture for 48hours. Wells were washed with KRB supplemented with 1mM glucose twice and then placed at room temperature. After 30min the KRB was replaced with dye loading solution (KRB +1mg/ml BSA +1.87µl/1ml 20% pluronic acid) containing 2µM of Fluo-4. After an additional 30min the wells were washed twice with KRB supplemented with 2mM glucose. Cells were left with 25% of the total volume of KRB as a starting point. A well of cells without Fluo-4-AM was used as an autofluorescence control for the data analysis. For stimulation, solutions were 1.17X higher than required with 75% of the total volume added from the solution plate per well. All measurements were made at room temperature

The BMG lab technologies Novastar plate reader was set up with the following parameters.

General Settings		*Concentrations / Volumes / Shaking*	
Positioning Delay	0.2s	*Volume*	
No of Kinetic Windows	2	Start Volume	0
Kinetic Window 1		Factor	1
Measurement Start Time	0.00	*Shaking Options*	
No of Intervals	5	Mode	Orbital
No of flashes per well and interval	10	Shaking Width	1mm
Interval time	1.00s	Additional Shaking	No shaking
End of kinetic Window 1	5s	*Pump*	

Filters and Integration		Pump speed	200µl/s
Fluorescence Intensity		Shaking time	0sec
Excitation filter	485-P		
Emission filter	520-P		
Gain	1640		

2.17 Confocal imaging of single cell GFP fluorescence

Coverslips were mounted on the stage of an Olympus FV300 scanning confocal microscope and left in 200µl KRB buffer as described in individual Figure legends. Application of agonists occurred via bath addition of 1.4X required concentration. All experiments were carried out at room temperature. Increases in intracellular and plasma membrane GFP fluorescence were detected by measuring regions of interest over time in those areas. Cells were excited using the 488nm laser line and 485nm excitation filter, and the emitted fluorescence was captured at wavelengths >520nm, with images collected at approximately 2sec intervals.The average pixel intensity over time for each cytosolic or membrane region of interest were monitored and fluorescence intensity analysed using the Fluoview Tiempo software (version 4.3), then divided by the initial fluorescence and expressed as F/F_0.

2.18 FRET imaging

ER luminal Ca^{2+} was imaged using the ER-localised FRET-based D1ER cameleon [463]. MIN6 cells were transfected with 4µg D1ER DNA using Lipofectamine then experimented upon 48hours later. Cells were starved of glucose and serum for 1hour in KRB prior to treatment. Application of agonists occurred via bath addition of 1.4X required concentration. All experiments were carried out at room temperature. Images were captured with the 20X objective of a Zeiss axiovert 200M epifluorescence microscope with a CCD camera controlled by Metafluor software. Emission ratio imaging of the cameleon was accomplished by using a 436DF20 excitation filter, 450-

nm dichroic mirror, and two emission filters (475/40 for enhanced CFP and 535/25 for citrine) controlled by a Lambda 10-2 filter changer. Exposure times were typically 100–1,000 ms and images were collected every 8–20 s.

2.19 Luciferase assay

48hours post-transfection MIN6 cells containing either the pFOXLUC410 insulin promoter construct [292] or GFP, as a marker of transfection, were starved overnight (16hours) in DMEM without serum or sodium pyruvate and containing 3mM glucose and 0.1% BSA. One well transfected with pFOXLUC410 was left in full media to act as a positive control for the assay. Cells were then washed and left in KRB for 1 hour at 37°C before treatments (as described in figure legends). Cells grown in 24-well plates were lysed in 50µl 1x passive lysis buffer, then luciferase expression determined using the luciferase assay system. A ratio of 1:5 ratio of lysate to luciferase assay reagent (LAR) was used. Relative light units were captured using the NOVOstar 96-well plate reader in luminescence mode. Data were expressed either as luminescence in relative light units or as % of control.

2.20 Quantification and statistical analysis

Immunoblot band intensities were quantified using the SynGene GeneGnome System with gene tools software. All data are given as mean +S.E.M., and statistical analysis were performed using a Student's t-test, one-way or two-way ANOVA followed by either Bonferroni's post hoc test or Dunnett's range test. To assess the EC_{50} values of concentration-response experiments values were fit to a curve using non-linear regression with a variable slope. Data were regarded as significant if $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

Chapter 3: Role of the L-type VGCC in GLP-1 stimulated ERK1/2 activation

3.1 Introduction

3.1.1 Voltage gated Ca^{2+} channels (VGCC)

The ubiquitous expression of VGCCs in prokaryotes and eukaryotes, coupled with their diversification in higher life forms, demonstrates the critical nature of these channels in cellular function. VGCCs are activated by a change in voltage across the plasma membrane. VGCCs have distinct voltage thouresholds and kinetics for opening and closing. A vast array of electrophysiological, biochemical and pharmacological work has been carried out confirming that multiple different channels exist with an array of distinct properties.

Historically Ca^{2+} channels were split into two classifications; high and low voltage activated channels (HVA and LVA). However, channels are currently classified according to the pore forming units pharmacological properties, and now uses the chemical symbol of the principal permeated ion (Ca^{2+}) with the principal physiological regulator (voltage) indicated as a subscript. Isoforms are grouped with L-type being 1, P/Q-, N- and R-type being 2 and T-type being 3 and each isoform has its own individual number as an identifier. This nomenclature is illustrated in Table 3.1.

The properties and kinetics of the different VGCC subfamilies and each of the individual isoforms can be found in Table 3.2. Table 3.2 highlights the voltage change required for activation, speed of inactivation, pharmacological agonists and antagonists as well as the expression profile of each of the isoforms in each subfamily. In β-cells the L-type $Ca_v1.2$ and $Ca_v1.3$ isoforms, the T-type $Ca_v3.1$ isoform, the P/Q –type $Ca_v2.1$ isoform, the N-type $Ca_v2.2$ isoform and the R-type $Ca_v2.3$ isoform have all been demonstrated to be expressed [468].

The functional VGCC can be composed of the pore forming α-subunit alone or with additional auxiliary subunits. The β, $\alpha_2\delta$ and/or γ auxiliary subunits can alter the pore's localisation and biophysical characteristics.

Voltage Activation	Subfamily	Isoform	Current Nomenclature
HVA	L-type	1_S	$Ca_v1.1$
		1_C	$Ca_v1.2$
		1_D	$Ca_v1.3$
		1_F	$Ca_v1.4$
HVA	P/Q-type	1_A	$Ca_v2.1$
HVA	N-type	1_B	$Ca_v2.2$
HVA	R-type	1_E	$Ca_v2.3$
LVA	T-type	1_G	$Ca_v3.1$
		1_H	$Ca_v3.2$
		1_I	$Ca_v3.3$

Table 3.1. Traditional and current voltage gated Ca^{2+} channel nomenclature. VGCC isoforms are listed with their traditional classification of either high voltage activation (HVA) or low voltage activation (LVA) and their corresponding current nomenclature.

ype VGCC

ne	**1.1 / 1_S**	**1.2 / 1_c**	**1.3 / 1_D**	**1.4 /1_F**
pression Profile	Skeletal Muscle	brain, heart, jejunum, ovary, **pancreatic β-cells**, vascular smooth muscle	**pancreatic β-cells**, hippocampus, basal ganglia, habenula, thalamus	skeletal muscle, retin
ivators	Bay-K	Bay-K	Bay-K	Bay-K
ckers	Dihydropyridine antagonists, e.g. nifedipine	Dihydropyridine antagonists, e.g. nifedipine	Dihydropyridine antagonists, e.g. nifedipine (less sensitivity than other L-type channels)	Dihydropyridine an e.g. nifedipine
nctional aracteristics	High voltage-activated Slow inactivation	High voltage-activated Slow inactivation- Ca^{2+} dependent	High voltage-activated Slow inactivation- Ca^{2+} dependent	High voltage-activat Slow inactivation independent

ype VGCC

ne	**3.1 / 1_G**	**3.2 / 1_H**	**3.3 / 1_I**
pression ofile	High expression in amygdala, subthalamic nuclei, cerebellum and thalamus, moderate expression in heart; low expression in placenta, kidney, lung, colon, bone marrow, tumoral cells. Highly expressed in fetal brain and peripheral fetal tissues (heart, kidney, lung), **pancreatic β-cells**	kidney, liver, heart, brain, testis	Brain Specific
ivators			
ckers	Mibefradil, low sens. to Ni^{2+}, kurtoxins	Mibefradil, high sens. to Ni^{2+}, kurtoxin	Mibefradil, low Ni^{2+}, kurtoxin
nctional aracteristics	Low voltage-activated Fast inactivation	Low voltage-activated Fast inactivation	Low voltage-act Moderate inacti

	P/Q-type	N-type	R_type
one	**2.1 / 1_A**	**2.2 / 1_B**	**2.3 / 1_E**
pression ofile	Brain Specific; P expressed in purkinje cells; Q expressed in cerebellar granule cells, **pancreatic β-cells**	CNS, **pancreatic β-cells**	neuronal tissues, **pancreatic β-cells**
ivators	Roscovitine(?)		
ckers	ω-Agatoxin IVA/IVB; ω-Conotoxin; MVIIC	ω-Conotoxin, MVIIC	SNX482 (peptide), hi
nctional aracteristics	Moderate Voltage Activated Moderate Inactivation	High Voltage Activated Moderate Inactivation	Moderate voltage-acti Fast inactivation

ble 3.2. Key features of VGCCs.

ormation from the Swis Prot database (website info), ALEXANDER, S.P.H., MATHIE, A. & PETERS, J.A. (2007). Guide to Rece annels (GRAC), 2nd edition (2007 revision). *Br. J. Pharmacol.*, **150** (Suppl. 1), S1–S168.

3.1.1.1 α_1 subunit

The α_1 subunit of all VGCCs forms the ion conducting pore of the channel, and in a similar structural organisation to voltage-gated sodium channels, consists of twenty four transmembrane segments organised into four homologous segments of six transmembrane domains (Figure 3.1). The homologous segments have voltage sensors within transmembrane domain four, whose movement under depolarisation, presumably due to a similar core of positive residues as found in the K^+ channel, causes a conformation change that opens and closes the pore. The membrane associated extracellular loop between transmembrane 5 and 6 forms the Ca^{2+} selectivity filter and the inner pore of the channel as it loops back into the lipid bilayer as demonstrated in Figure 3.1. The intracellular loops between segments and at the C-terminus provide sites of modulation for intracellular signalling molecules as well as regulation of the pore. The regulation of the Ca_v1 subfamily is thought to be primarily thourough protein phosphorylation of the channel via second messenger kinase systems. The Ca_v2 subfamily is regulated thourough direct interactions of SNAREs and G-proteins, whilst the Ca_v3 subfamily is regulated by phosphorylation and G-proteins [469].

3.1.1.2 β subunit

Four different genes exist for the β auxiliary subunit of the VGCC. These four genes, thouree of which are alternatively spliced, generate multiple isoforms of the β-subunit. The β-subunit is cytosolic, is predicted to consist of four α-helices and contains signalling domains that interact with both the pore forming α-subunit and signalling proteins within the cytosol. These domains include an SH3 domain, a PDZ domain and the β interaction domain (BID) as well as multiple phosphorylation motifs.

The BID domain associates with affinity to a corresponding motif on the α_1 subunit named the α-interaction domain (AID). This AID sequence is localised to the I-II intracellular linker of the α subunit [470, 471] and thourough the AID-BID interaction the ER retention signal on the α-subunit is masked and a larger proportion of the VGCC can localise in the plasma membrane.

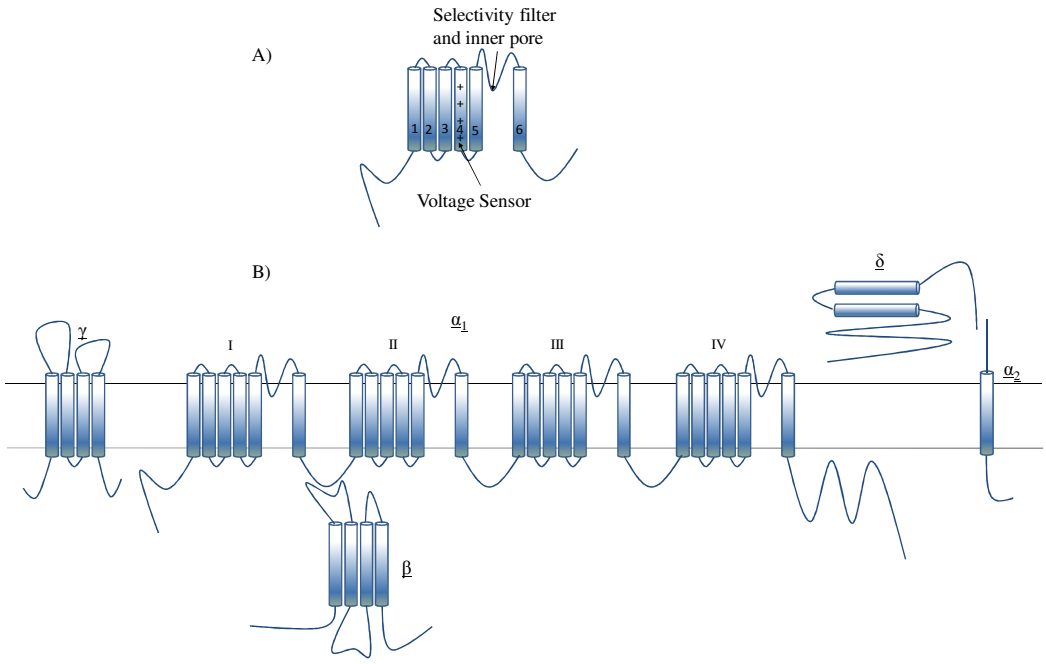


Figure 3.1. Molecular architecture of the VGCC. A) The α_1 pore forming subunit consists of 4 homologous repeats of the 6 transmembrane unit shown. S4 is the voltage sensor with positively charged amino acid residues within its structure and the extracellular loop between S5 and S6 forms the pore and selectivity filter for the channel. B) The pore forming α_1 subunit sits in the plasma membrane. The auxiliary subunits surround the pore directly interacting with it to alter its characteristics [472].

As well as being involved in the trafficking of the pore another major role of the β subunit is to modulate the α_1 subunits biophysical properties. The kinetics and voltage sensitivity have both been shown to be affected by the β-subunit. However, the multiple isoforms of the β- subunit evoke specific biophysical properties. One of these differential effects of β subunits is their effect on whole cell current density with the β_{1b} isoform having a greater effect than β_3 [473].

The presence of the β subunit also provides an additional point of modulation for the function of the pore. Grueter et al, 2006 have illustrated this showing that CaMKII phosphorylation of the β_{2a} subunit in cardiomyocytes leads to a facilitation of the L-type VGCC Ca^{2+} current, over and above that of the auxiliary subunit alone [242]. Other protein kinases, such as PKA, are also known to have phosphorylation sites in the β subunits but the functional relevance of these sites has yet to be understood.

3.1.1.3 $\alpha_2\delta$ subunit

The $\alpha_2\delta$ subunit is transcribed as a single polypeptide that is post-transcriptionally modified to give two polypeptides linked via extracellular disulphide bridges. Structure predictions suggest an extracellular α_2 subunit with the δ-subunit being membrane bound and possessing a short intracellular domain as depicted in Figure 3.1. Despite the α_2 subunit being totally extracellular it is this polypeptide rather than the membrane bound δ chain that interacts with the α_1 subunit [474].

As with the β-subunit the diversity of $\alpha_2\delta$ subunits arises from alternative splicing of a number of different genes. There are four distinct $\alpha_2\delta$ subunits (1-4) with $\alpha_2\delta$-1 being the most extensively characterised. The different isoforms have different tissue distributions with $\alpha_2\delta$-2 expression demonstrated in the pancreas [475].

The functional effects of the $\alpha_2\delta$ subunit include increasing membrane trafficking, shifting the activation and inactivation kinetics of the pore and increasing current amplitude of the VGCC. In some instances in order for the $\alpha_2\delta$ subunit to modulate the α_1 subunit the β-subunits presence is required. Additionally, the different isoforms of $\alpha_2\delta$ have different functions with the extensively characterised $\alpha_2\delta$-1 subunit having the greatest number of functions attributed to it.

3.1.1.4 γ subunit

Several different γ subunits have been identified [476-479] and consequently labelled from 1 to 8. The different isoforms of the γ subunit are predominantly expressed in neuronal and cardiac tissue, with no expression yet to be identified in the pancreas.

The predicted structure of the γ subunit includes four transmembrane domains with a PDZ-motif at the C-terminus. Interestingly, the γ subunit is the only auxiliary subunit known to traffic to the plasma membrane independently of the α_1 subunit however, the functional significance of this independent movement is currently unclear.

Unlike the β and $\alpha_2\delta$ subunits the γ subunit does not alter the trafficking of the pore but only modulates some biophysical properties of the channel, including the Ca^{2+} current [480] and activation and inactivation kinetics[481, 482].

3.1.1.5 Pathophysiology of VGCCs in diabetes

There are several accounts of changes in the expression, mutations or dysregulation of VGCCs in diabetic patients, models and cell lines. Across diabetic models there are different abnormalities in terms of VGCC function or expression but this is likely to be due to the changing role of VGCCs in the disease progression, or indeed further evidence that diabetes is a multi-faceted disease.

In β-cells isolated from streptozocin-induced diabetic (STZ) rats there is an upregulation of VGCC activity [483], but there is a significant decrease in DHP binding sites within the cells and decrease in gene expression [483, 484]. This suggests that a chouronic activation of a reduced level of VGCCs occurs in a diabetic state. In agreement, in the Goto-Kakizaki rat, which is a non-insulin-dependent model of diabetes, the activity of VGCCs was also increased [485]. However, other rodent models of diabetes display decreases in VGCC expression and activity. The Zucker diabetic fatty (ZDF) rat model of T2DM has approximately 50% reductions in the mRNA levels of L-type VGCCs and a drastic reduction in VGCC current. Furthermore, due to the lack of VGCC current, in these islets from these rats there is a severe reduction in glucose stimulated rises in Ca^{2+} and insulin secretion [486].

In humans, a subset of diabetic patients have been identified as having a gene polymorphism leading to an ATG repeat expansion, in the $Ca_v1.3$ L-type VGCC [487]. The incidence of the polymorphism is low, but could contribute to the pathogenesis of

the disease in this particular sub-group of patients. Another link to VGCC function, and diabetic onset, is the discovery that a neonatal diabetes linked locus, discovered in a family linkage analysis, maps to the position of the auxiliary β_2 subunit [488]. Although neonatal diabetes is generally thought to be transient, many of the individuals are predisposed to T2DM, and thus this polymorphism could be important in the pathophysiology of the disease for another subset of patients [489, 490].

T1DM is characterised by the destruction of β-cell mass. The use of type 1 diabetic serum on β-cells *in vitro* has demonstrated that this serum contains factors that hyper-activate VGCCs leading to Ca^{2+} overload and β-cell apoptosis [491-493]. This critical role of VGCCs in T1DM pathophysiology is illustrated by the prevention of apoptosis with L-type VGCC blockers [491]. There are several factors in the type 1 diabetic serum that could be mediating the effects on VGCCs, including Fas-specific antibodies, apolipoprotein CIII and inhibitors of G-proteins [491-493].

3.1.2 L-type VGCCs

The most recent nomenclature for the L-type VGCC have seen them classified as channels requiring a strong depolarisation for activation with a long lasting Ca^{2+} current [494]. However, there are multiple different isoforms of the L-type VGCC (Table 3.2) that convey distinct characteristics and cellular localisations to provide a multitude of functional possibilities for the cells in which they are expressed. As well as differences between the isoforms there are also many similarities in pharmacological and functional properties. All L-type VGCCs are sensitive to dihydropyridines (DHPs), although each separate isoform has its own distinct affinity, and all the L-type VGCCs are known to be sensitive to the activator Bay-K-8644 [495]. The different L-type VGCCs are expressed from 7 different genes but the isoforms can be grouped into 4 main types: $Ca_v1.1$, 1.2, 1.3 and 1.4.

3.1.2.1 $Ca_V1.1$

The $Ca_v1.1$ channel is predominantly localised to muscle cells but is expressed at low levels in other cell types. In skeletal muscle the excitation-contraction coupling mechanism between RyR1 and VGCC involves the $Ca_v1.1$ channel physically interacting with the RyR thourough its II-III loop [496] allowing CICR from internal stores [497]. The channel can also associate with other signalling molecules and has

multiple different phosphorylation sites within its C-terminus. For example, PKA phosphorylation mediates the potentiation of Ca^{2+} current thourough this $Ca_v1.1$ via an AKAP interaction [498-500]. Additionally, the C-terminal of the $Ca_v1.1$ channel can be cleaved upstream of the PKA phosphoylation site. The cleaved C-terminal remains associated with the channel [501] and can inhibit channel function dramatically [502].

3.1.2.2 $Ca_V1.2$

The $Ca_v1.2$ channel is near ubiquitously expressed in a range of different cell types including ovary, smooth muscle cells, endocrine cells, including β-cells, and specific brain areas. Due to this large expression profile this isoform is well characterised.

The full length $Ca_v1.2$ channel has been isolated from skeletal muscle and shown to be approximately 220kD, however multiple different isoforms of the channel have been isolated from as short as 160kDa [503].

The function of the $Ca_v1.2$ channel has historically been thought to be in membrane excitability, in allowing the Ca^{2+} influx into the cell in order to activate second messenger pathways. This appears to be correct in some circumstances as the channel can purely be a point of entry for Ca^{2+}. For example, in chouromaffin cells it has been shown that L-type VGCCs act merely as a conduit for Ca^{2+} entry and it is the subsequent global increase in Ca^{2+} that promotes cellular signalling [104]. However, recent work has shown that $Ca_v1.2$ VGCCs can not only provide a mechanism of entry but can, thourough interaction with signalling molecules, directly regulate cell signalling [460, 504]. The involvement of $Ca_v1.2$ in cellular signalling is discussed later (Section 3.3).

3.1.2.3 $Ca_V1.3$

The $Ca_v1.3$ channel has significantly different functional and pharmacological properties to the $Ca_v1.2$ and other L-type VGCCs [505]. For example, $Ca_v1.3$ has a lower thoureshold potential of -60 to -25mV and is less sensitive to DHPs than the other L-type VGCCs. In β-cells the majority of the L-type VGCC mRNA is of the $Ca_v1.3$ isoform despite the $Ca_v1.2$ and 1.3 channels having similar protein expression levels.

3.1.2.4 $Ca_v1.4$

$Ca_v1.4$ channels are the least well characterised of all the isoform of L-type VGCCs. They are thought to be retina specific, and display the greatest identity to the $Ca_v1.3$ isoform [484, 506]. The $Ca_v1.4$ VGCCs complexes are situated in the synaptic terminals of rod photoreceptors in the rat retina [507], and appear to be involved in the regulation of tonic glutamate release [508].

3.1.2.5 Mechanisms of inactivation

Many different experimental systems have shown that there are two mechanisms of inactivation for L-type and more specifically $Ca_v1.2$ VGCCs: Ca^{2+} dependent inactivation (CDI) and voltage dependent inactivation (VDI) [509-511]. These two mechanisms are characterised by the rate of channel inactivation, with slow inactivation which is sensitive to permeating Ca^{2+} being defined as CDI [512]. The effect of permeating Ca^{2+} in the inactivation of the $Ca_v1.2$ channels is independent of any auxiliary subunit [513], so the mechanism and therefore molecular determinants for CDI are found on the α_1 subunit itself.

There is a general agreement that there are two Ca^{2+} sensing domains (CSD) on the α_1 subunit required for CDI; the LA and IQ motifs. CaM binds to the LA motif in its Ca^{2+} independent apo-CaM isoform however, upon episodes of Ca^{2+} influx CaM binds to the IQ domain in a Ca^{2+} dependent mechanism [514-516]. CaM binding to the α-subunit appears to be a general requirement for CDI, as by mutating either the LA or IQ motifs or using a CaM_{1234} mutant with a diminished affinity for Ca^{2+}, CDI can be completely eliminated [516-519]. However the CSDs are not the single requirement for CDI as creating a chimeric channel of the T-type $Ca_v3.1$ channel with the C-terminal of the $Ca_v1.2$ channel failed to initiate CDI [520].

Mutations of four highly conserved amino acids in the S6 transmembrane region of the four homologous segments of the L-type VGCC impairs CDI independently of the CSDs [512]. This region has been described as the annular determinant of slow inactivation (ADSI) and these residues are situated at the cytoplasmic end of the pore. The hypothesis suggests that a mutual interaction between the ADSI and a CSD is required for CDI [521].

One hypothesis for the mechanism that prevents the movement of the C-terminal tail in the resting conformation, and thus allows channel opening, is the apo-CaM glue hypothesis. This hypothesis suggests that in its resting conformation the C-terminal tails of VGCCs are tethered together by the association of apo-CaM with the LA motif in the C-terminal. It is then Ca^{2+} influx thourough the channel that shifts the CaM to the IQ domain, releasing the C-terminal tails, and allowing a conformational change that moves the IQ domain and the Ca^{2+}-CaM towards its effector molecules, illustrated in Figure 3.2. Thus the CDI mechanism of switching CaM binding on the C-terminal tail upon Ca^{2+} influx also allows CaM signalling thourough the L-type VGCCs.

CDI has not been conclusively shown to occur with any other subfamily of VGCCs [512]. However, there are varied reports of CDI in both R-type and T-type channels. With CDI presumably functioning as a physiological feedback mechanism to prevent Ca^{2+} overload it is probable that a similar if not identical mechanism exists with alternative methods of Ca^{2+} entry. Indeed, CaM is found preassociated on the C-terminal domains of many of the different subfamilies [522] and the ADSI amino acids are highly conserved thouroughout the VGCC family.

Voltage dependent inactivation (VDI) of VGCCs is thought to require many of the same molecular determinants as CDI. The removal of the LA motif or mutation of CaM increases VDI rapidly [517] suggesting that the CDI mechanism of CaM interaction with the LA-motif puts a physiological brake on the natural inactivation of the channel [523]. The generally accepted theory for the mechanism of VDI involves the I-II loop of the channel and the adjacent 6^{th} transmembrane domain. Due to the observation that the removal of certain key residues in the I-II loop and the 6^{th} transmembrane domain led to the prevention of VDI it is believed that these residues provide a direct obstruction or constriction of the pore and lead to a block on Ca^{2+} influx [524-526].

The presence of CDI in only the L-type VGCCs explains the long-lasting current in this isoform as CDI by itself does not represent a true CDI but rather a Ca^{2+}-dependent brake for VDI. The long-lasting current of L-type VGCCs is therefore the physiological readout for the Ca^{2+} dependent brake on VDI.

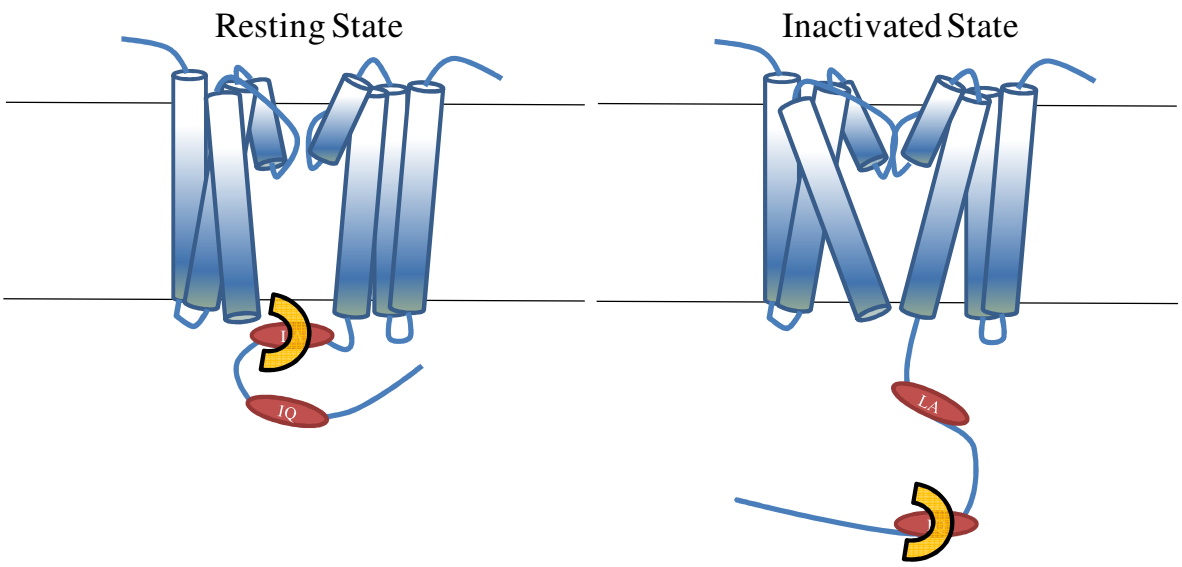


Figure 3.2. Mechanism of CDI in L-type VGCCs. The resting state of the L-type VGCC has apo-calmodulin bound to the LA motif within the pore of the channel. The position of CaM associated with the channel means that the Ca^{2+} binding sites on CaM are accessible to Ca^{2+} permeating thourough the channel. The inactivated state of the channel occurs after the removal of Ca^{2+}-bound CaM to the IQ motif and a constriction of the S6 segments which blocks the pore. The voltage-gated movement of the channel leads to the movement of the C-terminal tail with the movement of the Ca^{2+}-CaM to the IQ motif [521].

3.1.2.6 Tracking and targeting of the L-type VGCC

The insertion and removal of VGCCs from the plasma membrane can have a profound effect on the action of a particular depolarising agent. There are many proteins that have been identified to associate with L-type VGCCs to affect the trafficking and targeting of channels. However, there are also mechanisms, recently described, that suggest that the removal of activated VGCCs occurs based on agonist based signals in a similar fashion to GPCRs.

In pituitary cells it has been documented that prolonged depolarisation leads to the decrease in the number of L-type VGCC binding sites at the cell surface. Furthermore, the total number of binding sites in the whole cell lysate does not change suggesting that depolarisation mediates a removal of L-type VGCCs at the cell surface [527].

The interactions of the β and $\alpha_2\delta$ auxiliary subunits of the α_1 pore of VGCCs have been demonstrated to facilitate the trafficking of the channels to the plasma membrane [469-471]. However, a recently described interaction between the II-III loop of the $Ca_v1.2$ VGCC and eIF3e has been characterised to be important for the activity-dependent trafficking and internalisation of the channel in neurones [528].

3.1.2.7 Localisation of L-type VGCCs

The association of proteins with the L-type VGCC and their potential role in a large number of different signalling cascades, suggests that the L-type VGCC may form aggregates or signalosome complexes to mediate specific signalling. These complexes would need to be isolated to ensure only those specific signalosomes required would be activated by specific agonists. Several groups have identified this type of aggregation and isolation of L-type VGCCs.

Expression of a YFP-tagged $Ca_V1.2$ channel in HEK_{293} cells demonstrated that these channels form self-aggregating clusters of functional L-type VGCCs and that they cluster independently of the channels association with intracellular Ca^{2+} release channels [529]. Furthermore, a key sequence on the C-terminal tail of $Ca_V1.2$ has been described, that when mutated, prevented the clustering of the channel [530].

The clustering of L-type VGCCs has also been observed in arterial myocytes where persistent L-type VGCCs are active in non-uniform clusters across the cell membrane. However, the channels themselves had a uniform distribution but the observed

association with PKC led to the activation of specific clusters of channels [531]. In addition, clustering has been observed in hippocampal neuronal cultures where both $Ca_v1.2$ and $Ca_v1.3$ L-type VGCCs formed clusters on the membrane surface. Each type of L-type VGCC had various interacting partners that were not present in all signalosome complexes [532]. This suggests that not only the self-aggregation of L-type VGCCs results in a signalosome complex but their association with interacting partners affects their clustering and signalling properties.

One interaction that may help to achieve specific signalling with the generic components of the channel complex, is the association of $Ca_v1.2$ with caveolin-3, which has been demonstrated in myocytes [533] and in mouse skeletal muscle [534]. Caveolins are a marker of caveolae, which are specialised plasma membrane structures and the first type of lipid raft identified. Lipid rafts are specialised localisations of lipids, membrane bound proteins and glycoproteins that together allow for discrete and localised signalling.

3.1.3 Signalling via the L-type VGCC

Due to the ubiquitous nature of the $Ca_v1.2$ channel much of the work into the role of L-type VGCC in signalling has been studied with this isoform. $Ca_v1.2$ VGCCs have been shown to couple to multiple pathways. One way in which the channel does this is thourough its direct association with signalling molecules.

3.1.3.1 AKAP and PKA

One well characterised interaction is the association of the $Ca_v1.2$ channel with AKAPs. This interaction is known to affect the phosphorylation state of the channel, on specific cues, thourough the association of phosphatases and kinases associated with AKAP. Proteins known to interact with AKAPs include: PKA, PKC, calcineurin and CaMKII [244, 535-537]. PKA is known to phosphorylate the L-type VGCC and increase its Ca^{2+} current in HEK_{293} cells [211] as well as in neurones and ventricular myocytes [535, 538, 539]. For example, in primary neurones the membrane anchoring of PKA and calcineurin by AKAP79/150 leads to bidirectional regulation of the L-type current [535, 538].

The anchoring of PKA via AKAPs in β-cells has been shown to be required for the GLP-1 mediated increases in $[Ca^{2+}]_i$ [207]. This could be via AKAP150 as AKAP150 has been shown to be expressed and interact with PKA in β-cells [244].

3.1.3.2 CaM and CaMKII

L-type VGCC signalling to CREB and ERK1/2 has been shown to require CaM binding to the C-terminal domain of the channel. Elegant work by Dolmetsch and colleagues [460] using a nifedipine resistant $Ca_v1.2$ transiently expressed channel and mutant CaM proteins, showed that in neurones Ca^{2+} influx and CaM interaction with the IQ domain leads to a prolonged ERK1/2 and CREB activation [460]. This work suggests that $Ca_v1.2$ acts, via its association with CaM, like a plasma membrane receptor and it allows Ca^{2+}-dependent signalling to be initiated via the activation of proteins associated with the channel. The role of CaM and its binding to the CSD in the C-terminus of the L-type VGCC to mediate VGCC inactivation is discussed in Section 3.2.5. However, this mechanism is also required for signal transduction and as many isoforms of VGCCs possess prebound CaM it is conceivable that a generic mechanism exists that utilises this system for all channels to mediate Ca^{2+} signalling.

There are several reports of CaMKII being localised to, and specifically activated by, the L-type VGCC. In the denderitic spines of hippocampal pyramidal neurones CaMKII is activated, not thourough the global rise in $[Ca^{2+}]_i$, but specifically by a Ca^{2+} nanodomain surrounding the L-type VGCC [540]. In addition, in sympathetic superior cervical ganglions, the punctate staining of CaMKII at the plasma membrane requires L-type VGCC activation and indicates the phosphorylation and activation of the kinase requires L-type VGCC Ca^{2+} influx [541]. CaMKII has also been suggested to act as a local Ca^{2+} sensor for L-type VGCCs, decoding Ca^{2+} influx into signalling to the nucleus. One study suggests that Ca^{2+}-dependent activation of CaMKII leads to translocation of the kinase to the L-type VGCC and a tight coupling of the kinase and the channel [542]. CaMKII then acts as calcium spike frequency detector and is associated with the channel even after the return of global Ca^{2+} to basal levels [542].

3.1.3.3 ERK1/2

The activation and Ca^{2+} influx mediated by the L-type VGCCs is necessary for ERK1/2 activation in a number of cell types including fibroblasts [543], granulose cells [544], vascular smooth muscle cells [545], skeletal muscle cells [546] pancreatic β-cells [68,

129, 145] and neurones [460]. In neurones, the activation of ERK1/2 in response to depolarising conditions was demonstrated to require CaM pre-bound to the L-type VGCC [460]. The requirement for both L-type VGCC activity and CaM to mediate ERK1/2 activation can be seen in other cell types including pancreatic β-cells [68].

3.1.3.4 Transcription

Work has shown that in cortical neurones the $Ca_v1.2$ channel can initiate transcription alone thourough the cleavage of its C-terminal tail and its subsequent translocation into the nucleus [504]. The cleavage of the C-terminal of the channel (or the CCAT as the authors refer to it) is negatively regulated by Ca^{2+} so activation of the $Ca_v1.2$ VGCC decreases the transcriptional activity of the CCAT. The mechanism of how the C-terminus of the L-type VGCC acts as a transcription factor has also been characterised in cardiac myocytes where it auto-regulates the transcription of the L-type VGCC [547]. The cleavage and activity of $Ca_V1.2$ results in a negative feedback loop whereby the C-terminal fragment represses promoter activity and an overall decrease in long-term electrical activity. In other cell types the cleavage of the C-terminal tail of the L-type VGCC, by various different enzymes including calpain, has been observed [501, 548-550].

In addition to direct transcription by the channel, there is evidence that L-type VGCC stimulated Ca^{2+} influx can mediate Ca^{2+}-dependent transcription. In neurones, Ca^{2+} influx thourough L-type VGCCs has been shown to mediate CREB phosphorylation and CRE-dependent transcription [460, 551, 552]. In a variety of neuronal cell types the blockade of the L-type VGCC in depolarising conditions has a disproportional effect on gene transcription and global rises in $[Ca^{2+}]_i$. The blockade of the L-type VGCCs results in a small reduction in the global rise in Ca^{2+} but a large inhibitory effect on transcription is observed [552, 553]. In these studies the use of calcium chelators has also demonstrated that local Ca^{2+} signalling associated with the L-type VGCC is required for CRE-transcription [552]. The mechanism mediating CREB activation and CRE-transcription is thought to be mediated by CaM associated with the C-terminus of the L-type VGCC [460]. During L-type VGCC mediated Ca^{2+} influx CaM, which is pre-associated with the L-type VGCC at the LA motif within the C-terminal, binds Ca^{2+} and switches to a second domain within the C-terminal: the IQ domain. This translocation of CaM has been shown in neurones to be required for signalling to CREB

and CRE-dependent transcription [460]. However, other channel types constitutively bind CaM thus the presence of CaM alone cannot explain the ability of the channel to couple to CRE-dependent transcription. One proposed mechanism for mediating the specificity of the L-type VGCC is the interaction of the channel with proteins containing PDZ domains. The disruption of PDZ-dependent binding to the L-type VGCC via overexpression of the PDZ binding domain, or a mutated channel which no-long binds PDZ domains, disrupted L-type VGCC mediated CRE-dependent transcription [551].

There are a number of other Ca^{2+}-dependent transcription factors that are activated by Ca^{2+} influx thourough the L-type VGCC, including nuclear factor of activated T-cells (NFAT), and NF-κB [554-556]. The activation of a specific isoform of NFAT was found to be activated by Ca^{2+} influx via L-type VGCCs in neurones [554]. In a separate investigation the activation of NFAT was found to require the association of AKAP79/150 with the L-type VGCC [557] but other details of the mechanism underlying the activation of NFAT in this system is currently unknown [551]. In addition, L-type VGCC antagonists prevent the activation of the NFκB family members p65 and p50 after ischemic insult in the rat hippocampus [555] but again the mechanism involving the L-type VGCC in this pathway is unclear. However, the activation of NFκB-dependent transcription by depolarisation in β-cells has been demonstrated to require L-type VGCC activation and Ca^{2+} influx [556].

The distinct biophysical properties of L-type VGCC may lend this channel type to mediating electrical changes in gene transcription. The slow inactivation kinetics and the ability of a subpopulation of the L-type channel ($Ca_v1.3$) to be activated at lower membrane potentials may provide distinct cues that mediate gene expression [551, 558].

3.1.4 L-type VGCC in β-cells

The L-type VGCC is generally thought to be the most abundant and functionally relevant VGCC expressed in β-cells [559], however the relative expression and role of the L-type VGCC varies with species [448, 560-567]. Human β-cells have been shown to express L-type channels [564, 568, 569] however, the most recent investigation has shown that the $Ca_v1.3$ channel is the main L-type VGCC isoform to be expressed in human islets [564].

3.1.4.1 Physiological role of the L-type VGCC in β-cell

3.1.4.1.1 Insulin secretion

Glucose metabolism by β-cells leads to depolarisation and the activation of VGCCs in the plasma membrane. VGCCs are pivotal elements of the complex process mediating glucose and/or depolarisation stimulated insulin secretion [257]. In mouse β-cells glucose and secretagogue stimulated insulin release is almost completely abolished by DHP application [567] whereas in canine β-cells glucose stimulated insulin release is almost unaffected [566], but in β-cells of most species DHPs can block at least a proportion of secretagogue induced insulin release [448, 560-565]. Human β-cells have been shown to express L-type VGCCs that contribute to the electrical excitability and insulin secretion in response to glucose [564, 568, 569].

The importance of the L-type VGCC is illustrated in studies that perturb the functionality and expression of the $Ca_v1.2$ and $Ca_v1.3$ isoforms. Islets from β-cell selective $Ca_v1.2$ knockout mice have impaired capacitance measurements and reduced insulin exocytosis [563] demonstrating a key role for the L-type VGCC in insulin secretion. However, several lines of evidence suggest that the $Ca_v1.3$ L-type VGCC isoform has the predominate role in glucose stimulated insulin secretion in β-cells [560, 570]. In INS-1 cells $Ca_v1.3$ has been demonstrated to preferentially couple to glucose stimulated insulin secretion [560, 570]. This was demonstrated in INS-1 cells using DHP-insensitive $Ca_v1.2$ and $Ca_v1.3$ channels. Upon expression of $Ca_v1.2$ DHP-insensitive channels and simultaneous blockade of endogenous channels with nifedipine, insulin secretion was abolished. However, the expression of $Ca_v1.3$ DHP-insensitive channels in the presence of nifedipine did not significantly affect insulin secretion [570]. In addition, the $Ca_v1.3$ isoform of the L-type VGCC is less sensitive to DHP antagonism and a high concentration of DHP is required to inhibit glucose stimulated insulin secretion suggesting that $Ca_v1.3$ is mediating secretion [571-573]. However, the functional knockout of $Ca_v1.3$ isoforms of the L-type VGCC in β-cells led to no decreases in glucose stimulated insulin secretion perhaps due to a compensatory overexpression of $Ca_v1.2$ [574]. This suggests that both isoforms participate in the Ca^{2+}-response leading to glucose stimulated insulin secretion and perhaps some functional overlap exists between Ca^{2+}-dependent binding partners of the two channel isoforms.

Synaptotagmin, that binds to insulin containing vesicles, and syntaxin-1A and SNAP-25, which are involved in vesicle docking and associate with the plasma membrane, have been demonstrated to physically associate with the II-III loop of the L-type VGCC [575] in an area termed the synprint region [576]. It is believed that this association enables the vesicles docked to the L-type VGCC to be exposed to large concentrations of Ca^{2+} and fast exocytosis to occur [577]. Indeed, interruption of the interaction of the exocytosis machinery with the L-type VGCC, by the addition of a synprint peptide, causes a dramatic reduction in capacitance measurements indicating an attenuation of insulin secretion [575, 578]. This demonstrates that the L-type VGCC forms an excitatory complex at the plasma membrane and plays a pivotal role in the fast exocytosis mediated by glucose.

In β-cells L-type VGCCs are believed to generate Ca^{2+} microdomains that mediate Ca^{2+}-dependent signalling. These Ca^{2+} microdomains have a functional purpose indicated by the clustering of L-type VGCCs with large dense core vesicles [579]. The clustering of the channels and vesicles and the observation of Ca^{2+} hotspots at the plasma membrane demonstrates a role for the L-type VGCC in mediating insulin release [580, 581].

3.1.4.1.2 Insulin transcription

Glucose mediated insulin transcription has been documented to occur via several different mechanisms but several reports demonstrate a requirement for L-type VGCC activity. In dispersed rat islets glucose stimulated CAT expression under the control of a rat insulin promoter was significantly inhibited by the L-type VGCC blocker verapamil [582]. Furthermore, in rat islets glucose leads to the increase of preproinsulin mRNA levels and this could be inhibited by two chemically distinct L-type VGCC blockers [583]. In addition, several of the downstream targets of L-type VGCC mediated Ca^{2+} influx are known to mediate insulin transcription including CREB [291, 292] and MAPK [67, 143-145].

3.1.5 Aims of the chapter

GLP-1 has been shown to mediate ERK1/2 activation in a glucose dependent manner that requires Ca^{2+} influx via the L-type VGCC. However, the molecular mechanisms by which glucose and GLP-1 stimulate ERK1/2 phosphorylation in β-cells are not fully

understood. Therefore, the aim of this study was to identify the role of the L-type VGCC in mediating prolonged glucose and GLP-1 stimulated ERK1/2 phosphorylation in β-cells.

Initially this thesis will investigate the relationship between L-type VGCC mediated Ca^{2+} influx and ERK1/2 activation using depolarising concentrations of K^+ to activate the channels. Furthermore, I intend to investigate whether the temporal profile of ERK1/2 activation mediated by depolarising concentrations of K^+ is due to global levels of $[Ca^{2+}]_i$ or the specific involvement of the L-type VGCC.

To investigate the mechanisms mediating GLP-1 stimulated L-type VGCC-dependent ERK1/2 activation I intend to determine the temporal profile of GLP-1 stimulated ERK1/2 activation and rises in $[Ca^{2+}]_i$, and their dependence on the L-type VGCC. Furthermore, this thesis intends to confirm whether ERK1/2 activation is indeed Ca^{2+} dependent.

I also intend to investigate the physiological significance of L-type VGCC-dependent ERK1/2 activation by looking at the role of ERK1/2 and L-type VGCC activation in glucose and GLP-1 mediated insulin transcription.

3.2 Results

3.2.1 Characterisation of a temporal relationship between L-type VGCC-dependent increases in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation.

Previous work in β-cells has shown that Ca^{2+} entry via L-type VGCCs is necessary and sufficient [68] for the activation of ERK1/2 in response to glucose or GLP-1. In order to assess the relationship between increases in $[Ca^{2+}]_{i,}$ mediated via Ca^{2+} entry thourough L-type VGCCs and ERK1/2 activation, MIN6 cells were depolarised with increasing extracellular concentrations of K^+ and changes in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation determined. Increasing concentrations of K^+ resulted in a concentration-dependent increase in ERK1/2 phosphorylation at 5 min (Figure 3.3A) that paralleled increases in $[Ca^{2+}]_i$ (Figure 3.3B). The peak increases in $[Ca^{2+}]_i$ in response to all levels of K^+ mediated depolarisation were effectively blocked by nifedipine (Figure 3.3C). These results indicate that the magnitude of L-type VGCC dependent increases in $[Ca^{2+}]_i$ is proportional to the level of ERK1/2 phosphorylation.

To investigate the mechanism by which L-type VGCC activation leads to ERK1/2 activation we initially characterised the temporal relationship between L-type VGCC-dependent increases in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation in the β-cell line MIN6. Cells were incubated in two concentrations of depolarising K^+, 50mM potassium (K50) and 20mM potassium (K20), which are known to lead to the activation of L-type VGCCs [67, 68, 129, 145, 439]. The phosphorylation of ERK1/2 and changes in $[Ca^{2+}]_i$ were determined for up to 30 min in the presence or absence of the L-type VGCC blocker nifedipine. Incubation in both K20 and K50 induced the rapid phosphorylation of ERK1/2, which peaked at 5 min but which was still readily detectable at 30 min (Figure 3.3D and E). These increases in ERK1/2 phosphorylation were inhibited by nifedipine demonstrating, that under these conditions, activation of the L-type VGCC is necessary for ERK1/2 activation. This is in agreement with previous reports demonstrating that ERK1/2 activation induced by depolarising concentrations of K^+ require the influx of Ca^{2+} thourough L-type VGCCs in MIN6 cells [67, 68, 129, 145, 439].

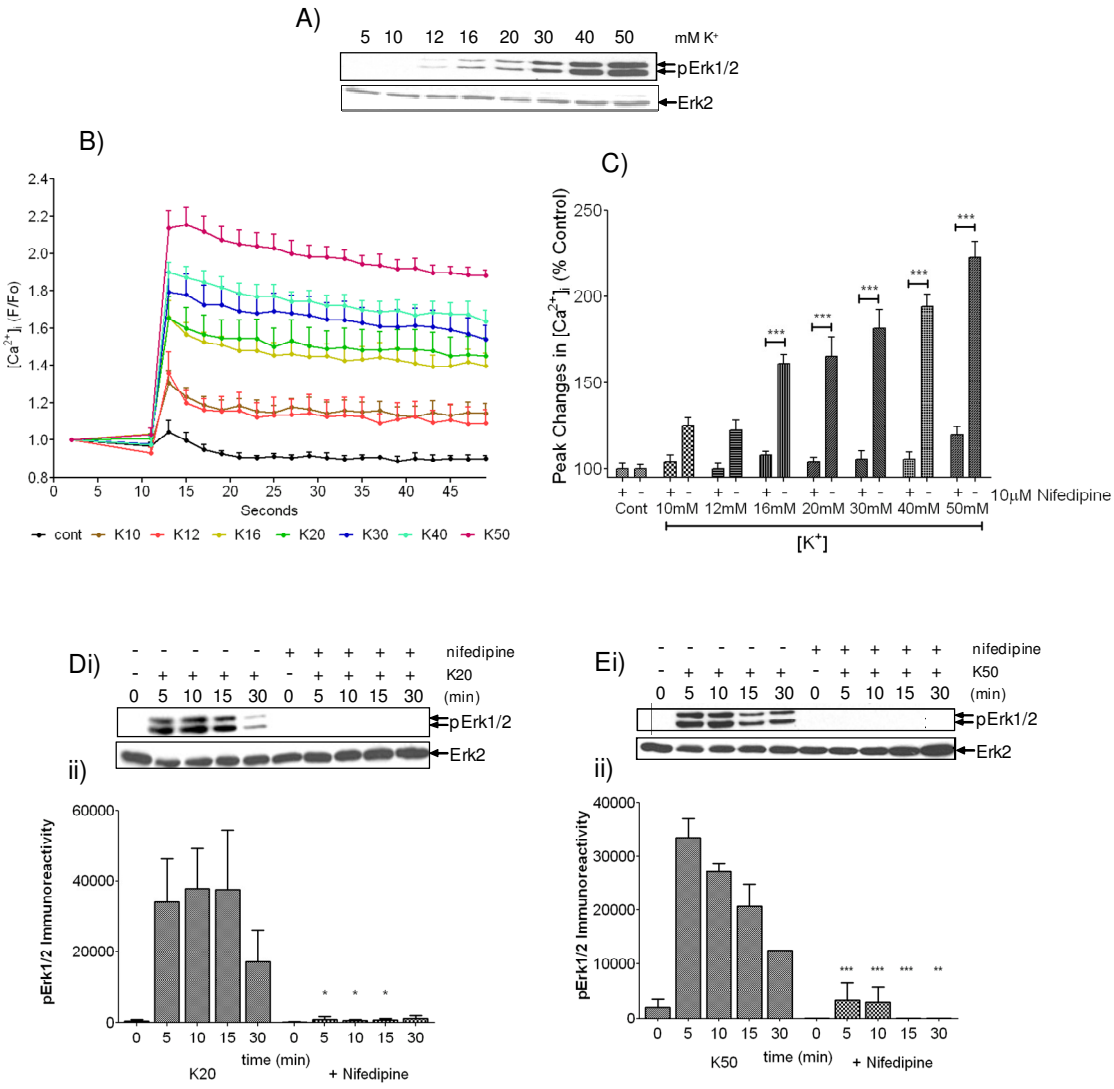


Figure 3.3. Characterisation of a temporal relationship between L-type VGCC dependent increases in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation. MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. A) i) MIN6 cells treated with indicated concentrations of K^+ in modified KRB for 5min and lysates analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown. B) MIN6 cells were loaded with 2μM Fluo-4-AM and $[Ca^{2+}]_i$ levels measured using NOVOstar Ca^{2+} imaging after treatment as in A) and then additionally in the presence of 10μM nifedipine C). Peak values as a percentage of control are plotted +S.E.M. and analysed via Student's t-test; ***, $P < 0.001$ (n=4). D and E) MIN6 cells were treated with either K20 or K50 respectively in the presence or absence of 10μM nifedipine for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry (ii) below +S.E.M. Statistical comparisons were via Student's t-test at each time point in the presence or absence of nifedipine; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=3).

3.2.2 Activation of ERK1/2 by depolarising concentrations of K^+ requires L-type VGCC activation

To establish whether the activation of the L-type VGCC is required thouroughout the sustained activation of ERK1/2 by depolarisation concentrations of K^+, MIN6 cells were treated with either K20 or K50 for up to 30 min and 10μM nifedipine applied to the cells at different times post-incubation. Nifedipine application at 0, 10 and 20 min subsequent to treatment with both depolarising concentrations of K^+ resulted in the attenuation of $[Ca^{2+}]_i$ (Figure 3.4A and C) and complete inhibition of ERK1/2 phosphorylation (Figure 3.4B and D) demonstrating that sustained L-type VGCC activation is required for sustained ERK1/2 activation upon stimulation with depolarising concentrations of K^+.

3.2.3 Global increases in $[Ca^{2+}]_i$ are not responsible for Ca^{2+}-mediated prolonged ERK1/2 activation in pancreatic β-cells

These data indicate that there is a close association between increases in $[Ca^{2+}]_i$ via L-type VGCC activation and ERK1/2 phosphorylation. Therefore, it is possible that the sustained L-type VGCC dependent phosphorylation of ERK1/2 observed upon depolarisation is due to the long-lasting current maintained by this channel and therefore its ability to maintain sustained elevations in $[Ca^{2+}]_i$ [494]. In order to investigate whether sustained increases in $[Ca^{2+}]_i$, independent of L-type VGCC activation, are sufficient for sustained ERK1/2 activation, I chouronically elevated $[Ca^{2+}]_i$ by treating the cells with ionomycin, a calcium ionophore. Although ionomycin caused a sustained elevation of $[Ca^{2+}]_i$ (Figure 3.5A), it was unable to induce the sustained activation of ERK1/2 (Figure 3.5B). However, ionomycin was able to cause the transient phosphorylation of ERK1/2 (Figure 3.5B) but the intensity of this phosphorylation was significantly less than that seen in response to K50, despite K50 inducing lower increases in $[Ca^{2+}]_i$. Together, these data provide evidence that L-type VGCC signalling to ERK1/2 occurs via a mechanism that is not solely dependent upon the sustained global increase in $[Ca^{2+}]_i$ but does require L-type VGCC activation.

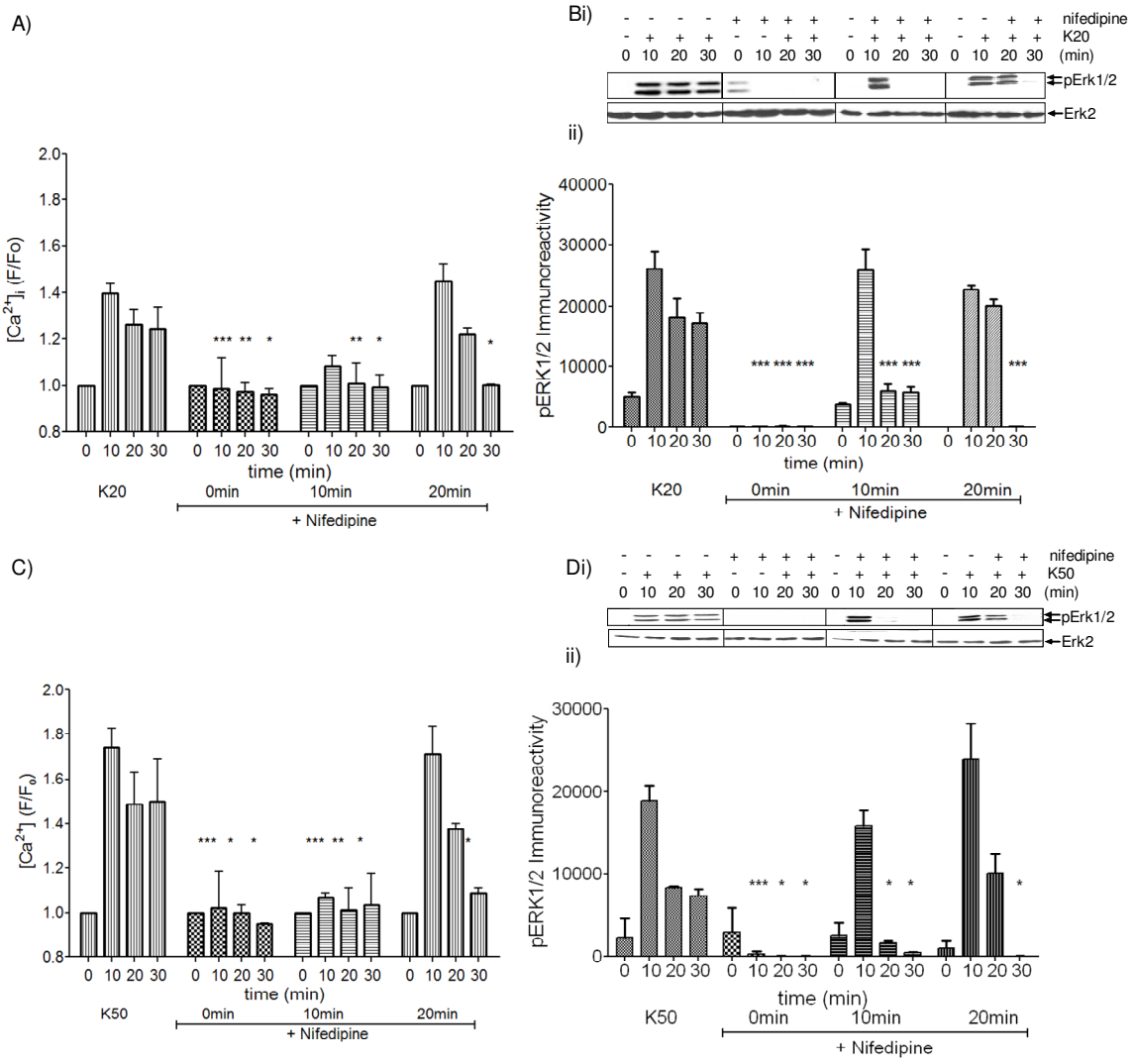


Figure 3.4. Activation of ERK1/2 by depolarising concentrations of K^+ requires L-type VGCC activation. MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. MIN6 cells were stimulated with either K20 or K50 for the times indicated and then treated with 10µM nifedipine at 0, 10, 20 and 30min post-stimulation. A and C) Cells were loaded with 2µM Fluo-4-AM and $[Ca^{2+}]_i$ levels measured using the NOVOstar.. Peak values are shown +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test, comparing K^+ alone to each treatment at matching time point; *, *P <0.05;* **, *P <0.01;* ***, $P < 0.001$ (n=3). B) and D) Cell lysates were also analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry (ii) below +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing K^+ alone to treatments at matching time points is marked on the graph, *, *P <0.05;* **, *P <0.01;* ***, $P < 0.001$ (n=3).

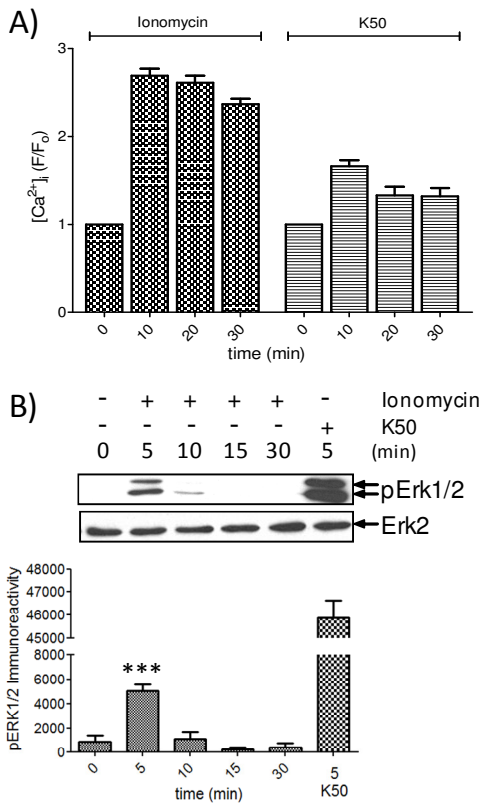


Figure 3.5. Global increases in $[Ca^{2+}]_i$ are not responsible for Ca^{2+} mediated prolonged ERK1/2 activation in pancreatic β-cells. MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. A) Cells were loaded with 2μM Fluo-4-AM and $[Ca^{2+}]_i$ levels measured using NOVOstar Ca^{2+} imaging, the cells were treated with either 100μM ionomycin or K50 for the times indicated (n=3). B) MIN6 cells were treated with 100μM ionomycin for the times indicated or K50 for 5min as reference. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M. (n=3). Statistical comparisons were via Student's t-test between K50 5min and ionomycin 5min; ***, $P < 0.001$ (n=3).

3.2.4 Coupling of the L-type VGCC to ERK1/2 phosphorylation is independent of Ca^{2+} release from intracellular stores.

This thesis has demonstrated that upon depolarisation with high extracellular K^+ that Ca^{2+} entry mediated via the L-type VGCCs is required for ERK1/2 activation. However, the increases in $[Ca^{2+}]_i$ may not be solely mediated thourough Ca^{2+} entry but by Ca^{2+} release from intracellular stores. In INS-1 cells it has been reported that an increase in $[Ca^{2+}]_i$ thourough release of intracellular stores may be the critical determinant in the activation of ERK1/2 [67, 251]. To investigate this possibility, MIN6 cells were pre-incubated with either 10μM dantrolene or 1μM ryanodine to inhibit the efflux of Ca^{2+} from intracellular stores, or 1μM thapsigargin to inhibit endoplasmic reticulum Ca^{2+}-ATPase allowing the stores to deplete. The effects of K20 or K50 on ERK1/2 phosphorylation were then investigated. Pre-treatment with the inhibitors had no significant effect on the ERK1/2 phosphorylation profiles over the 30 minute time course on stimulation with either K20 or K50 (Figure 3.6A and B). This demonstrates that Ca^{2+} release from the ER is not required for depolarising concentrations of K^+ to stimulate ERK1/2 activation in MIN6 cells.

3.2.5 GLP-1 and L-type VGCC-dependent ERK1/2 phosphorylation.

GLP-1 is a physiological agonist which signals to ERK1/2 via Ca^{2+} influx thourough L-type VGCCs in MIN6 cells [68]. In order to provide evidence that GLP-1 is mediating phosphorylation of ERK1/2 in primary β-cells a colleague, Dr Claire Moore, provided the following rat islet preparation and experiment. Rat islets were incubated with GLP-1 in the presence of glucose and in the presence or absence of 10μM nifedipine for the times indicated. Nifedipine effectively blocked ERK1/2 phosphorylation at both 10 and 30 min demonstrating a requirement of L-type VGCC activity in GLP-1 stimulated ERK1/2 activation (Figure 3.7A). In addition, MIN6 cells were incubated with glucose alone or GLP-1 in the presence glucose for 10 min to illustrate the potentiating effect of GLP-1 on glucose stimulated ERK1/2 activation in this model of β-cells (Figure 3.7B). To establish the role of the L-type VGCC in mediating ERK1/2 activation in MIN6 cells they were treated with GLP-1 for

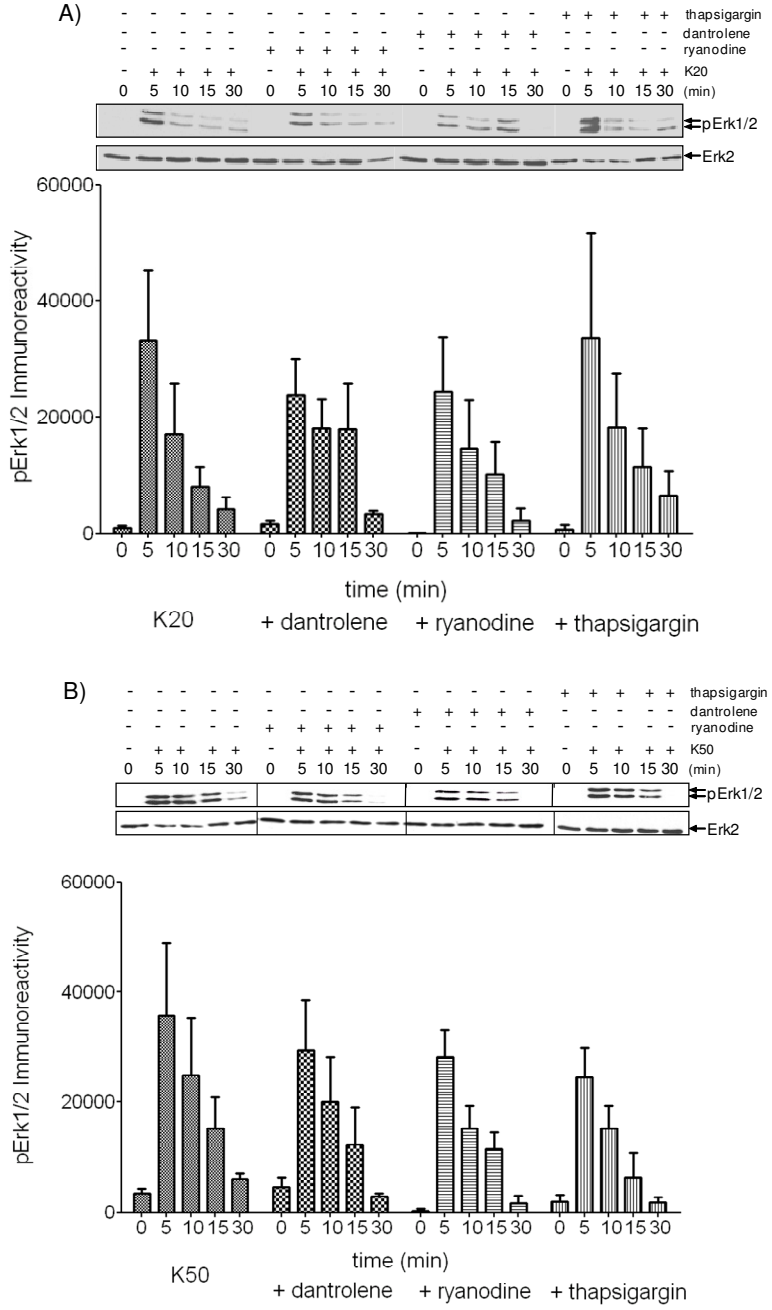


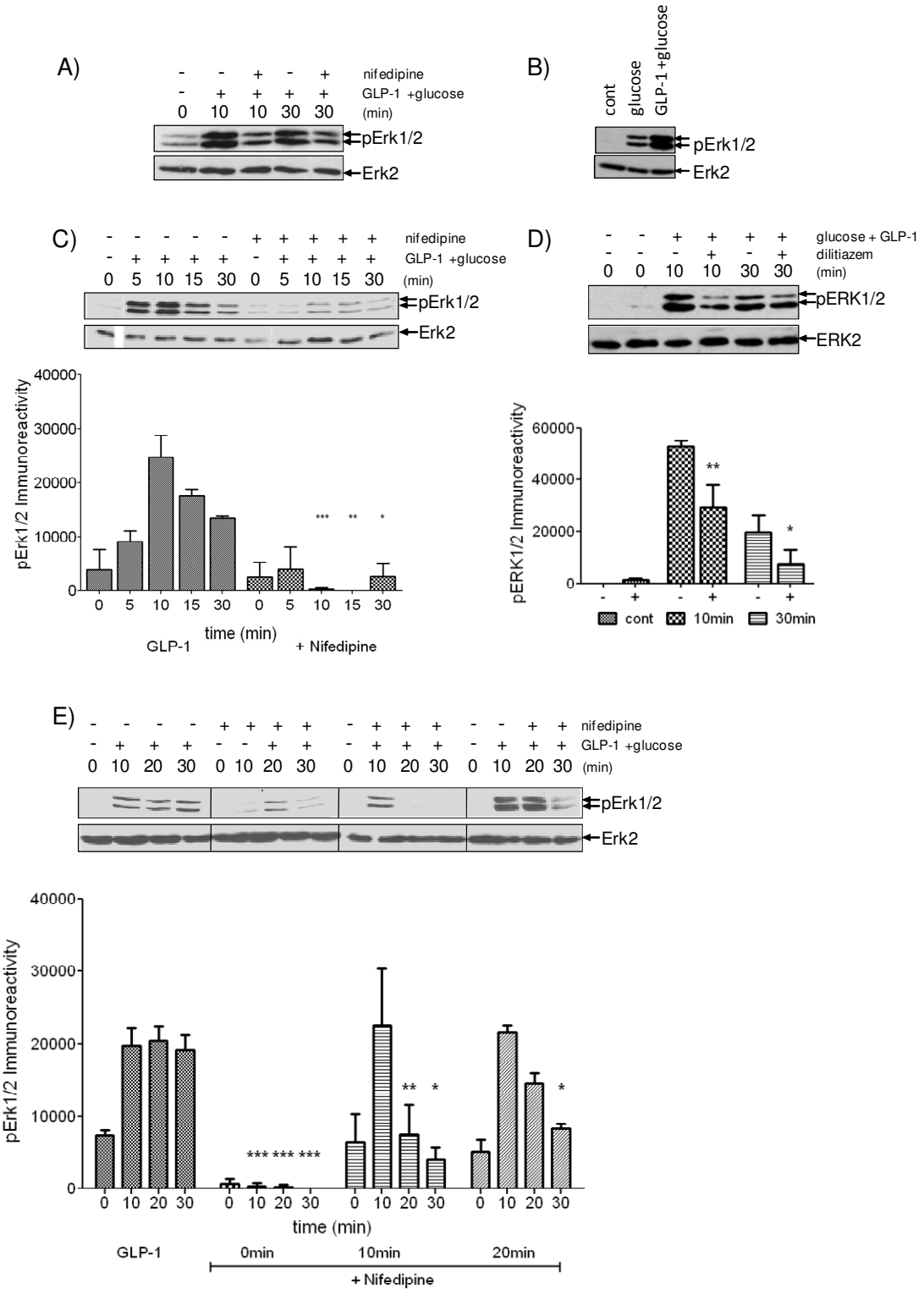
Figure 3.6. Coupling of the L-type VGCC to ERK1/2 phosphorylation is independent of Ca^{2+} release from intracellular stores. MIN6 cells were preincubated for 30min in KRB supplemented with 1mM glucose. Cells were then preincubated alone or with 1µM ryanodine, 1µM dantrolene and 1µM thapsigargin for 30min prior to treatment with A) K20 or B) K50 for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing K^+ alone to treatments at matching time points was evaluated (n=3)..

up to 30 min in the presence or absence of nifedipine and ERK1/2 phosphorylation determined by Western Blotting. GLP-1 treatment induced a rapid phosphorylation of ERK1/2 that was sustained up to 30 min and effectively inhibited by nifedipine at all time points tested (Figure 3.7C). In addition, a structurally distinct blocker of the L-type VGCC, dilitazem, was used to prevent L-type VGCC activity. Dilitazem effectively inhibited GLP-1 stimulated ERK1/2 activation (Figure 3.7D) confirming that MIN6 cells provide a good model to investigate GLP-1 signalling in β-cells.

To determine whether a sustained increase in $[Ca^{2+}]_i$ via the L-type VGCC is required for sustained ERK1/2 activation, MIN6 cells were incubated with GLP-1 for up to 30 min and 10μM nifedipine applied to the cells at different times post-incubation. Cells were treated with GLP-1 for up to 30 min and nifedipine applied to the cells at 0, 10 and 20 min following stimulation with GLP-1 (Figure 3.7E). Nifedipine application at all time points tested resulted in the complete inhibition of ERK1/2 phosphorylation demonstrating that sustained L-type VGCC activation is required for sustained ERK1/2 activation upon GLP-1 activation.

3.2.6 Coupling of the L-type VGCC to ERK1/2 phosphorylation is independent of Ca^{2+} release from intracellular stores.

We have demonstrated that upon treatment with GLP-1 that Ca^{2+} entry mediated via L-type VGCCs is required for ERK1/2 activation. However, the increases in $[Ca^{2+}]_i$ may not be solely mediated thourough Ca^{2+} entry but by Ca^{2+} release from intracellular stores. In INS-1 cells it has been reported that an increase in $[Ca^{2+}]_i$ thourough release of intracellular stores may be the critical determinant in the activation of ERK1/2 [67, 251]. To investigate this possibility, MIN6 cells were pre-incubated with either 10μM dantrolene or 1μM ryanodine to inhibit the efflux of Ca^{2+} from intracellular stores or 1μM thapsigargin to inhibit endoplasmic reticulum Ca^{2+}-ATPase and allow the stores to deplete. The effects of GLP-1 on ERK1/2 phosphorylation were then investigated. Pre-treatment with the inhibitors had no significant effect on the ERK1/2 phosphorylation profiles over the 30 minute time course on stimulation with GLP-1 (Figure 3.8). This demonstrates that Ca^{2+} release from the ER is not required for L-type VGCC signalling to ERK1/2 in MIN6 cells.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nifedipine
GLP-1 +glucose
0 10 10 30 30 (min)
pErk1/2
Erk2
B)
cont
glucose
GLP-1 +glucose
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Erk2
C)
nifedipine
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time (min)
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0min
10min
20min
+ Nifedipine

Figure 3.7. GLP-1, a physiological agonist of pancreatic β-cells, requires prolonged L-type VGCC activation to mediate ERK1/2 phosphorylation. A) Islets were preincubated in KRB supplemented with 1mM glucose for 1hour. Islets were then treated in KRB for 1hour with: 7.8mM glucose, 7.8mM glucose plus 10nM GLP-1 in the presence or absence of 10μM nifedipine as indicated. Proteins were resolved on SDS-PAGE and Western blotted using phospho-ERK1/2 and ERK2 antibodies. B) MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. Cells were stimulated with 16.7mM glucose in the presence or absence of 10nM GLP-1. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown. (n=3). C) MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. Cells were stimulated with 10nM GLP-1 plus 16.7mM glucose in the presence or absence of 10μM nifedipine for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M.. Statistical comparisons were via Student's t-test at each time point in the presence or absence of nifedipine; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=3). D) MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose. Cells were stimulated with 10nM GLP-1 plus 16.7mM glucose in the presence or absence of 50μM dilitiazem for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M.. Statistical comparisons were via Student's t-test at each time point in the presence or absence of nifedipine; *, $P < 0.05$; **, $P < 0.01$ (n=3). E) MIN6 cells were stimulated 16.7mM glucose and 10nM GLP-1 for the time course indicated then with 10μM nifedipine at 0, 10 and 20min post-stimulation. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing GLP-1 alone to treatments at matching time points is marked on the graph; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=3)..

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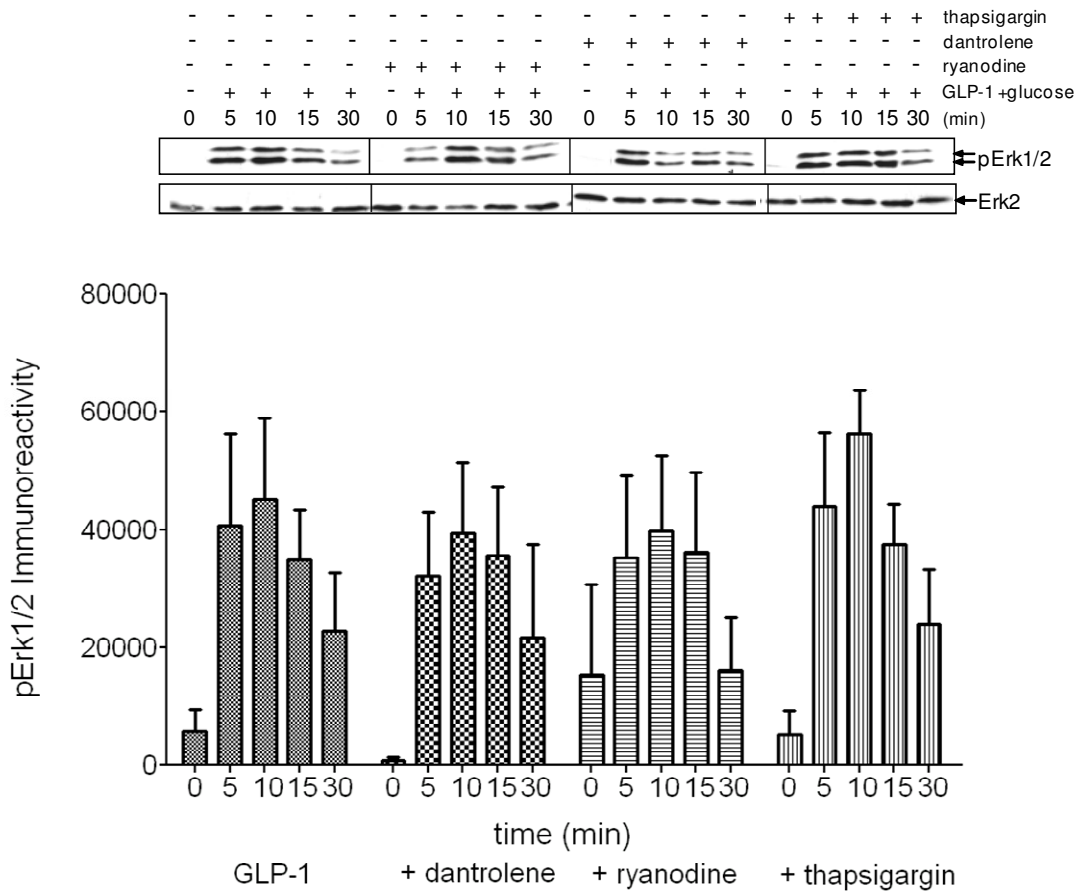


Figure 3.8. Coupling of the L-type VGCC to ERK1/2 phosphorylation is independent of Ca^{2+} release from intracellular stores. MIN6 cells were preincubated for 30min in KRB supplemented with 1mM glucose. Cells were then preincubated alone or with 1µM ryanodine, 1µM dantrolene and 1µM thapsigargin for 30min prior to treatment with 10nM GLP-1 plus 16.7mM glucose for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing GLP-1 alone to treatments at matching time was evaluated (n=3).

3.2.7 GLP-1 mediated L-type VGCC-dependent increases in $[Ca^{2+}]_i$.

In order to temporally correlate GLP-1 stimulated $[Ca^{2+}]_i$ and ERK1/2 activation, changes in $[Ca^{2+}]_i$ were assessed via single-cell epifluorescence microscopy. Application of 16.7mM glucose alone caused a rise in $[Ca^{2+}]_i$ initiating approximately 5min after application, whilst co-application of glucose and GLP-1 potentiates the magnitude and the onset of the $[Ca^{2+}]_i$ response (Figure 3.9A). This is illustrated by the area under the curve being significantly potentiated across the 30 min of GLP-1 treatment. Furthermore, at each individual time point GLP-1 can be seen to potentiate glucose mediated rises in $[Ca^{2}]_i$ and indeed, co-application of glucose and GLP-1 initiated rises in $[Ca^{2+}]_i$ before glucose alone (Figure 3.9B).

To confirm that L-type VGCCs participate in the $[Ca^{2+}]_i$ response to GLP-1 in MIN6 cells 10μM nifedipine was applied prior to stimulation with GLP-1 in the presence of glucose. Nifedipine application led to a significant attenuation in the $[Ca^{2+}]_i$ in response to GLP-1 stimulation across the 30 min (Figure 3.9A and C). These data indicate that L-type VGCCs are activated in response to GLP-1 stimulation and form the majority of the contribution to GLP-1 stimulated rises in $[Ca^{2+}]_i$.

3.2.8 Local Ca^{2+} influx within the microdomain of the L-type VGCC mediates GLP-1-stimulated prolonged ERK1/2 phosphorylation.

Our data indicate that there is an association between GLP-1 stimulated increases in $[Ca^{2+}]_i$ and the magnitude of ERK1/2 phosphorylation and that L-type VGCC activation is required for sustained ERK1/2 phosphorylation. Due to the involvement of the L-type VGCC it is possible that a local increase in $[Ca^{2+}]_i$ within the vicinity of the channel is important in the channel's ability to couple to the ERK1/2 signalling pathway. To assess this we used the Ca^{2+} chelators BAPTA and EGTA loaded into the cells in their AM forms. These chelators have different kinetics of Ca^{2+} binding with BAPTA binding Ca^{2+} at a faster rate than EGTA. Thus although both BAPTA and EGTA can prevent global Ca^{2+} signalling, BAPTA is more effective at preventing local increases in $[Ca^{2+}]_i$. These chelators can therefore reveal information regarding the spatial dynamics of Ca^{2+} signalling [552, 584-587].

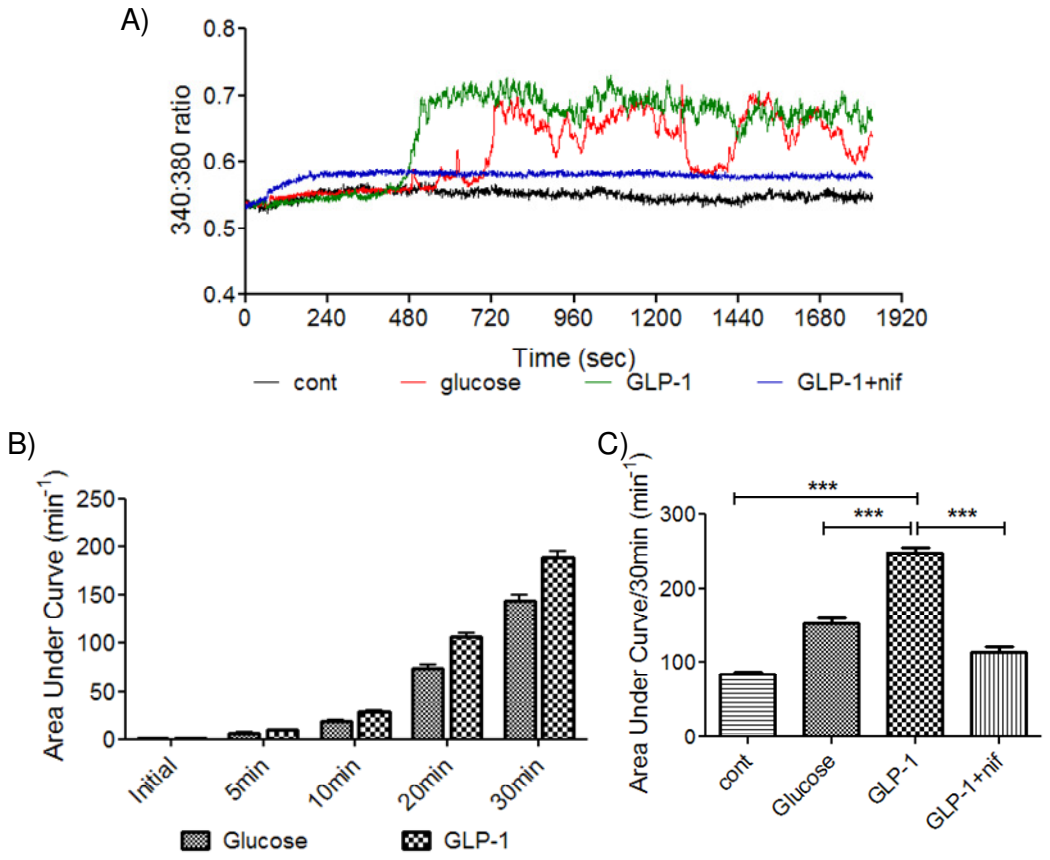
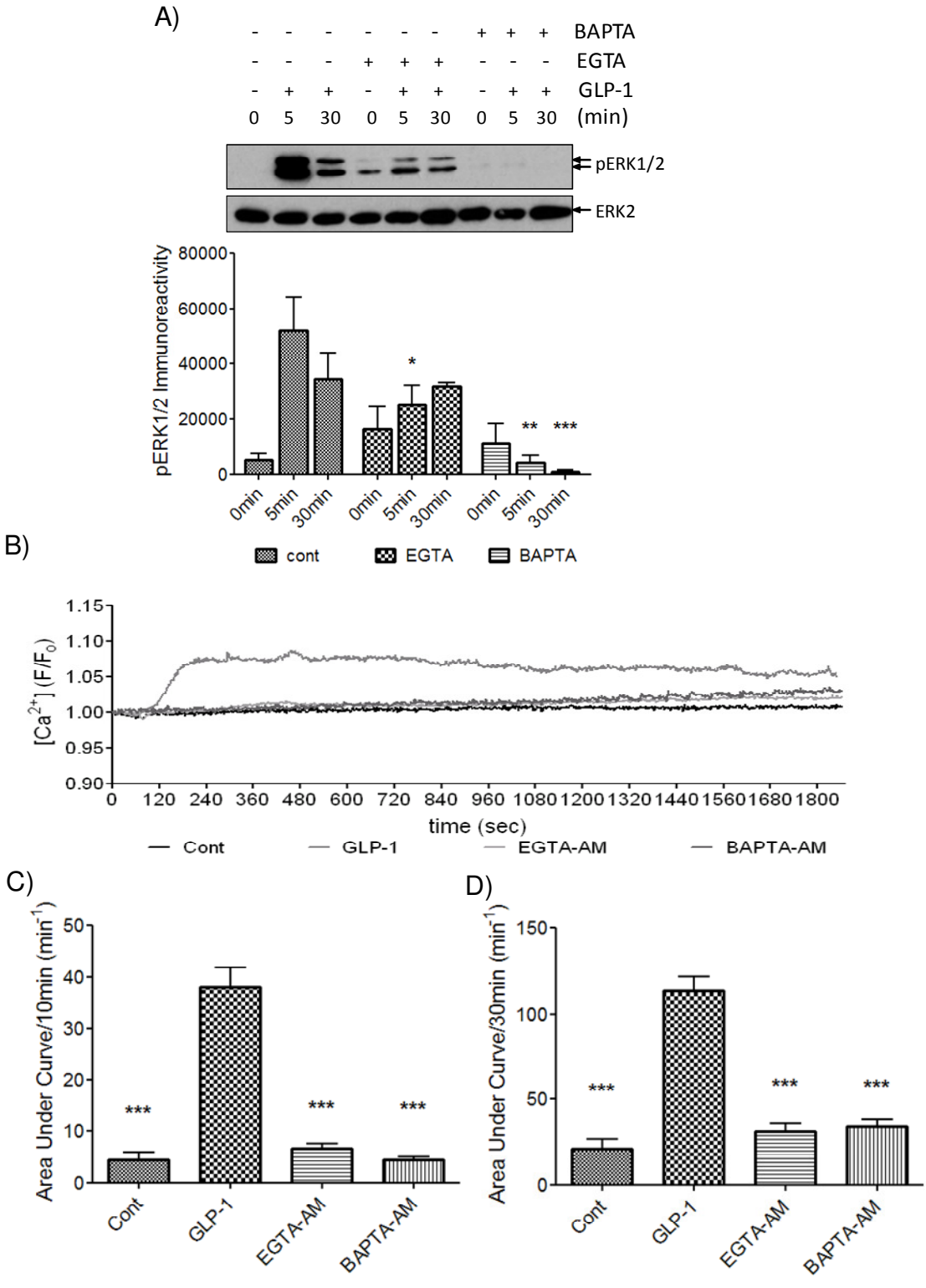


Figure 3.9. GLP-1, a physiological agonist of pancreatic β-cells, mediates sustained increases in global $[Ca^{2+}]_i$. A) MIN6 cells were loaded with 2μM Fura-2-AM and $[Ca^{2+}]_i$ levels measured using epifluorescence microscopy. Representative traces from single cells treated with either KRB, 16.7mM glucose, 16.7mM glucose plus 10nM GLP-1, or 16.7mM glucose plus 10nM GLP-1 and 10mM nifedipine. B) All cells on coverslips treated with either 16.7mM glucose or glucose plus 10nM GLP-1 where analysed at different time points across the 30min stimulation via area under the curve +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to glucose alone; ***, $P < 0.001$ (n>30). C) Analysis of area under the curve across the 30min treatments as A). Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to GLP-1 alone; ***, $P < 0.001$ (n>30).


A)
BAPTA
EGTA
GLP-1
(min)
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ERK2
pERK1/2 Immunoreactivity
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EGTA
BAPTA
B)
[Ca2+] (F/F0)
time (sec)
Cont
GLP-1
EGTA-AM
BAPTA-AM
C)
Area Under Curve/10min (min-1)
D)
Area Under Curve/30min (min-1)

Figure 3.10. Local calcium influx within the microdomain of the L-type VGCC mediates GLP-1 stimulated prolonged ERK1/2 phosphorylation. MIN6 cells were preincubated for 1hour alone or in the presence of 100µM EGTA-AM or BAPTA-AM in loading buffer at room temperature. A) MIN6 cells were treated with 10nM GLP-1 plus 16.7mM glucose at room temperature for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M.. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing GLP-1 alone to treatments at matching time points is marked on the graph; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, (n=4). For epifluorescence imaging MIN6 cells were loaded simultaneously with 2µM Fura-2-AM and EGTA-AM or BAPTA-AM and left to equilibrate for 10min before being stimulated at room temperature with 10nM GLP-1 plus 16.7mM glucose. B) Data shown represents mean increase in $[Ca^{2+}]_i$ for each treatment indicated. All cells on coverslips treated as in B) were analysed across the C) 10min and D) 30min stimulation via area under the curve +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to GLP-1 alone; ***, $P < 0.001$ (n>30).

MIN6 cells were incubated with BAPTA-AM or EGTA-AM prior to GLP-1 stimulation. ERK1/2 phosphorylation elicited by GLP-1 was abolished following incubation with BAPTA, whilst EGTA significantly attenuated ERK1/2 phosphorylation at 10 min whilst having no significant effect at 30 min (Figure 3.10A). However, both chelators prevented GLP-1 stimulated global increases in $[Ca^{2+}]_i$ (Figure 3.10B). This demonstrates that there are two phases of GLP-1 stimulated ERK1/2 activation mediated by two distinct Ca^{2+} dependent mechanisms. The initial phase of GLP-1 stimulated ERK1/2 activation (10 min) is dependent on global rises in Ca^{2+}, as it is EGTA sensitive, whilst the prolonged phase of ERK1/2 activation (30 min) occurs via a Ca^{2+}-dependent mechanism, within 20nm of the site of Ca^{2+} entry (i.e. the L-type VGCC).

3.2.9 Investigation into whether local Ca^{2+} influx within the microdomain of the L-type VGCC mediates K50 induced ERK1/2 phosphorylation

Due to the evidence that GLP-1 mediates ERK1/2 activation via Ca^{2+}-dependent signalling within the microdomain of the L-type VGCC, we investigated whether this mechanism mediates ERK1/2 activation upon stimulation with depolarising concentrations of K^+. MIN6 cells were loaded with concentrations of EGTA-AM and BAPTA-AM that inhibited GLP-1 stimulated rises in $[Ca^{2+}]_i$, prior to stimulation with K50.

The preincubation of MIN6 cells with BAPTA-AM abolished K50 mediated increases in $[Ca^{2+}]_i$ (Figure 3.11A), confirming its presence and buffering capacity within the cells. However, preincubation with EGTA-AM failed to inhibit the rises in $[Ca^{2+}]_i$ (Figure 3.11A) indicating that the buffering capacity of the chelator is not sufficient for this stimuli. Investigation of the $[Ca^{2+}]_i$ response in single cells indicated that the initial response of GLP-1 was blunted, but by 500sec there was no appreciable difference between control and EGTA loaded cells (Figure 3.11B). However, at 5 min post-stimulation with K50 a significant difference between cont and EGTA-AM loaded cells exists (Figure 3.1C). To investigate whether this buffering capacity at 5 min leads to any significant change in ERK1/2 phosphorylation, MIN6 cells were preincubated with

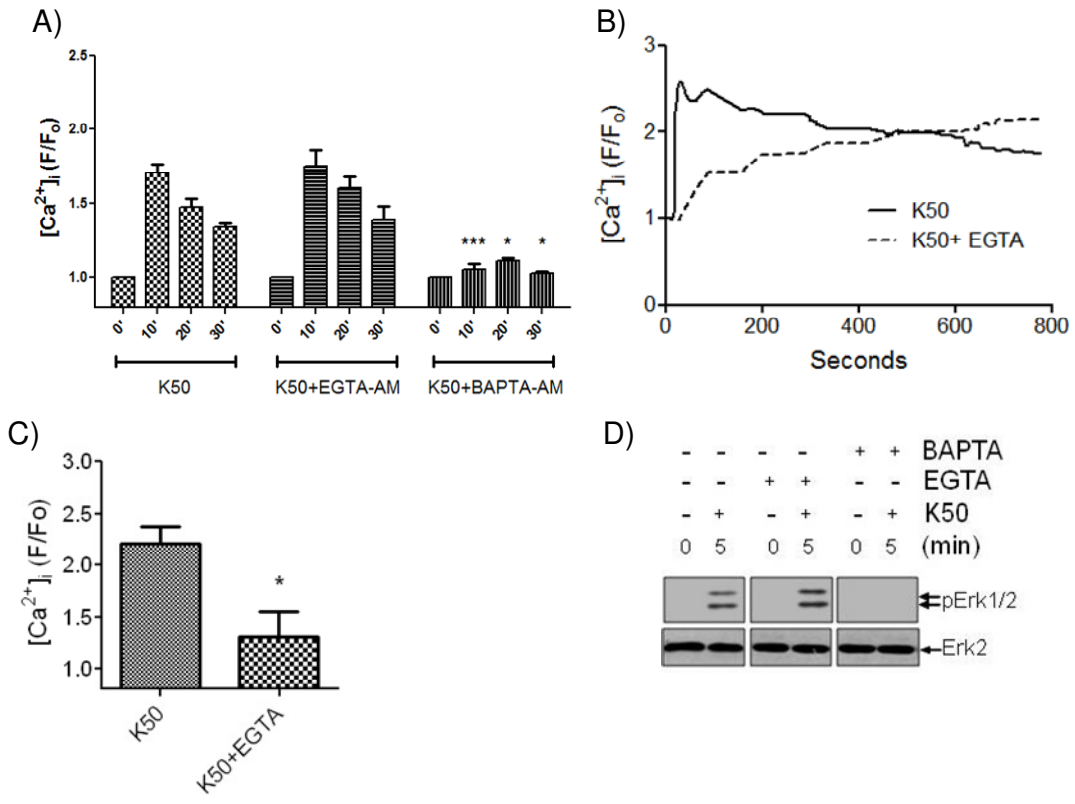


Figure 3.11. Evidence that local Ca^{2+} influx within the microdomain of the L-type VGCC mediates depolarising K^+ induced ERK1/2 phosphorylation. MIN6 cells were preincubated for 30min in KRB, then loaded with either 100µM EGTA-AM or BAPTA-AM in loading buffer at 37°C for 30min. A) Cells were loaded with 2µM Fluo-4-AM and $[Ca^{2+}]_i$ levels measured using the NOVOstar, the cells were treated with K50 alone or in the presence of intracellular EGTA or BAPTA for the times indicated. Data are Ca^{2+} level +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing K20 alone to treatments at matching time points is marked on the graph; *, $P < 0.05$; ***, $P < 0.001$, (n=3). .B) MIN6 cells were loaded with 2µM Fluo-4-AM, stimulated with K50 and $[Ca^{2+}]_i$ levels measured using single cell epifluorescent microscopy. Cells were treated with K50 alone or in the presence of intracellular EGTA (n=3). C) $[Ca^{2+}]_i$ levels at 5min post-stimulation with K50 alone or in the presence of intracellular EGTA taken from C). Statistical comparisons were via Student's t-test; *, $P < 0.05$, (n=3). .D) MIN6 cells were treated with K50 for 5min. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown (n=4).

EGTA-AM and ERK1/2 phosphorylation assessed at this time point. The presence of intracellular EGTA had no effect on ERK1/2 phosphorylation (Figure 3.11D) despite the buffering capacity of the chelator at this time point. Furthermore, the preincubation of BAPTA-AM abolished ERK1/2 activation (Figure 3.11D) confirming that K50 mediates the activation of ERK1/2 via a Ca^{2+}-dependent process.

3.2.10 Investigation into whether local Ca^{2+} influx within the microdomain of the L-type VGCC mediates K20 induced ERK1/2 phosphorylation.

Due to the evidence that EGTA-AM loaded MIN6 cells have not got the buffering capacity to prevent K50 stimulated rises in $[Ca^{2+}]_i$, a weaker depolarising stimulus of K20 was evaluated. MIN6 cells were loaded with 100μM EGTA-AM and BAPTA-AM, concentrations that inhibited GLP-1 stimulated rises in $[Ca^{2+}]_i$, prior to stimulation with K20.

The preincubation of MIN6 cells with BAPTA-AM abolished K20 mediated increases in $[Ca^{2+}]_i$ (Figure 3.12A) confirming its presence and buffering capacity within the cells. However, similarly to K50, preincubation with EGTA-AM failed to inhibit the rises in $[Ca^{2+}]_i$ mediated by K20 (Figure 3.12A) indicating that the buffering capacity of the chelator is not sufficient for this stimulus. Investigation of the $[Ca^{2+}]_i$ response in single cells indicated that, like with K50, the initial response was blunted with comparable levels of $[Ca^{2+}]_i$ between control and EGTA loaded cells after 500secs of stimulation (Figure 3.12B). However, at 5 min post-stimulation a significant difference between K20 and EGTA-AM loaded cells exists (Figure 3.12C).

To investigate whether this buffering capacity at 5 min leads to any significant change in ERK1/2 phosphorylation upon K20 stimulation MIN6 cells were preincubated with EGTA-AM and ERK1/2 phosphorylation assessed. The presence of intracellular EGTA had no effect on ERK1/2 phosphorylation (Figure 3.12D) despite the buffering capacity of the chelator at this time point. Furthermore, the preincubation of BAPTA-AM abolished ERK1/2 activation (Figure 3.12D) confirming that K20 mediates the activation of ERK1/2 via a Ca^{2+}-dependent process.

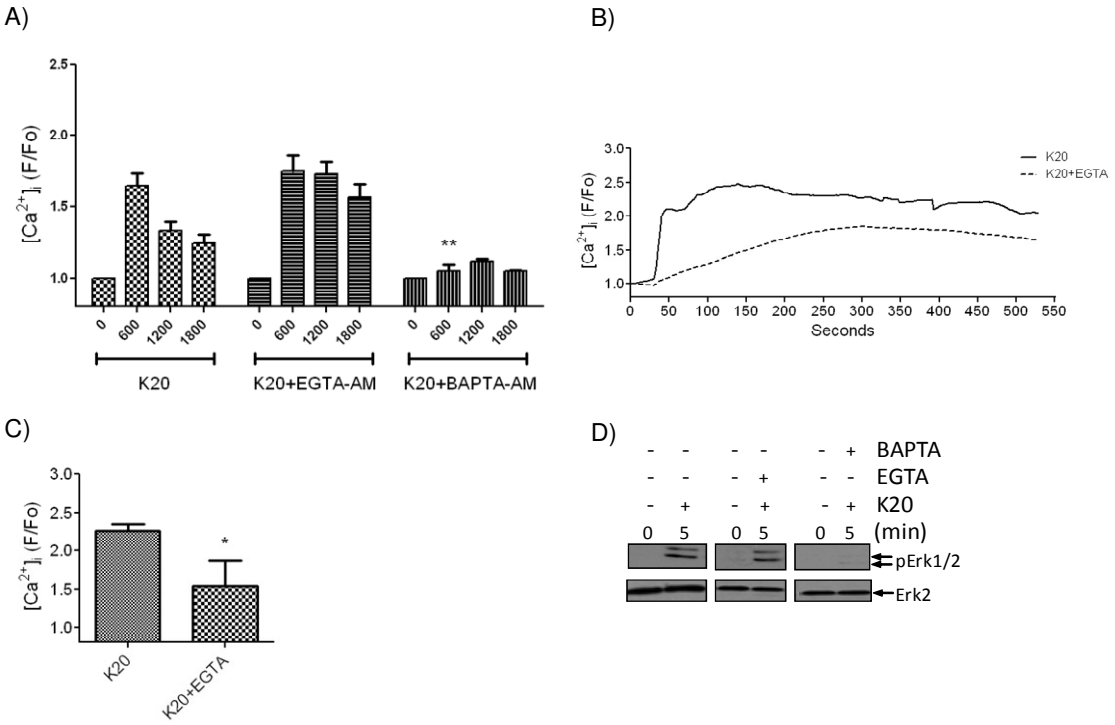


Figure 3.12. Evidence that Local Ca^{2+} Influx within the microdomain of the L-type VGCC mediates depolarising K^+ induced ERK1/2 phosphorylation. MIN6 cells were preincubated for 30min in KRB, then loaded with either 100µM EGTA-AM or BAPTA-AM in loading buffer at 37°C for 30min. A) Cells were loaded with 2µM Fluo-4-AM and $[Ca^{2+}]_i$ levels measured using NOVOstar Ca^{2+} imaging, the cells were treated with K20 alone or in the presence of intracellular EGTA or BAPTA for the times indicated. Data are Ca^{2+} level +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing K20 alone to treatments at matching time points is marked on the graph; **, $P < 0.01$, (n=3). .B) MIN6 cells were loaded with 2µM Fluo-4-AM, stimulated with K20 and $[Ca^{2+}]_i$ levels measured using single cell epifluorescent microscopy. Cells were treated with K50 alone or in the presence of intracellular EGTA (n=3). C) $[Ca^{2+}]_i$ levels at 5min post-stimulation with K20 alone or in the presence of intracellular EGTA taken from C). Statistical comparisons were via Student's t-test; *, $P < 0.05$, (n=3). .D) MIN6 cells were treated with K20 for 5min. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown (n=4).

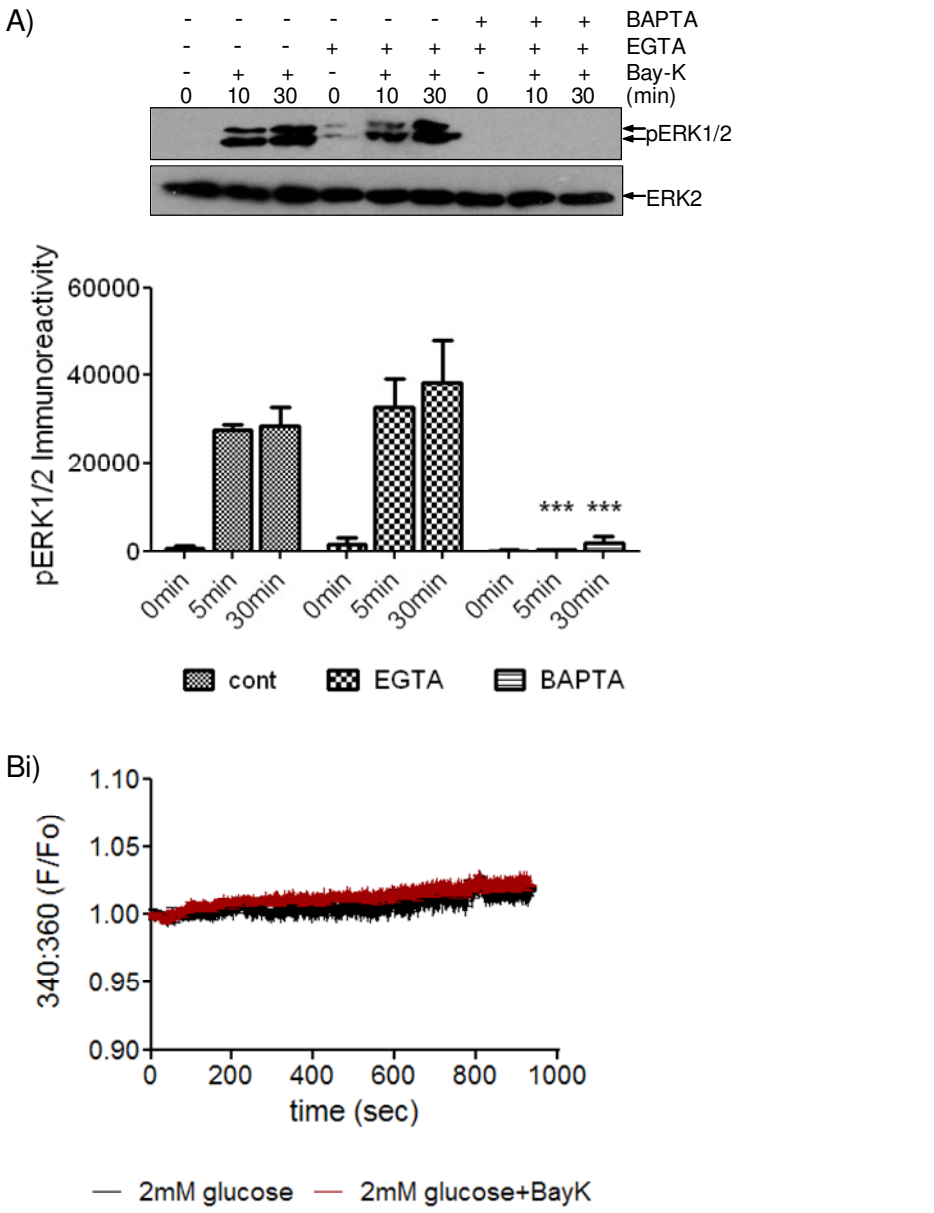


Figure 3.13. L-type channel activation is sufficient to mediate sustained ERK1/2 phosphorylation in MIN6 cells via local Ca^{2+} signalling. MIN6 cells were preincubated for 1hour in loading buffer with 2mM glucose alone or in the presence of 100µM EGTA-AM or BAPTA-AM in loading buffer at room temperature. A) MIN6 cells were treated with 10µM Bay-K-8644 at room temperature for the times indicated. Cell lysates were analysed by SDS-PAGE and Western blotting using phospho-ERK1/2 and ERK2 antibodies. A representative blot is shown with densitometry below +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing Bay-K-8644 alone to treatments at matching time points is marked on the graph; ***, $P < 0.001$ (n=4). B) MIN6 cells were loaded with 2µM Fura-2-AM, treated with 10µM Bay-K-8644 and $[Ca^{2+}]_i$ levels measured using epifluorescence microscopy (n>30).

3.2.11 Bay-K8644, an artificial activator of the L-type VGCC, mediates ERK1/2 activation via local rises in $[Ca^{2+}]_i$

Bay-K8644 is an artificial activator of the L-type VGCC that shifts the voltage-dependent activation of the channel to the left by approximately 10mV. To assess whether Ca^{2+} influx via L-type VGCCs is sufficient to mediate ERK1/2 activation, as previously reported [68], and whether this ERK1/2 activation requires local Ca^{2+} signalling, I again utilized EGTA-AM and BAPTA-AM.

Bay-K8644 stimulated sustained ERK1/2 activation (Figure 3.13A) for up to 30 min. The presence of intracellularly loaded BAPTA prevented ERK1/2 activation confirming the Ca^{2+}-dependence of L-type VGCC signalling to ERK1/2, despite no global changes in $[Ca^{2+}]_i$ being observed upon stimulation with Bay-K8644 (Figure 3.13B). However, unlike upon GLP-1 stimulation, in the presence of intracellular EGTA there is no effect on Bay-K8644 stimulated ERK1/2 activation. This demonstrates that L-type VGCC mediated ERK1/2 activation in MIN6 cells requires local Ca^{2+} signalling within the microdomain of the channel.

3.2.12 GLP-1 but not glucose, stimulates ERK1/2 independent CREB phosphorylation in MIN6 cells

In order to demonstrate a physiological role for the local Ca^{2+}-mediated ERK1/2 activation I looked at proteins downstream of ERK1/2. The transcription factor CREB has been shown to be activated downstream of ERK1/2 in β-cells in response to a variety of stimuli [142, 152, 153, 421, 588]. To see if CREB is activated downstream of ERK1/2 in response to GLP-1 stimulation, MIN6 cells were incubated with glucose alone or GLP-1 in the presence of glucose for up to 30 min and the phosphorylation of CREB and ERK1/2 assessed by Western Blotting. Despite ERK1/2 phosphorylation in both conditions, CREB phosphorylation at S133 was only stimulated by GLP-1 (Figure 3.14A). This suggests that CREB is being activated by a signalling cascade only activated by GLP-1, and as we have demonstrated that Ca^{2+}-dependent glucose mediated ERK1/2 activation is potentiated by GLP-1 this suggests that CREB is not downstream of ERK1/2. To test this hypothesis we treated MIN6 cells with two different MEK inhibitors PD098059 and UO126, prior to stimulation with GLP-1, and

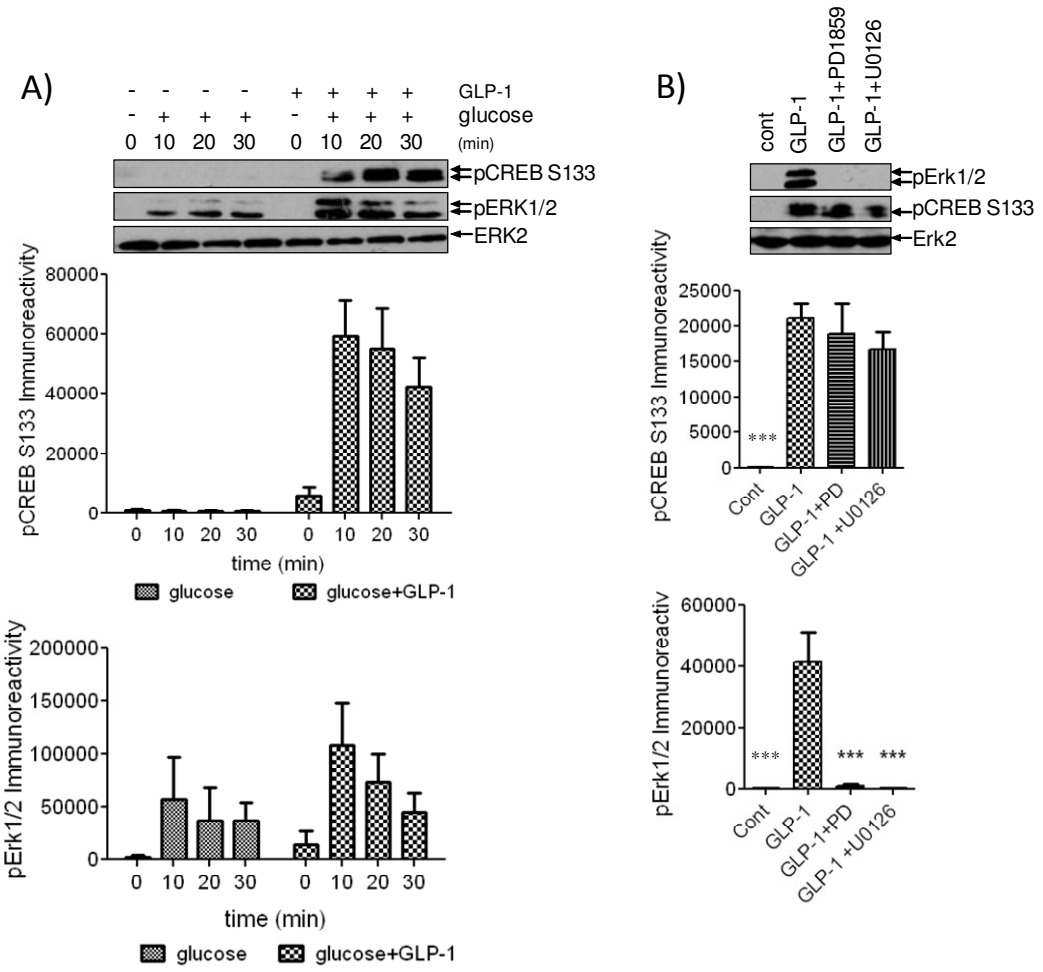


Figure 3.14. GLP-1 but not glucose, stimulates ERK1/2 independent CREB phosphorylation in MIN6 cells. A) MIN6 cells preincubated for 1hour with KRB supplemented with 1mM glucose were treated with 16.7mM glucose in the presence or absence of 10nM GLP-1 for the times stated. ERK1/2 and CREB phosphorylation was assessed by Western blotting using phospho-specific antibodies. A representative blot is shown above with densitometry below +S.E.M. (n=3). B) MIN6 cells preincubated for 1hour in KRB supplemented with 1mM glucose and then treated with 10nM GLP-1 with 16.7mM glucose for 10min. Cells were pre-treated alone or in the presence of 1μM PD098059 or 20μM U0126 for 30min. ERK1/2 and CREB phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to GLP-1 alone; ***, $P < 0.001$ (n=6).

assessed ERK1/2 and CREB phosphorylation. As expected, the MEK inhibitors abolished GLP-1 stimulated ERK1/2 activation but failed to attenuate CREB phosphorylation (Figure 3.14B), thus confirming that CREB activity is not downstream of ERK1/2 activation in MIN6 cells.

3.2.13 GLP-1 does not require prolonged L-type VGCC activity to stimulate CREB phosphorylation.

As in neurones the activity of the L-type VGCC is required to mediate CREB activation [460], I assessed the role of the channel in CREB activation in β-cells. To determine whether the L-type VGCC was involved in GLP-1 mediated CREB phosphorylation I used nifedipine to block channel activity. MIN6 cells were pre-treated with nifedipine, stimulated with GLP-1 and CREB and ERK1/2 phosphorylation assessed. The preincubation of nifedipine inhibited GLP-1 stimulated CREB and ERK1/2 phosphorylation (Figure 3.15) confirming the requirement for L-type mediated Ca^{2+} influx for CREB phosphorylation.

In order to assess if a sustained requirement for Ca^{2+} influx via the L-type VGCC is required for sustained CREB phosphorylation, nifedipine was applied post-stimulation with GLP-1. Nifedipine application post-stimulation had no effect at 10min but application at 20min led to a significant decrease in CREB phosphorylation at 30min (Figure 3.15). ERK1/2 phosphorylation was inhibited as previously reported (Figure 3.7E and Figure 3.15).

3.2.14 GLP-1 stimulated pre-proinsulin transcription requires local Ca^{2+} and ERK1/2 activation

ERK1/2 activity in β-cells has been show to stimulate phosphorylation and activation of a variety of different proteins which mediate preproinsulin gene transcription [144, 147]. Furthermore, glucose and GLP-1 can mediate preproinsulin gene transcription via a variety of different mechanisms [67, 143-145, 292]. In order to investigate whether the local Ca^{2+}-signalling within the microdomain of the L-type VGCC, stimulated by GLP-1, is the mechanism leading to GLP-1 stimulated preproinsulin transcription I employed a construct expressing luciferase under the control of the rat insulin II promoter [292].

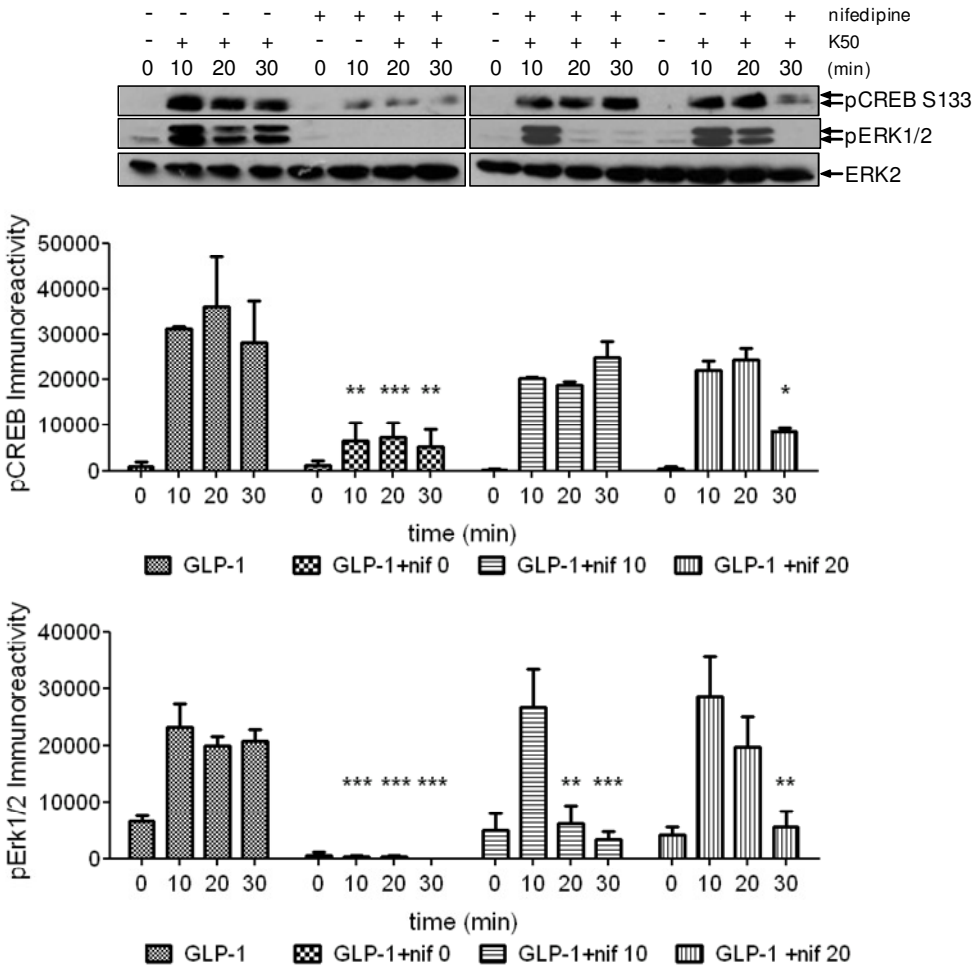


Figure 3.15. GLP-1 does not require a prolonged L-type VGCC activation to mediate CREB phosphorylation. A) MIN6 cells were preincubated for 1hour in KRB supplemented with 1mM glucose then stimulated 16.7mM glucose and 10nM GLP-1 for the time course indicated then with 10μM nifedipine at 0, 10 and 20min post-stimulation. ERK1/2 and CREB phosphorylation was determined via Western blotting using phospho-specific antibodies. A representative blot is shown with densitometry below +S.E.M. Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing GLP-1 alone to treatments at matching time points are marked on the graph; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=3).

MIN6 cells were starved overnight (16 hours) of serum and left in DMEM without sodium pyruvate and containing 3mM glucose. Initially, MIN6 cells were treated with either glucose alone or GLP-1 in the presence of glucose for 4 hours. GLP-1 potentiated the increase in luciferase expression mediated by glucose (Figure 3.16A) indicating that both stimuli can mediate preproinsulin transcription. To assess whether ERK1/2 and Ca^{2+} influx thourough the L-type channel is required to mediate GLP-1 stimulated insulin gene transcription, the MEK inhibitor PD098059 and nifedipine where used to block ERK1/2 and L-type VGCC activity. Both MEK and nifedipine application significantly attenuated GLP-1 mediated transcription (Figure 3.16B) demonstrating that insulin gene transcription is mediated by a mechanism requiring local Ca^{2+}-dependent ERK1/2 activation.

In order to confirm that the assay is measuring changes in transcription, rather than an increase in luciferase stability, GLP-1 stimulation was carried out in the presence of actinomycin D, a transcriptional inhibitor. Actinomycin D abolished the GLP-1 stimulated luciferase expression (Figure 3.16C) confirming that GLP-1 stimulates an increase in transcription of luciferase via the rat insulin II promoter.

To order to assess whether activation of the L-type channel and generation of a Ca^{2+} microdomain surrounding the L-type VGCC alone, is sufficient to mediate insulin gene transcription, MIN6 cells were treated with the artificial activator of the L-type VGCC Bay-K8644. Treatment of MIN6 cells with Bay-K8644 for 4 hours did not stimulate luciferase expression (Figure 3.16D) suggesting that Ca^{2+} influx via the L-type VGCC alone is insufficient to mediate insulin gene transcription and a variety of different signalling components are required for glucose and GLP-1 stimulated transcription.

In order to assess whether a sustained activation of the L-type VGCC and therefore, the local Ca^{2+} to ERK1/2 by the channel, is required for GLP-1 mediated insulin transcription, nifedipine was applied 15 and 20 min post-stimulation with either glucose alone (Figure 3.17A) GLP-1 in the presence of glucose (Figure 3.17B), a treatment which is known to abolish ERK1/2 activation (Figure 3.7D). Both glucose and GLP-1 stimulated luciferase expression where significantly attenuated with nifedipine application post-stimulation. This indicates that a sustained activation of the L-type VGCC and Ca^{2+} signalling within the microdomain of the channel lead to ERK1/2 activation and insulin gene transcription.

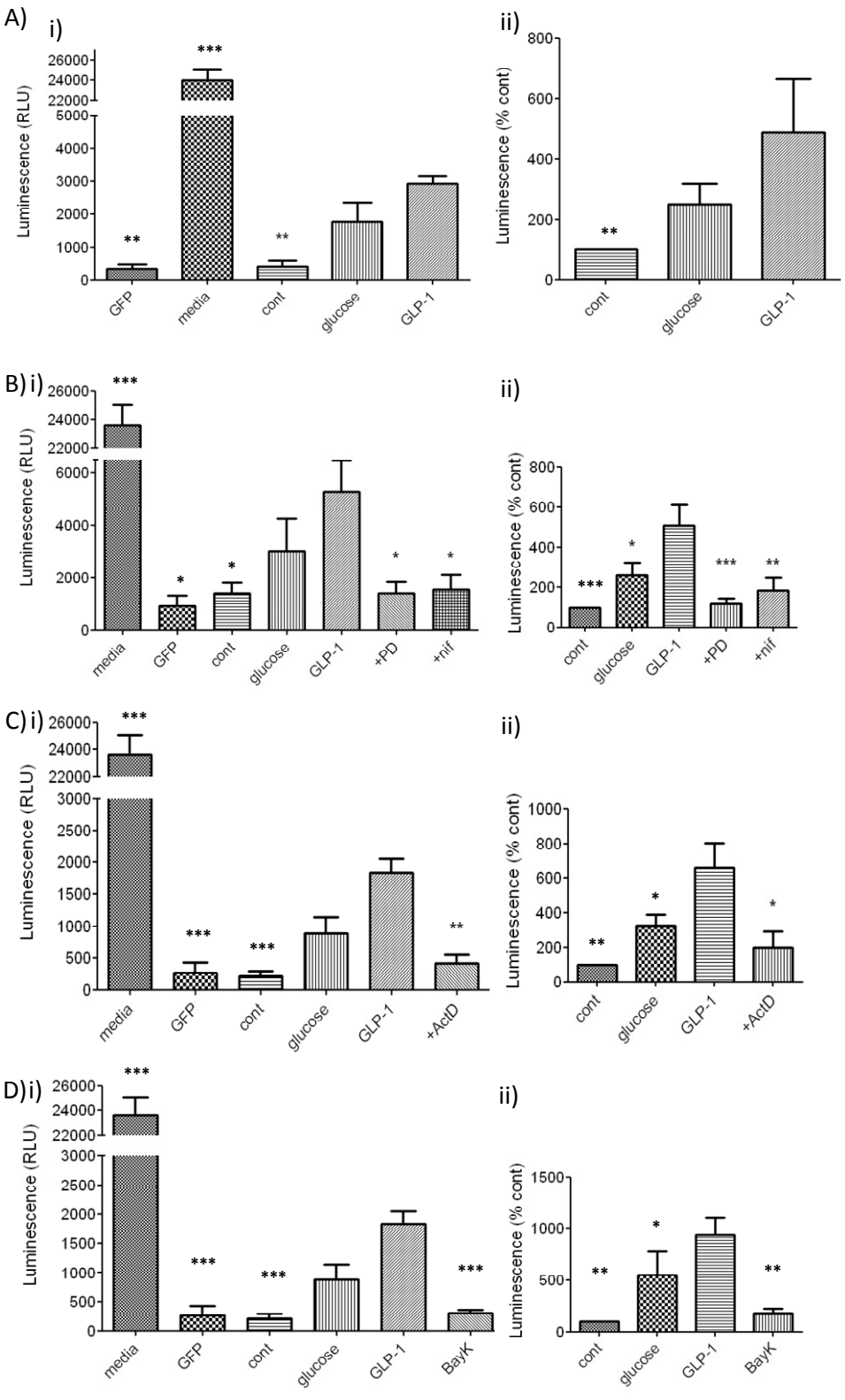

A)
i)
ii)
Luminescence (RLU)
Luminescence (% cont)
GFP
media
cont
glucose
GLP-1
B)
+PD
+nif
C)
+ActD
D)
BayK

Figure 3.16. GLP-1 stimulated pre-proinsulin transcription requires local Ca^{2+} and ERK1/2 activation. MIN6 cells were transfected with 0.8µg of DNA as indicated. pFOXLUC410 contains the rat insulin promoter (-410 to +1bp) in the pFOXLUC vector. Cells were cultured overnight (16hours) in serum-free media containing low glucose (3mM) and 0.1% BSA. A) Cells were then placed in KRB supplemented with 1mM glucose for 1 hour then left unstimulated (cont) or treated with either 16.7mM glucose alone or in the presence of 10nM GLP-1 for 4hours B) Cells were then placed in KRB supplemented with 1mM glucose for 1 hour and left unstimulated (cont) or treated with either 16.7mM glucose alone or with 10nM GLP-1 for 4 hours. In addition cells were simultaneously treated with either 1µM PD183459 or 10µM nifedipine. Whole cell extracts were prepared using Promega Dual luciferase assay kit (Cat No E1910) and lysed in passive lysis buffer, according to the manufacturer's instructions, prior to measuring firefly luciferase activity. C) Cells were treated as in B) but using 1µg/µl actinomycin D as an inhibitor of transcriptional responses. D) Cells were treated as in B) or with 10µM Bay-K-8644 for 4hours. All data are mean +S.E.M.. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to GLP-1; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=4)..

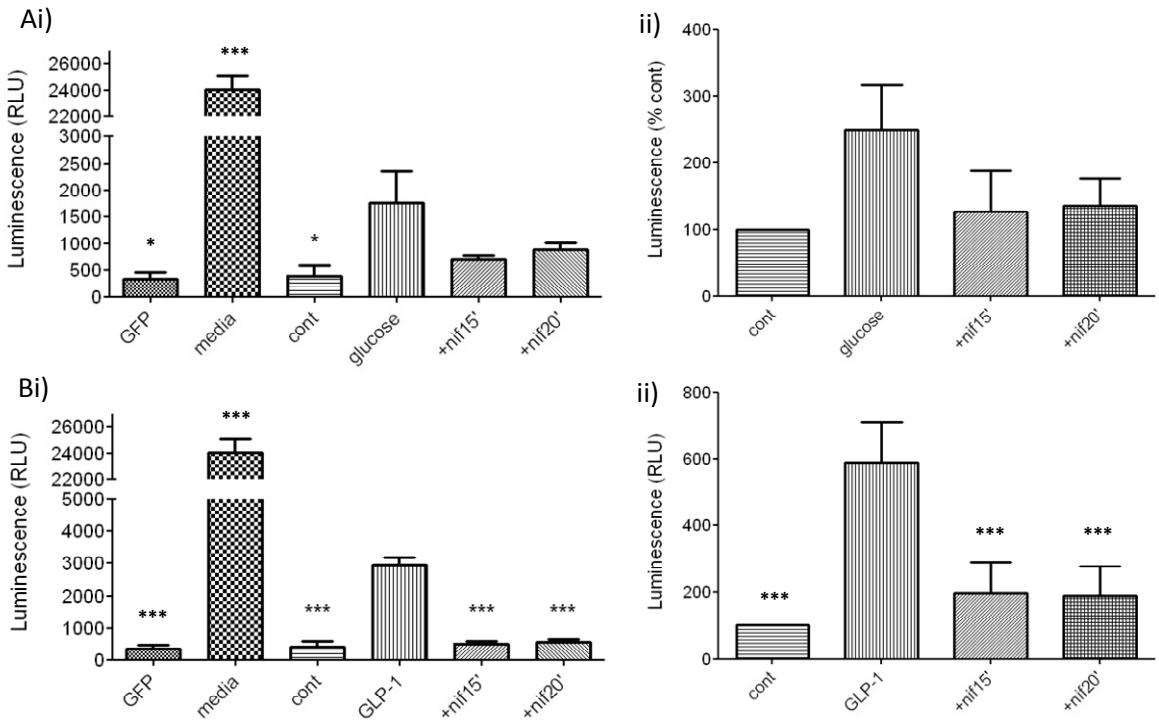


Figure 3.17. Glucose and GLP-1 stimulated pre-proinsulin transcription requires local Ca^{2+}. MIN6 cells were transfected with 0.8µg of DNA as indicated. Cells were cultured overnight (16h) in serum-free media containing low glucose (3mM) and 0.1% BSA.. A and B) Cells where treated with either 16,7mM glucose alone (A) or in the presence of 10nM GLP-1 (B) alone and then treated with 10µM nifedipine at the time points indicated. Whole cell extracts were prepared using Promega Dual luciferase assay kit (Cat No E1910) and lysed in passive lysis buffer, according to the manufacturer's instructions, prior to measuring firefly luciferase activity. All data are mean +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to either glucose (A) or GLP-1 (B); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (n=4)..

3.3 Discussion

In this thesis I demonstrate that upon stimulation of β-cells with either depolarising concentrations of K^+ or GLP-1 in the presence of glucose, causes a sustained activation of ERK1/2. Both agents require the sustained activation of the L-type VGCC for sustained ERK1/2 activation and ERK1/2 activation is Ca^{2+}-dependent. Although it is possible for Ca^{2+} entry to evoke Ca^{2+}-induced Ca^{2+} release (CICR) at either ryanodine or IP_3 receptors, these mechanisms did not play a role in ERK1/2 activation with either agonist. Using Ca^{2+} chelators I have demonstrated that a local Ca^{2+} signal within the microdomain of the L-type VGCC mediates sustained time points of ERK1/2 activation in response to GLP-1 stimulation and that this signalling is required for GLP-1 to mediate insulin gene transcription.

3.3.1 Mode of Ca^{2+} entry affects signalling outcome

Global increases in $[Ca^{2+}]_i$ independent of L-type VGCCs are sufficient to stimulate ERK1/2 activation as ionomycin stimulates ERK1/2 phosphorylation. However, ionomycin is unable to stimulate the sustained activation of ERK1/2, despite being able to induce a sustained increase in $[Ca^{2+}]_i$. In contrast, depolarising concentrations of K^+, which result in a lower global change in $[Ca^{2+}]_i$ to that observed in ionomycin-treated cells, causes greater ERK1/2 phosphorylation than ionomycin and stimulate sustained ERK1/2 activation (Figure 3.5). These results provide evidence that the mode of Ca^{2+} entry, rather than a global rise in $[Ca^{2+}]_{i,}$ is important in coupling Ca^{2+} elevations to the ERK1/2 signalling pathway. The importance of the mode of Ca^{2+} entry can be observed for other signalling cascades in β-cells and from other cell types [460, 579].

In β-cells, Ca^{2+}-dependent insulin secretion is specifically mediated by L-type VGCCs. In rodent models, insulin secretory vesicles are associated with L-type VGCCs via adaptor and fusion proteins, allowing signalling thourough the L-type VGCC to specifically mediate insulin secretion. When there is a large global level of Ca^{2+} in the β-cell, non-specific but Ca^{2+}-dependent insulin secretion can occur. The rise in $[Ca^{2+}]_i$ will spread thouroughout the β-cell acting upon vesicles that are not coupled to the channel but still have the exocytotic machinery attached. Therefore, it is the localisation of the components rather than their ability to respond which renders them coupled to L-type VGCCs [579]. This specific coupling could be a mechanism that is occurring in the

L-type VGCC-dependent signalling to ERK1/2 observed in this study. ERK1/2 is able to respond to global rises in $[Ca^{2+}]_i$ as demonstrated by ionomycin treatment (Figure 3.5), but the specific localisation of the signalling machinery to the L-type VGCC at time points where only local Ca^{2+} can act upon them enables a specific coupling mechanism to exist.

In other cell types, Ca^{2+} dependent signalling to ERK1/2 has been shown to be mediated by specific Ca^{2+}-entry mechanisms as well as global changes in Ca^{2+}. In cultured cortical neurones ERK1/2 activation by depolarising concentrations of K^+ is mediated specifically by Ca^{2+} influx via the L-type VGCC, as the downstream effector, CaM, is specifically tethered to the channel [460]. However, in chouromaffin cells depolarisation-induced ERK1/2 phosphorylation depends on the cytosolic Ca^{2+} level rather than on the Ca^{2+} channel subtype mediating rises in $[Ca^{2+}]_i$ [104]. This suggests that Ca^{2+}-dependent signalling to ERK1/2 is dependent on the cell type.

In addition to the mode of Ca^{2+} entry affecting ERK1/2 activation, the mode of entry can also dictate the pattern of gene expression. In human vascular smooth muscle cells (VSMCs) VGCC and SOC mediated Ca^{2+} influx differentially regulate the transcription of CRE-containing genes [589]. The transcription of c-fos in VSMC can be mediated by both depolarisation stimulated L-type VGCC Ca^{2+} influx or rises in $[Ca^{2+}]_i$ from ER store depletion. However, the intensity and duration of c-fos accumulation, as well as the mechanism leading to transcription, is different between Ca^{2+} influx and Ca^{2+} release [589]. Moreover, the depletion of Ca^{2+} from ER stores and the subsequent increase in SOC activity stimulated an increase in MKP-1 expression which was not observed upon the activity of L-type VGCCs [589]. Conversely, Egr-1 expression was stimulated by L-type VGCC activity whilst ER store-depletion had no effect on Egr-1 transcription [589].

In neurones there are several documented examples of local rises in $[Ca^{2+}]_i$ or Ca^{2+} microdomains being responsible for changes in gene transcription. Bicucullin mediated inhibition of GABAA receptors in neurones activates action potential firing and Ca^{2+} transients thourough NMDA receptors which leads to ERK1/2 and CREB activation [590]. The application of EGTA-AM to the cells has no effect on ERK1/2 or CREB activation suggesting that a microdomain of Ca^{2+} surrounding NMDA receptors mediates this signalling [590]. A similar study demonstrated that the transcription factor

NF-κB is also activated by submembranous calcium in hippocampal neurones [591]. Furthermore, Ca^{2+} influx via the L-type VGCC, as opposed to Ca^{2+} entry thourough NMDA receptors, leads to the activation of the transcription factor NFAT and gene transcription in hippocampal neurones [554].

In the present study insulin gene transcription stimulated by glucose and GLP-1 was shown to be activated by sustained Ca^{2+} influx via the L-type VGCC. The sustained activation of the channel, as well as the activity of ERK1/2, mediated by local Ca^{2+}, was required for gene transcription. This suggests that local Ca^{2+} within the microdomain of the L-type VGCC is responsible for glucose and GLP-1 stimulated insulin transcription.

3.3.2 Two phases of GLP-1 stimulated ERK1/2 activation

This work demonstrates that there are two phases to GLP-1 stimulated ERK1/2 activation (Figure 3.10). Both of these phases are Ca^{2+}-dependent as incubation with BAPTA prevents ERK1/2 activation (Figure 3.10), and both phases require the activation of the L-type VGCC (Figure 3.7). However, the initial phase of ERK1/2 activation was sensitive to the presence of intracellular EGTA (Figure 3.10). Due to BAPTA binding Ca^{2+} at a faster rate than EGTA, BAPTA is more effective at preventing local increases in $[Ca^{2+}]_{i.}$ and therefore EGTA sensitivity can reveal information regarding the spatial dynamics of Ca^{2+} signalling [552, 584-587]. The attenuation of the initial phase of GLP-1 mediated ERK1/2 activation in the presence of EGTA (10min time point), provides evidence that this ERK1/2 activation is being mediated by global rises of $[Ca^{2+}]_i$ outside of the microdomain of the point of Ca^{2+} entry (i.e. the L-type VGCC). Conversely, the lack of sensitivity to intracellular EGTA of the sustained phase of GLP-1 stimulated ERK1/2 activation (30min time point) demonstrates that at these prolonged time points ERK1/2 activation is occurring within the microdomain of the L-type VGCC. However, the initial phase of ERK1/2 activation is only decreased in the presence of EGTA to the relative level of ERK1/2 activation observed after 30 min of GLP-1 stimulation. This suggests that perhaps two mechanisms of ERK1/2 activation are occurring during this initial time point (10 minute); the EGTA sensitive, global Ca^{2+}-dependent ERK1/2 activation and the EGTA-insensitive Ca^{2+} microdomain signalling observed in the latter time point.

Two phases of GLP-1 mediated ERK1/2 activation has been documented previously. In INS-1 cells GLP-1 stimulated ERK1/2 activation was shown to initially be dependent

upon a cAMP/PKA dependent mechanism and the prolonged phase of ERK1/2 activation to require β-arrestin recruitment to the GLP-1R [592]. However, these experiments in INS-1 cells were completed in the absence of glucose and thus are unlikely to be physiologically relevant. However, the potential involvement of β-arrestin in the local Ca^{2+}-dependent mechanisms is discussed below.

3.3.3 Generation of microdomains in pancreatic β-cells

These data demonstrates that an increase in Ca^{2+} within the microdomain of the L-type VGCC leads to ERK1/2 activation subsequent to GLP-1 stimulation. The presence of Ca^{2+} microdomain signalling at L-type VGCCs has been previously observed in β-cells. Ca^{2+} dependent insulin secretion has been visualised at Ca^{2+} hot-spots in the plasma membrane [580, 581] and L-type VGCCs and dense core vesicles cluster together at the membrane [575].

Glucose is able to mediate $[Ca^{2+}]_i$ gradients in the cytoplasm of mouse islets. The Ca^{2+} gradients are more polarised with increasing glucose concentrations, however depolarising concentrations of K^+ are unable to ellicit these gradients and mediate a uniform global rise in $[Ca^{2+}]_i$ [581, 593].

The sustained rises in $[Ca^{2+}]_i$ stimulated by two depolarising concentrations of K^+ (K20 and K50) were unable to be fully prevented in the presence of the intracellular Ca^{2+} chelator EGTA (Figure 3.11 and 3.12). There a several potential reasons for this. Firstly the sustained rise in Ca^{2+} elicited by K20 and K50 as opposed to the oscillatory nature of the GLP-1 signal may have overwhelmed the buffering capacity of the chelators. Another possible reason is that glucose and GLP-1 mediate a graded Ca^{2+} response with the bulk of Ca^{2+} close to the membrane, probably within the microdomain of the site of entry, the L-type channel, whereas K^+ gives a uniform Ca^{2+} response [581, 593]. This difference in spatial Ca^{2+} distribution may play a role in the inability of EGTA to prevent K^+ evoked changes in $[Ca^{2+}]_i$. Finally, the intracellular concentration of EGTA may be different in the GLP-1 and K^+ stimulated cells. Although the cells were treated equivalently and the chelators loaded in the same manner, perhaps treatment with depolarising concentrations of K^+ led to greater extrusion of the intracellular EGTA from the cell, preventing the chelator from inhibiting global rises in $[Ca^{2+}]_i$.

3.3.4 Potential mechanisms by which local Ca^{2+} mediates ERK1/2 activation

3.3.4.1 CaM tethering to the L-type VGCC

In cultured cortical neurones the L-type VGCC has been implicated in the activation of ERK1/2 via a mechanism that requires calmodulin (CaM) binding to the α_1 pore forming subunit of the L-type VGCC [460]. CaM is thought to bind to the LA motif on the C-terminal tail, within the pore of the α1 subunit, in a Ca^{2+}-independent fashion [469]. Upon membrane depolarisation and the opening of the L-type VGCC channel, the increase in Ca^{2+} concentration activates the bound CaM, which is then displaced from the LA motif and re-binds to a second Ca^{2+}-calmodulin binding site (the IQ motif) within the intracellular C-terminal tail [469]. The bound-CaM then directly activates other signalling molecules and causes a conformational change in the channel which helps it recruit and perhaps activate other signalling molecules [460]. Interestingly, GLP-1-mediated ERK1/2 activation in β-cells is also dependent on CaM as W7, a competitive inhibitor of CaM, prevents GLP-1-mediated ERK1/2 activation in MIN6 and INS-1Ecells [67, 68]. Therefore, the mechanism by which L-type VGCC activation leads to ERK1/2 activation in β-cells is likely to be similar to that observed in neurones. Although this mechanism described in neurones provides evidence for a requirement for CaM the role of local increases in Ca^{2+} was not directly investigated. Using an alternative experimental approach we provide evidence that local increases in $[Ca^{2+}]$ at the point of entry provides the cue for L-type VGCC signalling to ERK1/2 (Figure 3.11).

3.3.4.2 Downstream signalling components

Despite confirming that the Ca^{2+}-dependent activation of ERK1/2 occurs within the microdomain of the L-type VGCC this study has not identified any additional molecules responsible for mediating the signalling. The tethering of CaM to the IQ domain of the L-type VGCC is a likely mechanism for linking Ca^{2+} to ERK1/2 especially given that inhibitors of CaM have been demonstrated to inhibit GLP-1 stimulated ERK1/2 activation in both MIN6 and INS-1 cells [67, 68]. There are a large number of different interacting domains and multiple phosphorylation sites on the $Ca_v1.2$ channel suggesting that the channel has a large number of interactions with intracellular

signalling molecules, all of which have the potential to mediate downstream signalling events due to Ca^{2+} influx thourough the L-type VGCC.

Presuming CaM is involved in GLP-1 stimulated ERK1/2 phosphorylation there are several downstream effectors of CaM, which may in turn activate ERK1/2, including the CaMKs [590]. Indeed, there have been two reports indicating that the CAMKII inhibitors KN62 and KN93 prevent GLP-1-mediated ERK1/2 activation in β-cells [67, 68]. However, these inhibitors also inhibit VGCCs [594] and therefore, the conclusions of these studies must be treated with some caution.

3.3.3 Mechanism may be true for other agonists of L-type VGCC including glucose and K^+

I have demonstrated in this study that GLP-1 and depolarising concentrations of K^+ mediate ERK1/2 activation by a nifedipine sensitive pathway. However, glucose-stimulated ERK1/2 activation in β-cells is also mediated by a nifedipine-sensitive mechanism [67, 261] and GLP-1 potentiates glucose-stimulated increases in $[Ca^{2+}]_i$ and ERK1/2 activation [246, 284, 469, 595]. Therefore, it is likely that glucose mediated ERK1/2 activation is mediated by a similar mechanism to that of GLP-1, via the local increases in Ca^{2+} at the cytoplasmic mouth of the L-type VGCC. Furthermore, any metabolite that mediates depolarisation such as leucine or pyruvate is also likely to be capable of mediating ERK1/2 via local Ca^{2+} mechanism. However, as we have been unable to definitely prove that depolarising concentrations of K^+ mediate ERK1/2 activation via local increases in Ca^{2+} it is possible that GLP-1 provides an additional signalling component allowing this localised signalling. Although, this appears unlikely as L-type VGCC activation via Bay-K8644 is sufficient to mediate ERK1/2 activation via a local Ca^{2+}-dependent mechanism (Figure 3.12).

3.3.6 GLP-1 mediated CREB activation is independent of ERK1/2 but requires L-type VGCC activation

Depolarising agents in β-cells such as glucose, GLP-1 and depolarising concentrations of K^+ have been demonstrated to mediate CREB phosphorylation via the activation of ERK1/2 in MIN6 cells [152, 153, 292]. However, recent work has suggested that the mechanism of CREB phosphorylation is dependent on the stimuli in MIN6 cells [421]. The MEK inhibitor PD098059, which prevents ERK1/2 activation, has been shown to

partially inhibit glucose mediated CREB phosphorylation whilst having no effect on GLP-1 mediated CREB phosphorylation [421]. This would suggest that glucose and GLP-1 are not acting additively and are signalling to CREB via distinct pathways [421]. In the present study, MEK inhibitors failed to prevent GLP-1 stimulated CREB activation in MIN6 (Figure 3.14) however CREB activation by GLP-1 did require Ca^{2+} influx thourough L-type VGCCs (Figure 3.15). PKA is also known to phosphorylate and activate CREB at S133 and this could be the mechanism mediating GLP-1 stimulated CREB activation. The requirement of activated L-type VGCCs could be due to the necessity for a Ca^{2+}-activated AC to mediate additional cAMP/PKA signalling to the Ca^{2+}-independent cAMP accumulation initiated by GLP-1R stimulation leading to CREB activation.

3.3.7 Insulin gene transcription via GLP-1 stimulated L-type VGCC Ca^{2+} influx and ERK1/2 activation

This study has demonstrated that glucose and GLP-1 mediate transcription at the rat insulin promoter in MIN6 cells and this requires sustained Ca^{2+} influx thourough the L-type VGCC and ERK1/2 activation stimulated within the microdomain of the channel (Figures 3.16 and 3.17). Several previous reports demonstrate a role for Ca^{2+} in mediating insulin transcription. In dispersed rat islets an L-type channel blocker verapamil inhibited glucose stimulated CAT expression under the control of a rat insulin promoter [582] whilst in cultured rat islets an increase in prepro-insulin mRNA levels by glucose was prevented with two chemically distinct L-type VGCC blockers [583].

GLP-1 has previously been demonstrated to mediate insulin transcription in a variety of different β-cell lines [196, 286, 290]. Thourough the use of luciferase promoter constructs the CRE site in the rodent insulin promoter as well as one of the CRE sites in the human insulin promoter appear to mediate GLP-1 stimulated insulin transcription. Transcription from the CRE sites is believed to occur via GLP-1 activated bZIP transcription factors such as CREB [291]. However, in this study CREB was shown not to be downstream of ERK1/2 upon GLP-1 stimulation. CREB activation was however stimulated by GLP-1 and nifedipine sensitive (Figure 3.15). This suggests that GLP-1 stimulated insulin gene transcription is likely to require both CREB and ERK1/2 activation independently and other transcription factors are likely to be involved.

ERK1/2 has previously been implicated in insulin transcription thourough its ability to activate several transcription factors known to act on the insulin promoter [144, 145, 147] and perhaps these transcription factors are mediating the effect of GLP-1, local Ca^{2+} and ERK1/2 at the insulin promoter.

3.3.8 Final conclusions

In summary this work has provided an insight into the mechanism mediating GLP-1-stimulated ERK1/2 activation and preproinsulin transcription. The mechanism has been shown to be 1) be Ca^{2+}-dependent, 2) require a sustained activation of the L-type VGCC and; 3) be mediated by a localised Ca^{2+} signal within the microdomain of the L-type VGCC.

Chapter 4: GLP-1 stimulated Ca^{2+}-dependent signalling to ERK1/2 in pancreatic β-cells

4.1 Introduction

4.1.1 Background

In the first chapter of this thesis I identified that the Ca^{2+}-dependent signalling mechanism mediating GLP-1 stimulated ERK1/2 activation is initiated within the microdomain of the L-type VGCC. Previous published work and unpublished observations (Herbert Lab) have identified a number of potential signalling components that may be mediating the Ca^{2+}-dependent signalling from the L-type VGCC to ERK1/2. These include the Ca^{2+} dependent proteins CaM [67, 68], calcineurin and CaMKII [67, 68, 239], the protein scaffold β-arrestin [262, 592] and IKKβ and Tpl-2 components of the NFκB pathway [239]. In addition, several MAPKKK have been demonstrated not to be required for GLP-1 signalling to ERK1/2 in MIN6 including Ras, Raf and Rap [239, 261], whilst apparently required in other β-cells including INS-1 cells and human islets [66, 67]. This illustrates that the mechanism by which GLP-1 stimulates ERK1/2 activation is unclear. Therefore, the aim of this study was to further investigate the molecular mechanism of GLP-1 signalling to ERK1/2 via the L-type VGCC in MIN6 cells.

4.1.2 Ca^{2+}-dependent signalling to MAPKKK and ERK

Unpublished data from our laboratory has identified a MEK kinase activity in lysates from β-cells stimulated with GLP-1 that co-elutes with Tpl-2 but not Raf isoforms [239]. Furthermore, inhibitors of Tpl-2 or one of its upstream activators, IKKβ, significantly attenuated GLP-1 stimulated ERK1/2 phosphorylation [239]. This suggests that Tpl-2 and IKKβ may play a role in mediating GLP-1 stimulated ERK1/2 activation. Therefore, the mechanisms of activation of these proteins will be discussed below. In addition, the Ca^{2+}-dependent proteins that have been identified to mediate IKKβ activation in a number of cell types will be discussed [556, 596, 597]. These are summarised in Figure 4.1.

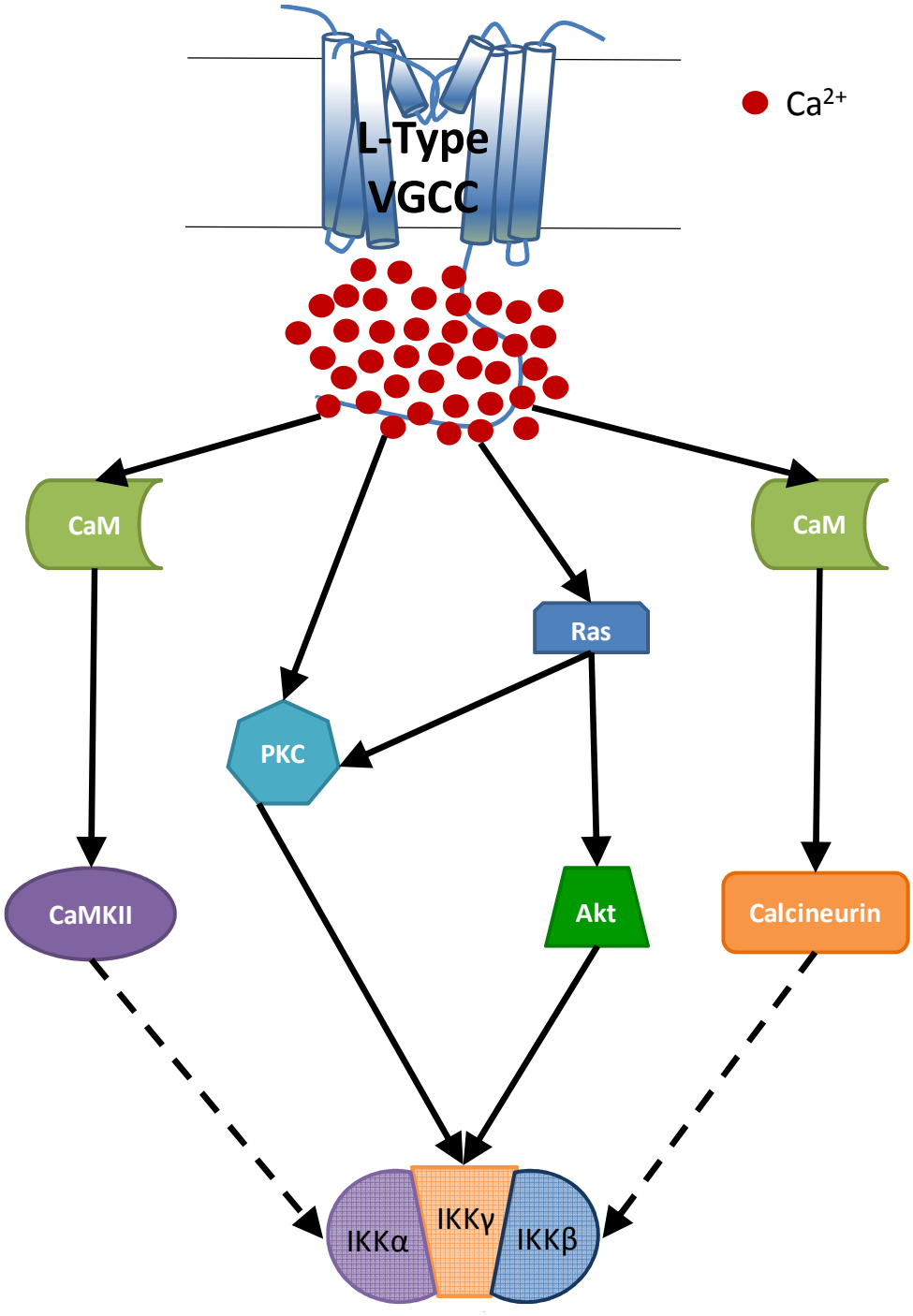


Figure 4.1. Ca^{2+}-dependent signalling to the IKK complex. Ca^{2+} entry into cells leads to the activation of various Ca^{2+}-dependent binding proteins. There have been various Ca^{2+}-dependent pathways identified, primarily in neurones, that activate the IKK complex and NFκB signalling. calmodulin has been found to activate both CaMKII and calcineurin to mediate IKK complex activation via an unknown mechanism. Ca^{2+} also activates PKC to directly phosphorylate components of the IKK complex leading to its activation.The Ca^{2+}-dependent activation of Ras and subsequently PKB and PKC have both been shown to activate the IKK complex.

4.1.2.1 IKKβ

The IKK complex has been implicated in glucose signalling in several cellular contexts. Chouronic exposure to elevated concentrations of glucose leads to IKKβ activation and the impairment of insulin signalling in endothelial cells [597]. Pharmacological inhibition and expression of a dominant negative form of IKKβ demonstrated that active IKKβ was required for glucose to mediate defects in insulin signalling [597]. In p53 deficient MEF cells IKK activity is attenuated by inhibitors of glycolysis but restored by GLUT-3 expression [598]. The authors of this work suggest that glycolysis drives IKK and NFκB activity and that loss of p53 leads to an oncogenic phenotype [598]. The mechanism by which glucose activates IKKβ in endothelial cells and MEFs is not understood at present.

The IKK complex is capable of mediating phosphorylation, activation and the subsequent degradation of NFκB proteins. The activation of the IKK / NFκB signalling pathway as assessed by the degradation of IκBα has been demonstrated to occur in β-cells in response to TNFα, glucose or a depolarising concentration of K^+ [556]. Furthermore, degradation of IκBα correlated with NFκB mediated luciferase gene expression, which was dependent on both L-type VGCC mediated Ca^{2+} influx and ERK1/2 activation [556].

4.1.2.2 Tpl-2

In a quiescent state Tpl-2 is bound to the NFκB protein p105 which is required for Tpl-2s stabilisation as well as its inhibition [73-75]. The activation of IKKβ leads to the phosphorylation of p105, its disassociation from Tpl-2 and its subsequent degradation [74, 76, 77, 80]. IKKβ also phosphorylates Tpl-2 on Thour290 which is required for full Tpl-2 kinase activity [80].

Tpl-2 is also phosphorylated at another C-terminal site, S400. In Jurkat cells this is mediated by PKB [599]. In these cells a S400A mutation of Tpl-2 has no effect on kinase activity but diminishes the ability of Tpl-2 to mediate NFκB transcriptional activity [599]. This suggests that S400 phosphorylation may play a role in another function of Tpl-2. The authors suggest that the phosphorylation may initiate or relieve an association with other proteins leading to NFκB activity, especially as protein association is known to play a role in the activation of NFκB proteins [599]. However, a more recent paper suggests that S400 is required for Tpl-2-dependent, LPS mediated,

ERK1/2 phosphorylation in RAW264.7 cells [600]. The phosphorylation of S400 occurred independently of T290 phosphorylation, of Tpl-2 kinase activity and only occurred when coupled with the release of Tpl-2 from p105 [600]. However, the phosphorylation of S400 in response to LPS stimulation was not mediated by either PKB or the IKK complex, as inhibitors of these signalling molecules failed to attenuate phosphorylation or ERK1/2 activation. Therefore, in macrophages Tpl-2 is phosphorylated and activated to mediate ERK1/2 activation via a mechanism independent of PKB and IKK, but the release of p105 from Tpl-2 is required for its activity.

Cytokine mediated activation of Tpl-2 has previously been demonstrated to require the activation of IRAK-1, a protein kinase associated with cytokine receptors, which mediates IKKβ activity and subsequent phosphorylation of p105 and the release of Tpl-2. However, in IRAK-1 null cells phosphorylation of T290, S400 and autophosphorylation of S62 in Tpl-2 still occurs by a mechanism that is independent of IKKβ but is sensitive to the src protein kinase inhibitor PP2 [84]. This suggests that there is an alternative mechanism of Tpl-2 activation which has yet to be determined.

4.1.2.3 CaM

The activation of CaM occurs thourough the binding of Ca^{2+} to one of its 4 EF hand domains. The ability of CaM to activate ERK1/2 has been documented in several cell types [67, 68, 601-603]. One important example is in hippocampal neurones, where the activation of L-type VGCCs, by depolarising concentrations of K^+, leads to the activation of CaM tethered to the channel and subsequent ERK1/2 activation [460]. L-type VGCC tethered CaM is a potential mechanism for how local Ca^{2+} mediates GLP-1 stimulated L-type VGCC-dependent ERK1/2 activation in β-cells. CaM dependency of glucose or GLP-1 mediated ERK1/2 activation has been demonstrated with W7, a calmodulin inhibitor [67, 68]. In addition, there have been several calmodulin dependent proteins implicated in GLP-1 stimulated ERK1/2 activation, including calcineurin [67, 239] and CaMKs [67, 68].

4.1.2.4 CaMKs

The Ca^{2+}/calmodulin dependent protein kinases (CaMKs) are activated by Ca^{2+}/CaM in response to an increase in Ca^{2+} and have been documented to activate ERK1/2 in several different cell types including: hippocampal and cortical neurones [604-607],

vascular smooth muscles [608] and colon cells [609]. CaMKI activation induced by CaMKK, is required for the Ca^{2+}-dependent activation of ERK1/2 in response to depolarisation in hippocampal neurones and the neuroblastoma cell line NG108 [604-606, 609]. CaMKIV has also been reported to participate in ERK1/2 activation [607, 610]. CaMKIV in cortical neurons can phosphorylate the small G protein Rap1 [607], which increases ERK1/2 activation via B-Raf. Furthermore, the activation of CaMKII has been implicated in ERK1/2 activation [68, 608, 609]. In vascular smooth muscle cells CaMKII can activate ERK1/2 via association with PYK2 kinase [608]. In human colon cells it has been reported that CaMKII can directly phosphorylate and interact with MEK bypassing MAPKKK pathways [609]. In neurones CaMKII has been demonstrated to directly phosphorylate and activate IKKβ [596], making CaMKII a potential mediator of GLP-1 stimulated ERK1/2 activation.

There is evidence that GLP-1 stimulated ERK1/2 activation in β-cells requires CaMKII activation [68]. However, these conclusions were based on pharmacological inhibitors of CaMKII, KN62 and KN93 and they have subsequently been demonstrated to interfere with Ca^{2+} channel activity [594]. Therefore, the role of CaMKII in mediating ERK1/2 activation remains unclear. However, CaMKII has been demonstrated to be activated by submembraneous Ca^{2+} in a variety of different cell types including hippocampal neuronal cultures [241, 591, 611], HEK_{293} cells [542] and superior cervical ganglion neurones [541]541]. In addition, as CaMKII has also been shown to directly activate IKK [596, 612], it is a candidate for mediating GLP-1 stimulated local Ca^{2+}-dependent ERK1/2 activation via Tpl-2 at the mouth of the L-type VGCC.

4.1.2.5 Ras and Raf

The activation of Raf isoforms requires their dimerisation and in HeLa cells this has been demonstrated to be mediated by rises in $[Ca^{2+}]_i$ [613]. In addition, rises in $[Ca^{2+}]_i$ can mediate the activation of specific guanine nucleotide exchange factors (GEFs) for the G-protein Ras, which include Ras-GRP and Ras-GRF [614, 615]. Ras-GRF is activated by the binding of Ca^{2+} and calmodulin (CaM) [615]. Once bound, the activated Ras-GRF facilitates GTP exchange and Ras activation [615]. Ca^{2+}/CaM also regulates the ERK1/2 pathway by regulating synGAP, which is a neuronal specific GTPase activating protein [616]. synGAP can be inhibited by CaMKII mediated phosphorylation which is activated by Ca^{2+}/CaM [617].

There is much debate in the literature about the role of the different MAPKKK isoforms in GLP-1 stimulated ERK1/2 activation [66-68, 261]. The expression of a dominant negative form of Raf-1 (RafC4B) blocked glucose stimulated ERK1/2 activation in INS-1 cells [67] but in MIN6 cells neither A-Raf, B-Raf or C-Raf activity was stimulated by GLP-1 [68]. Conversely, a study in human islets showed that glucose and GLP-1 promoted the association of Rap with B-Raf [66] indicating that B-Raf is activated by glucose and GLP-1. In MIN6 cells glucose and GLP-1 stimulated ERK1/2 activation has also been demonstrated to be independent of Ras activation thourough the use of overexpressed dominant negative RasN17 constructs [68, 261]. However, the expression of a different dominant negative form of Ras blocked glucose stimulated ERK1/2 activation in INS-1 cells [67]. Therefore, the role of Ras and Raf isoforms in glucose and GLP-1 mediated ERK1/2 activation remains unclear.

4.1.2.6 PKC

PKC can modulate the ERK1/2 pathway in a number of ways. Conventional and novel PKC isoforms have been demonstrated to activate C-Raf. One mechanism by which PKC activates C-Raf is thourough releasing it from its Raf kinase inhibitor protein (RKIP). PKC has been reported to phosphorylate RKIP on Ser152 allowing the dissociation of C-Raf and subsequent MEK and ERK1/2 activation [618]. In PC12 cells, PKC-dependent phosphorylation of RKIP results in the prolonged activation of ERK1/2 in response to NGF stimulation [619]. However, the PKC isoform responsible for this signalling is unknown. Other roles for PKC include the activation of Ras in order to mediate C-Raf activations [620] and the direct activation of MEK by the atypical PKCζ [621].

The role of PKC in mediating glucose and GLP-1 stimulated ERK1/2 activation has been investigated previously both in INS-1 and MIN6 cells [145, 622]. The use of PKC inhibitors in INS-1 cells demonstrated that PKC was not required for glucose stimulated ERK1/2 activation [145]. In addition, the downregulation of DAG-dependent PKC isoforms with prolonged phorbol ester treatment had no effect on glucose stimulated ERK1/2 activity in INS-1 cells [622]. However, in MIN6 cells extended phorbol ester treatment abolished glucose stimulated ERK1/2 activation [145]. Therefore, the role of PKC in glucose and GLP-1 stimulated ERK1/2 activation is unclear.

4.1.3 Aims of the chapter

The aim of this study was to investigate the molecular mechanism by which the L-type VGCC mediates GLP-1 stimulated ERK1/2 activation in MIN6 cells. Unpublished observations from our laboratory and the evidence presented in this thesis demonstrate that local Ca^{2+} signalling is required for GLP-1 stimulated ERK1/2 activation, and therefore in my opinion, the pre-associated CaM on the C-terminal tail of the L-type VGCC is mediating ERK1/2 activation.

To investigate this hypothesis I initially intended to use DHP resistant L-type VGCCs which were gifted to us by two independent groups, the Hockerman group who provided the GFP-tagged channel [560] and the Dolmetsch group who provided us with the His-tagged channel [460]. Both constructs are WT $Ca_v1.2$ L-type VGCCs with a point mutation at T1039Y rendering it resistant to DHPs. In addition to the L-type VGCCs I have DHP-resistant His-tagged channels with a second mutation within the C-terminal IQ domains. The IQ mutation renders the channel incapable of mediating CaM tethering and therefore CaM-dependent signalling. To utilise these channels I will transfect cells and treat with depolarising agents to stimulate ERK1/2 activation in the presence of a DHP. This will inhibit endogenous L-type VGCCs and allow signalling to ERK1/2 to only occur thourough the mutated channels (Figure 4.2A). Once the DHP resistant channel has been characterised, I intend to use the channels with the additional point mutations in the IQ motif alongside to assess the effect of CaM binding disruption on GLP-1 stimulated ERK1/2 activation. In addition, I intend to utilise a $CaM_{1,2,3,4}$ construct which is incapable of binding Ca^{2+} and therefore, incapable of switching to the IQ motif of the L-type VGCC. This will allow me to assess the role of CaM binding to the channel in mediating GLP-1 stimulated ERK1/2 activation

In addition I wish to investigate how changes in local Ca^{2+} mediate ERK1/2 activation by determining whether known Ca^{2+}-dependent proteins are required. I intend to achieve this by:

1. Using a dominant negative CaMKII adenovirus to assess the involvement of this known activator of IKKβ and upstream activators of ERK1/2.

2. Utilising PKC inhibitors and chouronic downregulation of DAG-dependent PKC isoforms to assess the role of PKC in mediating ERK1/2 activation.

3. Using a dominant negative Ras adenovirus to assess if this known Ca^{2+}-dependent upstream activator of ERK1/2 is involved in L-type dependent ERK1/2 activation.

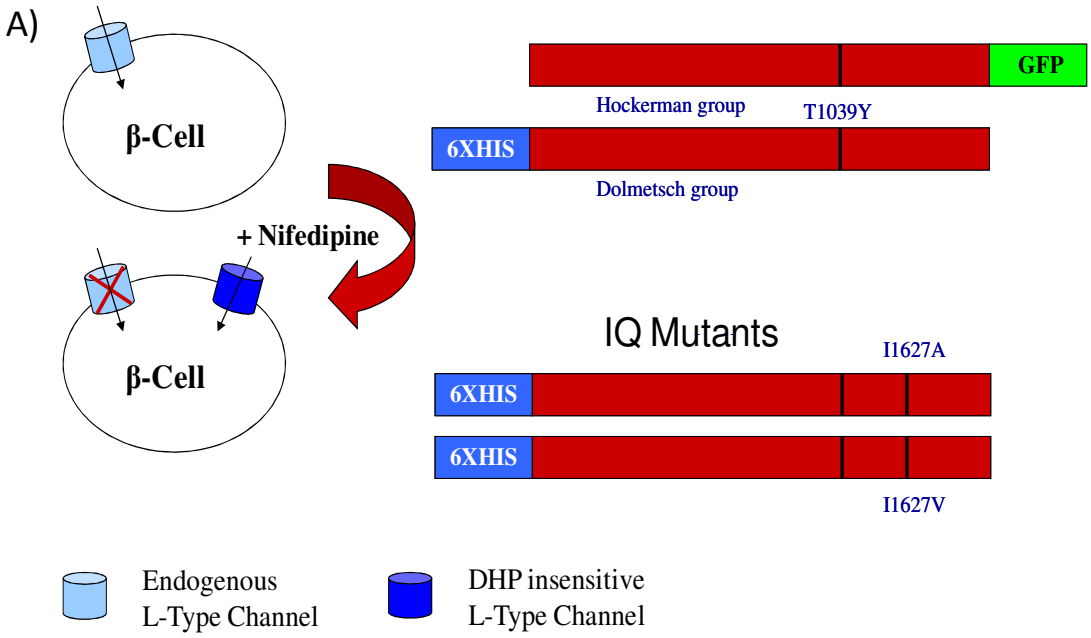
4. Assessing the involvement of Tpl-2 in GLP-1 mediated ERK1/2 activation by utilising siRNA constructs and investigating the activation of parallel pathways.

4.2 Results

4.2.1 Characterisation of nifedipine resistant L-type VGCC constructs

To initially characterise the DHP resistant L-type VGCC and to confirm their functionality the GFP-tagged $Ca_v1.2$ DHP resistant channel was expressed in HEK_{293} cells with or without the β_3 subunit, which has been demonstrated to increase the trafficking of the channel to the cell surface [623-628]. GFP-$Ca_v1.2$ was found to localize at the cell surface and expression of the β_3 auxiliary subunit appeared to enhance the cell surface expression (Figure 4.2Bi). Expression of GFP-$Ca_v1.2$ was also confirmed by Western blotting (Figure 4.2Bii). To confirm the functionality of the constructs and their DHP resistance, HEK_{293} cells were loaded with Fura-Red AM to measure Ca^{2+} currents in response to a depolarising K^+ concentration. The presence of the channel significantly prevented the inhibitory effect of nifedipine to the $[Ca^{2+}]_i$ response and the expression of the β_3 auxiliary subunit led to a further attenuation of the inhibitory effect of nifedipine on the rise in $[Ca^{2+}]_i$ (Figure 4.2Biii). These results confirm that the $Ca_v1.2$ construct is expressed at the cell surface and is functional in HEK_{293} cells.

The GFP-$Ca_v1.2$ plasmid construct was then transfected into MIN6 cells. The transfection efficiency for the GFP-$Ca_v1.2$ construct was very low (~5%) and the GFP appeared to be intracellularly localised. Co-transfection of the GFP-$Ca_v1.2$ with dsred-tagged calreticulin, a protein that residues within the ER, demonstrated that the GFP-$Ca_v1.2$ was localised to the ER (Figure 4.3Ai). Due to the poor transfection efficiency and the intracellular localisation of the channel the $Ca_v1.2$ and auxiliary subunits, β_3 and $\alpha_2\delta$, were sub-cloned and packaged into an adenoviruses by Dr Edith Gomez (Dr Herbert's Lab).


A)
β-Cell
+ Nifedipine
β-Cell
GFP
Hockerman group
T1039Y
6XHIS
Dolmetsch group
IQ Mutants
I1627A
6XHIS
6XHIS
I1627V
Endogenous
L-Type Channel
DHP insensitive
L-Type Channel

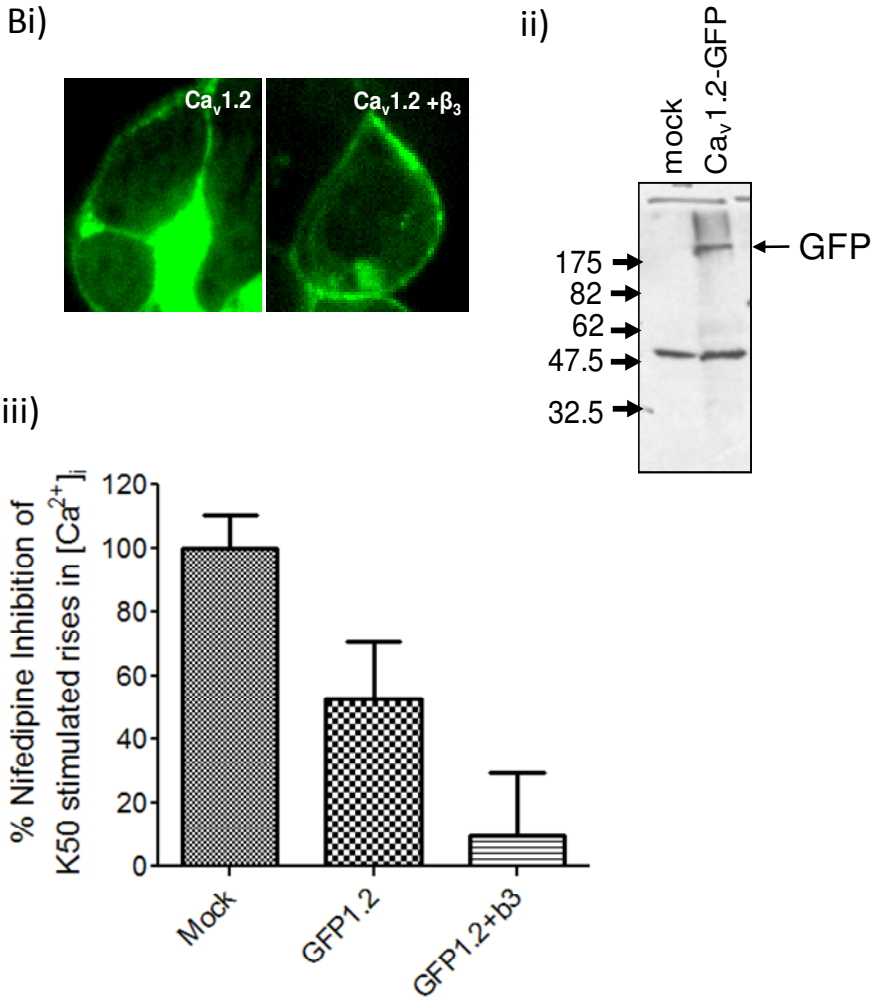

Bi)
Cav1.2
Cav1.2 +β3
ii)
mock
Cav1.2-GFP
GFP
175
82
62
47.5
32.5
iii)
% Nifedipine Inhibition of K50 stimulated rises in [Ca2+]i
120
100
80
60
40
20
0
Mock
GFP1.2
GFP1.2+b3

Figure 4.2. Expression of DHP-resistant L-type VGCCs in HEK_{293} cells. A) Schematic representation of the DHP resistant constructs used in these studies. i) To investigate the involvement of the IQ domain of the L-type VGCC in signalling to ERK1/2 I transfected DHP-resistant constructs into cells and then switched off endogenous signalling using nifedipine. ii) The constructs used for this work are $Ca_v1.2$ L-type VGCCs with either a GFP-tag at the C-terminal or a 6xHIS tag at the N-terminus. A single point mutation T1309Y is required to render the L-type VGCC insensitive to DHPs. In addition, there are channels with double mutations, the T1309Y for DHP-resistance and an additional point mutation at I1627 within the IQ motif of the channel to prevent CaM signalling at the IQ motif. Bi) DHP resistant GFP-$Ca_v1.2$ was transfected in HEK_{293} using the $CaCl_2$ methodology and GFP localisation assessed using confocal microscopy. ii) Expression of DHP resistant GFP-$Ca_v1.2$ was assessed via Western blotting of whole cell lysates using an anti-GFP antibody. iii) Single cell Ca^{2+}-imaging of DHP resistant GFP-$Ca_v1.2$ transfected HEK_{293} cells loaded with 5µM Fura-Red-AM in the presence or absence of the β_3 auxillary subunit. Cells were treated with K50 in the presence or absence of 10µM nifedipine and the Ca^{2+} response analysed in terms of % inhibition upon nifedipine application (n=3).

MIN6 cells were infected with the GFP-$Ca_v1.2$ DHP resistant adenovirus and the localisation of GFP determined by confocal microscopy. However, the GFP did not localize to the cell membrane (Figure 4.3Aii) indicating that the channel was unable to be trafficked to the membrane. Due to the ability of the β_3 subunit to increase the signal at the plasma membrane and confer more nifedipine resistance in HEK_{293} cells (Figure 4.2B), the adenovirus expressing the non-tagged β_3 auxiliary subunit was co-infected with the GFP-$Ca_v1.2$. However, GFP-$Ca_v1.2$ was still localized intracellularly (Figure 4.3Aii). In parallel the functionality and expression of the GFP-$Ca_v1.2$ channel was tested. Cells were treated with depolarising concentrations of K^+ and $[Ca^{2+}]_i$ responses recorded. The overexpression of GFP-$Ca_v1.2$ alone or co-expression of the β_3 auxiliary subunit did not lead to any significant nifedipine resistant $[Ca^{2+}]_i$ response (Figure 4.3Aiv). This suggests that the channel is either not-functional or not expressed at the cell surface. Protein expression was also analysed by Western Blotting using a GFP antibody. This analysis indicated a major product of 60kD, a 47kD product and a product at approximately 25kD (Figure 4.3Aiii). This indicates that the N-terminal of the channel may have been deleted or removed. Sequence analysis however, revealed that both ends of the coding sequence for the channel remained intact. This would suggest that the channel is being proteolytically cleaved within the MIN6 cell.

As this apparent proteolytic cleavage of GFP-$Ca_v1.2$ occurred in MIN6 cells it may be a cell-specific phenomenon. Therefore, I tested the adenoviruses in another β-cell line, INS-1Ecells. The localisation of the GFP-$Ca_v1.2$ channel in INS-1E cells was also determined by confocal microscopy. The GFP signal in INS-1E cells did not localize to the cell membrane but was intracellular (Figure 4.3Bi). In addition, the overexpression of GFP-$Ca_v1.2$ alone or co-expression of the β_3 auxiliary subunit did not lead to any

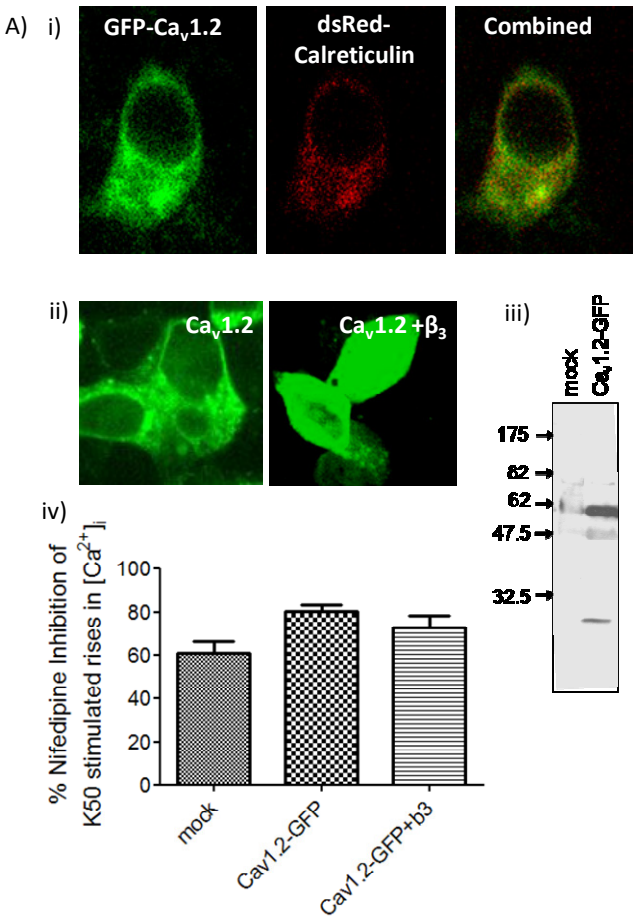

A) i)
GFP-Cav1.2
dsRed-Calreticulin
Combined
ii)
Cav1.2
Cav1.2 +β3
iii)
mock
Cav1.2-GFP
175
82
62
47.5
32.5
iv)
% Nifedipine Inhibition of K50 stimulated rises in [Ca2+]i
100
80
60
40
20
0
mock
Cav1.2-GFP
Cav1.2-GFP+b3
B)
i)
Cav1.2
Cav1.2 +β3
ii)
mock
Cav1.2-GFP
175
82
62
47.5
32.5
iii)
% Nifedipine Inhibition of K50 stimulated rises in [Ca2+]i
100
80
60
40
20
0
Mock
GFP1.2
GFP1.2+b3
C) i)
Cav1.2
Cav1.2 +β3

Figure 4.3. DHP resistant constructs in MIN6 and INS-1E cells. Ai) The $Ca_v1.2$-GFP and dsred-calreticulin plasmids were co-transfected into MIN6 cells using Lipofectamine and GFP localisation determined by confocal microscopy. ii) The $Ca_v1.2$-GFP adenovirus was infected into MIN6 cells and GFP localisation assessed using confocal microscopy. ii) Expression of $Ca_v1.2$-GFP adenovirus constructs was assessed via Western blotting of whole cell lysates using an anti-GFP antibody. iii) Single cell Ca^{2+}-imaging of $Ca_v1.2$-GFP infected MIN6 cells in the presence or absence of the β_3 auxillary subunit were loaded with 5μM Fura-Red-AM. Cells were treated with K50 in the presence or absence of 10μM nifedipine and the Ca^{2+} response analysed in terms of % change upon nifedipine application. Bi) The $Ca_v1.2$-GFP adenovirus was infected into INS-1Ecells and GFP localisation assessed using confocal microscopy. ii) Expression of $Ca_v1.2$-GFP constructs was assessed via Western blotting of whole cell lysates using an anti-GFP antibody. iii) Single cell Ca^{2+}-imaging of $Ca_v1.2$-GFP infected INS-1Ecells in the presence or absence of the β_3 auxillary subunit were loaded with 5μM Fura-Red-AM Cells were treated with K50 in the presence or absence of 10μM nifedipine and the Ca^{2+} response analysed in terms of % change upon nifedipine application. C) The $Ca_v1.2$-GFP adenovirus was infected into CHO cells and GFP localisation assessed using confocal microscopy

significant nifedipine resistant $[Ca^{2+}]_i$ response (Figure 4.3Biii). Western blotting confirmed that the GFP-$Ca_v1.2$ channel also appears to be truncated in INS-1Ecells (Figure 4.3Bii). Therefore, adenoviral-mediated expression of the GFP-$Ca_v1.2$ DHP resistant channels does not lead to the presence of a functional channel at the cell surface in β-cells.

CHO cells were also infected with adenoviral GFP-$Ca_v1.2$ DHP resistant channels. However, no cell surface expression of the $Ca_v1.2$ was detected (Figure 4.3C). For this reason I have concluded that the virus is defective and no further work has been carried out with it. Therefore, it was concluded to terminate this line of investigation.

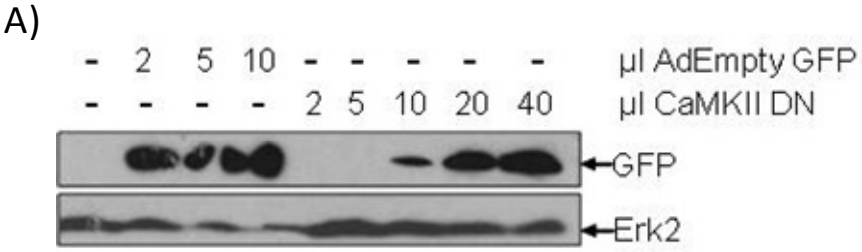
4.2.2 Investigation into Ca^{2+}-dependent signalling to ERK1/2

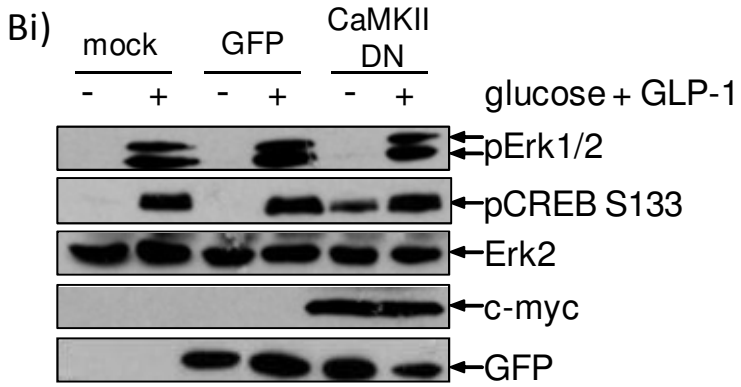
To investigate the mechanism mediating L-type VGCC signalling to ERK1/2 I have investigated a variety of different signalling components that have been implicated in Ca^{2+}-dependent ERK1/2 activation.

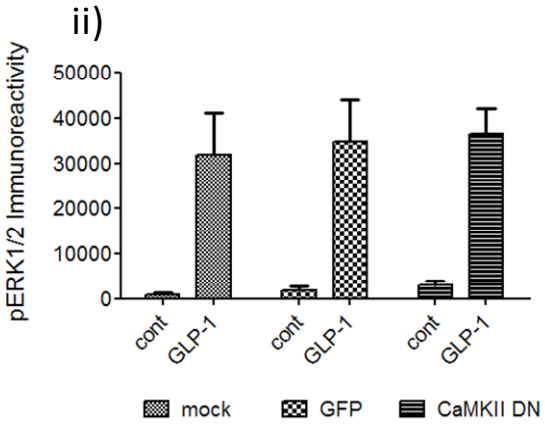
4.2.2.1 CaMKIIδ is not required for GLP-1 mediated ERK1/2 activation

Previous studies have indicated thourough the inhibitors of CaMKII that this protein may be required for GLP-1 stimulated ERK1/2 activation [67, 68]. However, the inhibitors used have been shown to interfere with Ca^{2+} influx in β-cells. Therefore, to further investigate whether CaMKII has a role in Ca^{2+}-dependent ERK1/2 activation, I employed a dominant negative CaMKIIδ adenovirus with a point mutation (K43A) which prevents ATP binding ($CaMKII\delta^{DN}$), made by Dr Claire Moore (Dr Herbert's lab). To ensure equal expression of the control and $CaMKII^{DN}$ virus MIN6 cells were infected with increasing amounts of Ad-Empty-GFP and $AdCaMKII^{DN}$ for 48h. The $CaMKII^{DN}$ protein is expressed under the same promoter as GFP and therefore, the efficiency of infection was monitored by GFP expression. The use of 2µl AdEmpty-GFP and 40ul $CaMKII^{DN}$ gave equal GFP expression (Figure 4.4A) and thus these volumes were used for future experiments.

MIN6 cells were infected with either Ad-Empty-GFP or $AdCaMKII^{DN}$ for 48h. Then following an hour starvation of glucose and serum in KRB supplemented with 1mM glucose, the cells were treated with GLP-1 in the presence of glucose for 10 min and ERK1/2 phosphorylation assessed via Western blotting. GLP-1 stimulated ERK1/2 phosphorylation was unaffected by overexpression of $CaMKII\delta^{DN}$ (Figure 4.4B).


A)
- 2 5 10 - - - - -
µl AdEmpty GFP
- - - - 2 5 10 20 40
µl CaMKII DN
GFP
Erk2


Bi)
mock
GFP
CaMKII DN
- + - + - +
glucose + GLP-1
pErk1/2
pCREB S133
Erk2
c-myc
GFP


ii)
pERK1/2 Immunoreactivity
50000
40000
30000
20000
10000
0
cont
GLP-1
mock
GFP
CaMKII DN

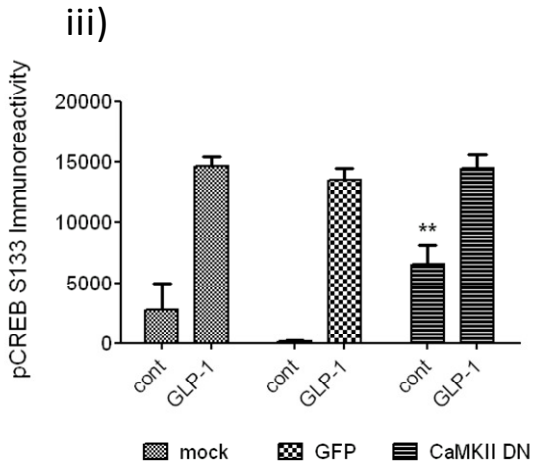

iii)
pCREB S133 Immunoreactivity
20000
15000
10000
5000
0
**
cont
GLP-1
mock
GFP
CaMKII DN

Figure 4.4. CaMKIIδ is not required for GLP-1 mediated ERK1/2 activation but has a role in maintaining CREB in a dephosphorylated state. A) MIN6 cells were infected with increasing volumes of either CsCl purified AdEmpty GFP adenovirus or CsCl purified CAMKIIδ DN adenovirus. GFP expression as a marker of infectivity, and ERK2 levels as a marker of protein loading were determined via Western blotting using specific antibodies. B) MIN6 cells were either infected with 1µl of AdEmpty GFP virus, 40µl CaMKIIδ DN or mock infected. 48h post-infection MIN6 cells were starved in KRB supplemented with 1mM glucose for 1hour then left unstimulated or treated with 10nM GLP-1 plus 16.7mM glucose for 10min. ERK1/2 and CREB phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blots are shown with densitometry below ii) pERK1/2 densitometry, iii) pCREB S133 densitometry. Data are mean +S.e.m. (n=4). Statistical comparisons were by two-way ANOVA with Bonferroni's multiple comparison test. Only significance comparing cont and GLP-1 in the mock condition to other groups are marked on the graph; **, $P < 0.01$.

CaMKII has been demonstrated to phosphorylate CREB at both S133 and S142 *in vitro* [629, 630]. The phosphorylation of CREB at S142 inhibits CREB activity even in the presence of S133 phosphorylation [629]. As CaMKII has the potential to mediate phosphorylation of CREB at S133 it was used as a potential control for the dominant negative action of the CaMKII. However, no decrease in CREB phosphorylation was observed upon GLP-1 stimulation, but $CaMKII^{DN}$ caused an increase in basal CREB S133 phosphorylation (Figure 4.4B). This indicated that CaMKII negatively regulates basal CREB phosphorylation at S133 in β-cells but does not effect GLP-1 stimulated ERK1/2 or CREB S133 activation (Figure 4.4).

4.2.2.2 Role of PKC in GLP-1 mediated ERK1/2 activation

One of the Ca^{2+}-dependent pathways leading to IKKβ activation is the direct phosphorylation of IKKβ by PKC [596]. In order to determine the role of PKC in mediating GLP-1 stimulated ERK1/2 activation in β-cells, MIN6 cells were pre-treated for 30 min with BIM I, Ro320432 and Gö6976 to selectively inhibit classical and novel PKC isoforms [631, 632]. In addition, MIN6 cells were subjected to chouronic stimulaton with TPA, which is known to down-regulate all DAG-dependent PKCs [633]. None of the PKC inhibitors affected GLP-1 stimulated ERK1/2 activation (Figure 4.5A) but chouronic TPA treatment, which down-regulates DAG-dependent PKCs [633], significantly attenuated ERK1/2 activation.

4.2.2.3 Role of Ras in Ca^{2+}-mediated ERK1/2 activation in MIN6 cells

The role of Ras in glucose and GLP-1 stimulated ERK1/2 phosphorylation in β-cells has been investigated previously in a variety of cell lines with differing conclusions [67, 68, 261]. In MIN6 cells GLP-1 stimulated ERK1/2 activation has been demonstrated to be independent of Ras activation thourough the use of overexpressed dominant negative RasN17 [68, 261]. However, the expression of a different dominant negative form of Ras blocked glucose stimulated ERK1/2 activation in INS-1 cells [67]. However, these studies evaluated the effect of the Ras dominant negative protein on peak GLP-1 mediated ERK1/2 activation, at 10min post-stimulation in MIN6 cells and 30min post-stimulation in INS-1 cells. From the first chapter of this thesis I have demonstrated that two Ca^{2+}-dependent phases of ERK1/2 activation occur, one initiated by global rises in $[Ca^{2+}]_i$ at 10min, and a sustained phase of ERK1/2 activation (30min) mediated by local

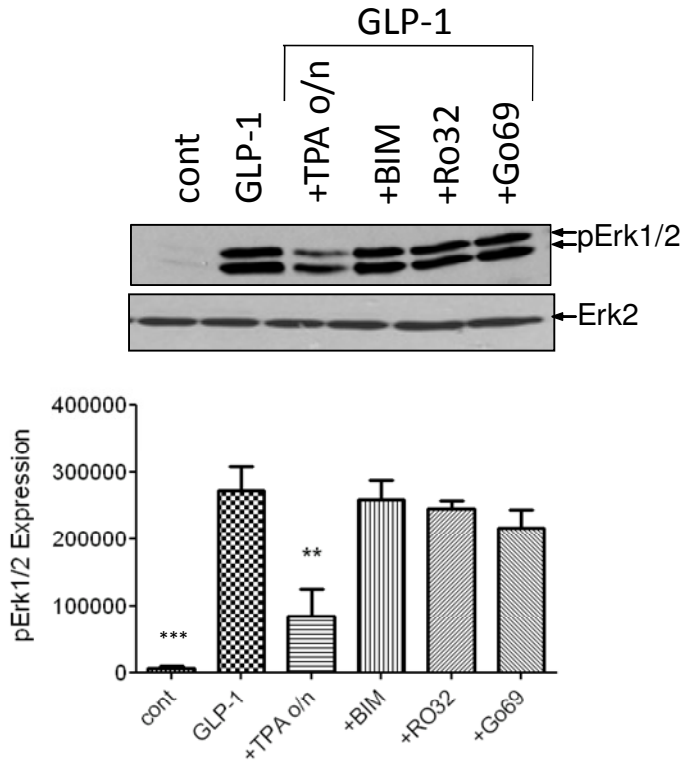


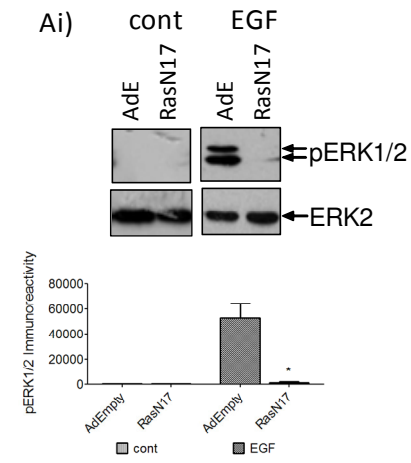
Figure 4.5. PKC is not required for GLP-1 mediated ERK1/2 activation but a DAG-sensitive protein is implicated. MIN6 cells were preincubated with KRB with 1mM glucose for 1hour prior to treatment with 10nM GLP-1 plus 16.7mM glucose for 10min. Cells were additionally pretreated for 30min alone or in the presence of various PKC inhibitors as described at 1μM. In addition to the inhibitors, MIN6 cells were pretreated with 1μM TPA (a phorbol ester) for 16hours in order to downregulate DAG-sensitive proteins. Phospho-ERK1/2 and ERK2 levels were analysed by Western blotting using antibodies. A representative blot is shown with densitometry below +S.E.M. Statistical comparisons were by one-way ANOVA and Dunnett's range test compared to GLP-1 (n=3). **, $P < 0.01$; ***, $P < 0.001$.

Ca^{2+}. It is likely that as the two phases require different Ca^{2+} inputs or ERK1/2 activation they will require different Ca^{2+}-dependent proteins. Therefore, the disparity between cell models, the ability of Ras to be activated by Ca^{2+}, and my work demonstrating that there are two distinct phases to GLP-1 stimulated L-type VGCC dependent ERK1/2 activation, led us to re-examine the effects of blocking Ras by adenoviral over-expression of the dominant negative RasN17 protein.

MIN6 cells were infected with either an empty virus (AdE) or AdRasN17 48hours prior to treatment. As a control for the effects of the dominant negative RasN17 adenovirus the cells were treated for 5 and 30min with EGF. The over-expression of the dominant negative Ras effectively inhibited EGF stimulated ERK1/2 activation at both 5 and 30min (Figure 4.6Ai and Bi). The treatment of the cells with glucose alone or GLP-1 in the presence of glucose was unaffected by the Ras dominant negative adenovirus at 10min (Figure 4.6aii) as previously reported [68, 261]. The treatment of MIN6 cells for 30min with glucose alone or GLP-1 in the presence of glucose was also unaffected by overexpression of the dominant negative Ras protein (Figure 4.6Bii).

To assess whether L-type VGCC activation using depolarising concentrations of K^+ also activated ERK1/2 phosphorylation via Ras-independent mechanism MIN6 cells were treated with two different concentrations of K^+, K20 and K50, in the presence of the dominant negative Ras protein. The treatment of MIN6 cells with K20, which leads to a similar level of ERK1/2 phosphorylation to glucose, is unaffected by the presence of the dominant negative Ras protein at either 10 or 30 min (Figure 4.6Aiii and Biii). However, the stronger depolarising stimulus of K50 stimulated ERK1/2 phosphorylation that is significantly inhibited by the presence of AdRasN17 at both time points (Figure 4.6Aii and Bii). However, the level of ERK1/2 activation was only reduced to the level of K20 stimulated ERK1/2 phosphorylation (Figure 4.6Aii and Bii).

This suggests that due to the greater magnitude of depolarisation mediated by K50 in relation to K20 ERK1/2 activation switches from Ras-independent to Ras-dependent. K50 also stimulates a much greater global $[Ca^{2+}]_i$ response than K20 as demonstrated in Figure 3.2. This suggests that a thoureshold of global Ca^{2+} exists that mediates the switch to a Ras-dependent pathway that mediates Ca^{2+}-dependent ERK1/2 activation.


Ai)
cont
EGF
AdE
RasN17
pERK1/2
ERK2
pERK1/2 Immunoreactivity
AdEmpty
RasN17
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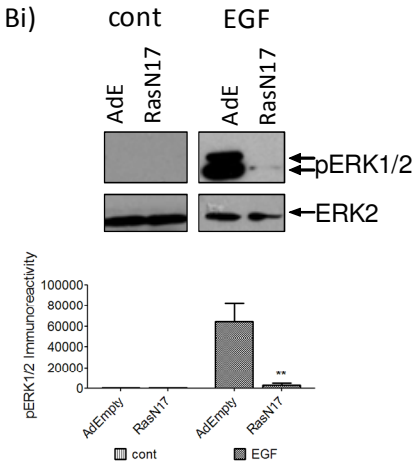
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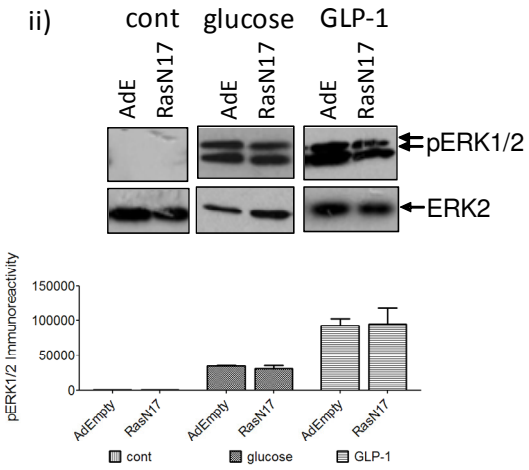
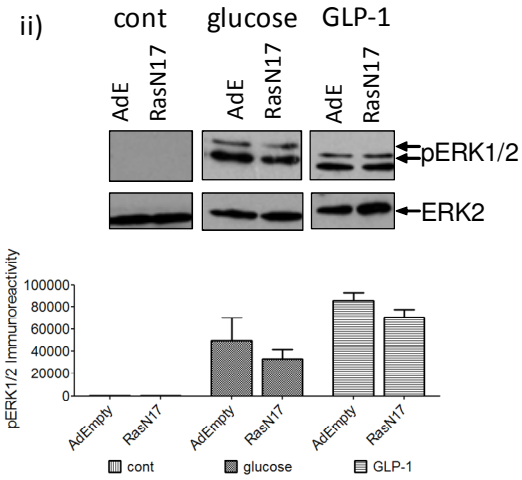
pERK1/2
ERK2
pERK1/2 Immunoreactivity
AdEmpty
RasN17
cont
EGF


ii)
cont
glucose
GLP-1
AdE
RasN17
pERK1/2
ERK2
pERK1/2 Immunoreactivity
AdEmpty
RasN17
cont
glucose
GLP-1


ii)
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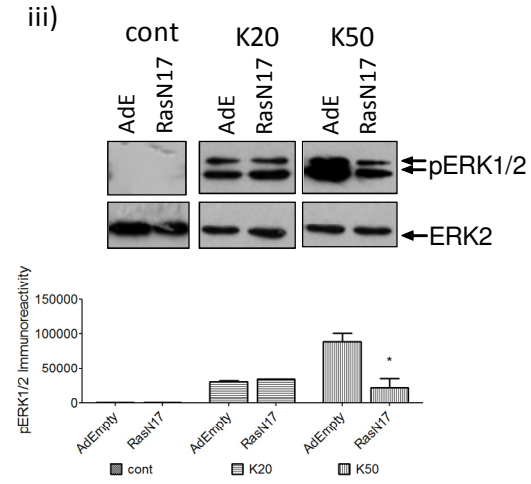
iii)
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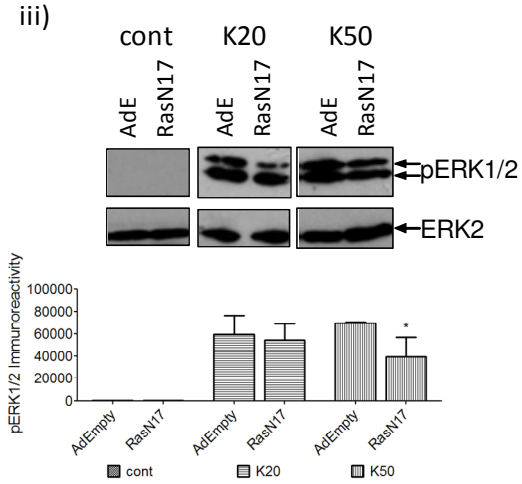
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K50

Figure 4.6. Stimuli that mediate large global changes in $[Ca^{2+}]_i$ mediate ERK1/2 activation via a Ras-dependent mechanism. MIN6 cells were infected with either the control adenovirus AdEmpty-EGFP, or infected with recombinant adenovirus expressing RasN17 (AdRasN17). 48h post-infection, the cells were preincubated for 1hour in KRB supplemented with 1mM glucose prior to treatment with either Ai) 20ng/ml EGF for 5min as a control ii) 16.7mM glucose alone for 10min or 10nM GLP-1 plus 16.7mM glucose for 10min iii), K20 KRB for 5min or K50 KRB for 5min. B) Cells were also treated equally but for 30min. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Statistical comparisons were by t-test within each treatment; *, $P < 0.05$ (n=3).

4.2.3 Role of Tpl-2 and the potential mechanism of activation of ERK1/2 in response to GLP-1 stimulation

4.2.3.1 Investigation into the role of Tpl-2 in GLP-1 mediated ERK1/2 activation

I have demonstrated that GLP-1 mediated ERK1/2 activation is Ras-independent (Figure 4.6) and therefore, a different MAPKKK must be responsible for ERK1/2 activation. Previous unpublished work in our lab has identified Tpl-2 as a candidate for the MAPKKK responsible for GLP-1 stimulated ERK1/2 activation. Therefore, to confirm the role of Tpl-2 in GLP-1 stimulated ERK1/2 activation MIN6 cells were transfected with either one of the 4 independent siRNAs targeted against the mouse Tpl-2 gene or a scrambled siRNA. In order to try and achieve siRNA mediating silencing of the Tpl-2 gene I used 3 commercially available and a designed siRNA construct targeted against Tpl-2 (Figure 4.7A). However, the introduction of these siRNAs failed to inhibit the expression of the Tpl-2 protein or prevent GLP-1 stimulated ERK1/2 activation (Figure 4.7Bi and ii) despite the FITC-conjugated siRNA demonstrating high transfection efficiency (Figure 4.7Biii).

4.2.3.2 L-type VGCC-dependent activation of the NF-κB pathway

The activation of Tpl-2 in macrophages has been shown to occur via the phosphorylation and degradation of p105, the NFκB binding partner of Tpl-2 [74]. p105 is a member of the NFκB pathway and its degradation via proteolysis is due to its phosphorylation by IKKβ [74]. To assess IKKβ and Tpl-2 activity indirectly, two markers of the NFκB pathway were used: IκBα and p65. The degradation of IκBα occurs due to the phosphorylation of the protein by activated IKKβ and subsequent ubiquitination and processing by the proteosome. p65 is an NFκB protein bound in the basal state by an IκB inhibitor protein. The release by p65 by IKK mediated phosphorylation of IκBα causes p65's phosphorylation. Therefore, IκBα degradation acts as a direct readout for IKK activity whilst p65 phosphorylation is an indirect readout of IKK activity.

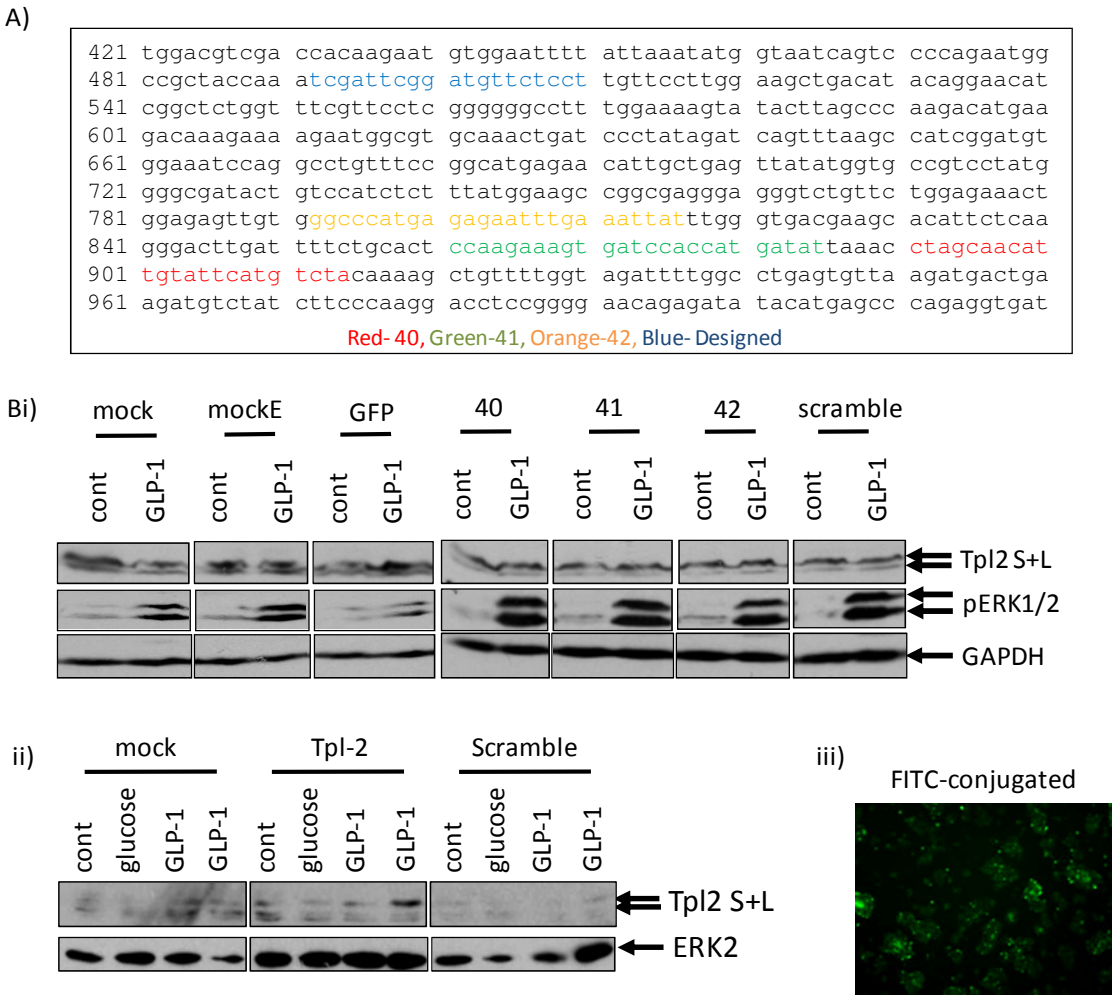


Figure 4.7. Commercially available and designed mouse siRNA oligos siRNA against Tpl-2 are ineffective at knocking down Tpl-2 expression in MIN6 cells. A) Section of the Tpl-2 nucleotide CDS with the nucleotides highlighted that have been targeted by the various siRNA oligos. B) MIN6 cells were electroporated with siRNA targeted against Tpl-2/MAP3K8 gene and appropriate controls. Cells were counted and $9x10^5$ cells/well were transfected with 20ng siRNA. i) 3 independent stealth siRNA constructs purchased from Invitrogen against the mouse gene were used alongside a scrambled siRNA as a negative control, a FITC-conjugated siRNA as a marker of transfection efficiency and 2 mock conditions, mock and mock with electric current. MIN6 cells were preincubated with KRB supplemented with 1mM glucose for 1hour. Each siRNA condition was then treated with either GLP-1 plus glucose or left as control. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. ii) The designed siRNA against Tpl-2 was tested as in i). iii) At 72hours post-transfection the transfection efficiency was assessed by observing the FITC signal conjugated to the siRNA.

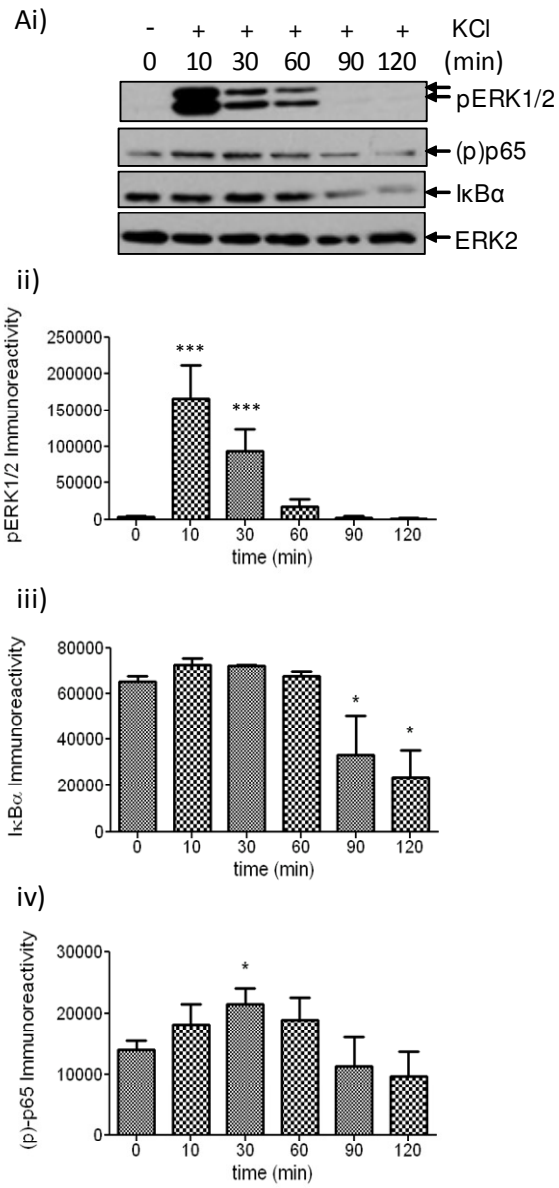


Figure 4.8. Markers of NFκB pathway activation parallels ERK1/2 phosphorylation upon stimulation with depolarising concentrations of K^+ in MIN6 cells. A) MIN6 cells were preincubated for 1hour with KRB supplemented with 1mM glucose then treated for the times indicated with K50. ERK1/2 and p65 phosphorylation, and total levels of IκBα and ERK2 were determined via Western blotting using specific antibodies. Representative blots are shown with densitometry below ii) pERK1/2 densitometry, iii) IκBα densitometry, iv) (p)-p65 densitometry. Data are mean +S.e.m. (n=3). Statistical comparisions were by one-way ANOVA with Dunnett's range test compared to cont; *, $P < 0.05$

Initially, to assess whether L-type VGCC actvation was sufficient to activate the NFκB pathway, MIN6 cells were incubated with K50 and the phosphorylation of ERK1/2 and p65 as well as total levels of IκBα were assessed by Western blotting. K50 modestly but statistically significantly stimulates the phosphorylation of p65. Furthermore, there is a significant decrease in the total level of IκBα by 90 min (Figure 4.8Aiii). This suggests that global rises in $[Ca^{2+}]_i$ via influx thourough the L-type VGCC are sufficient to stimulate NFκB pathway activation.

I then investigated whether the IKKβ and NFκB pathway is activated upon GLP-1 stimulation in MIN6 cells with glucose stimulation alone or GLP-1 in the presence of glucose for up to 30 min. Both glucose and GLP-1 in the presence of glucose, had a tendency to increase the phosphorylation of p65 and the temporal profile of p65 phosphorylation was similar to that of ERK1/2 phosphorylation (Figure 4.9A). Furthermore, although not significantly decreased, the total level of IκBα had a tendency to be lower at 30 min of stimulation with GLP-1 (Figure 4.9Aiii). However, glucose alone failed to stimulate IκBα degradation (Figure 4.9Aiii). MIN6 cells were also stimulated with GLP-1 in the presence of glucose for up to 120 min to assess whether IκBα levels were decreased at later time points. ERK1/2 phosphorylation was maintained thouroughout the 120 minute time period but despite the tendency of lower levels of total IκBα, after 60 min of stimulation no statistically significant decrease in IκBα was observed (Figure 4.9B).

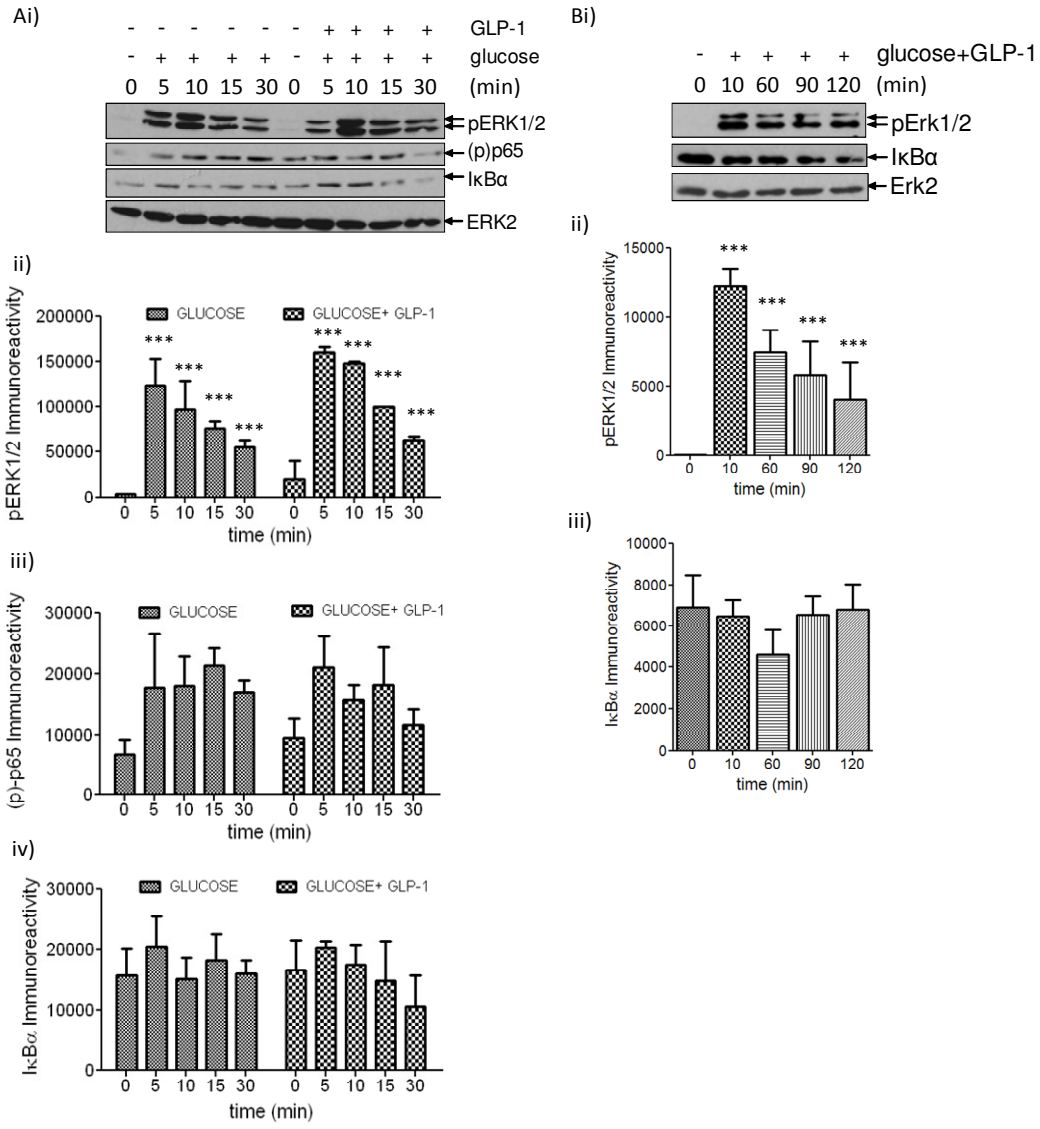


Figure 4.9. Investigation into markers of NFκB pathway activation upon glucose and GLP-1 stimulation of MIN6 cells. Ai) MIN6 cells were preincubated for 1hour with KRB supplemented with 1mM glucose then treated for the times indicated with either 16.7mM glucose alone or in the presence of 10nM GLP-1. ERK1/2 and p65 phosphorylation, and total levels of IκBα and ERK1/22 were determined via Western blotting using specific antibodies. Representative blots are shown with densitometry below ii) pERK1/2 densitometry, iii) (p)-p65 densitometry, iv) IκBα densitometry. Data are mean +S.e.m. (n=3). Statistical comparisions were by one-way ANOVA with Dunnett's range test compared to basal levels (0min); *, $P < 0.05$. B) MIN6 cells were preincubated for 1hour with KRB supplemented with 1mM glucose then treated for the times indicated with 16.7mM glucose in the presence of 10nM GLP-1. ERK1/2 phosphorylation and total levels of IκBα and ERK2 were determined via Western blotting using specific antibodies. Representative blots are shown with densitometry below ii) pERK1/2 densitometry, iii) IκBα densitometry. Data are mean + s.e.m. (n=3). Statistical comparisions were by one-way ANOVA with Dunnett's range test compared to basal levels (0min); *, $P < 0.05$.

4.3 Discussion

4.3.1 Summary

The aim of this study was to investigate the mechanisms mediating the Ca^{2+}-dependent signalling between the L-type VGCC and ERK1/2 upon GLP-1 stimulation. My attempts to utilise the DHP-resistant L-type VGCC constructs to investigate the involvement of CaM pre-bound to the channel were unsuccessful due to the inability to express the channel at the cell surface in β-cell lines. However, I went on to investigate the involvement of potential Ca^{2+}-dependent proteins. Using dominant negative adenoviruses I demonstrated that GLP-1 stimulated ERK1/2 activation is independent of CaMKII and Ras activation. Furthermore, I demonstrated that GLP-1 stimulated ERK1/2 activation is independent of classical and novel isoforms of PKC. However, the chouronic treatment of MIN6 cells with phorbol ester, which leads to the downregulation of DAG-dependent PKC isoforms, significantly inhibited ERK1/2 activation. I have also demonstrated that upon treatment of MIN6 cells with depolarising concentrations of K^+ markers of the NFκB pathway are activated or degraded which indicates an activation of IKKβ.

4.3.2 The role of Tpl-2 in GLP-1 mediated ERK1/2 activation.

In order to assess the involvement of Tpl-2 in GLP-1 mediated ERK1/2 activation I utilised thouree commercially available and one designed siRNA molecule targeted against Tpl-2. None of these siRNAs successfully inhibited the expression of Tpl-2 or prevented ERK1/2 activation (Figure 4.7). The lack of success with the siRNA led to the acquisition of islets from Tpl-2 KO mice. At present the investigation into the role of Tpl-2 in GLP-1 mediated ERK1/2 activation in β-cells is continuing with these KO mice.

I have demonstrated in this thesis that GLP-1 mediated ERK1/2 activation is independent of Ras and our laboratory has evidence that Tpl-2 is the kinase responsible for mediating MEK and ERK1/2 phosphorylation upon GLP-1 stimulation. If this is the case then it is possible that this is mediated by the Ca^{2+}-dependent activation of IKKβ. Indeed, I have demonstrated that K50 increases the degradation of IκBα and the phosphorylation of p65 indirectly indicating the activation of the IKK complex. Although GLP-1 did not show any statistically significant effect on the NFκB pathway

markers there was a trend towards a decrease in IκBα levels and an increase in p65 phosphorylation.

4.3.3 NFκB signalling correlates with ERK1/2 phosphorylation upon GLP-1 stimulation

This study has demonstrated that only K50 is able to stimulate the phosphorylation of p65 and mediate the degradation of IκB (Figure 4.8 and 4.9), indirectly indicating the activation of the IKK complex. The difference in signalling between GLP-1 and K50 may be due to the strength of stimulation. K50 stimulates global changes in $[Ca^{2+}]_i$ at a much greater magnitude to GLP-1, therefore the changes in IκBα levels may be due to the localisation or differences in their ability to elicit rises in $[Ca^{2+}]_i$. We know that there is a local Ca^{2+} signal mediating ERK1/2 phosphorylation and perhaps this is activating only a specifically localised signalling cascade. However, with K50, all generic Ca^{2+}-dependent signalling pathways may be activated within the β-cell and many different Ca^{2+}-dependent spatially localised signalling mechanisms may be activated as a consequence of such a large rise in Ca^{2+} leading to a greater magnitude of overall signalling.

The localisation of IKKβ at the plasma membrane does occur in β-cells thourough its interaction with ELKS and thus a local Ca^{2+} signal could mediate IKKβ activation [634]. ELKS is expressed in β-cells and is a structural related protein to the active zone protein CAST, which defines the site of exocytosis for neurotransmitters in neurones [635]. In β-cells ELKS is found localised with insulin granules and syntaxin-1, and has been suggested to have a role in regulating insulin exocytosis [634]. ELKS has also been demonstrated to act as a scaffold protein presenting IκBα proteins to the IKK complex [636]. With ELKS localised with insulin granules it is likely that at least a proportion of the cellular ELKS protein is within the microdomain of the L-type VGCC. Therefore, ELKS could be a candidate protein for linking localised Ca^{2+} signalling and IKKβ function.

4.3.4 Role of Ras in GLP-1 stimulated ERK1/2 activation

In many systems Ras activation is required for the activation of ERK1/2 in response to depolarisation and mitogenic signals. In neuronal cell types, depolarisation induces ERK1/2 activation thourough Ca^{2+} influx and the activation of Ras via the Ras specific

GEFs [637, 638]. Furthermore, Ca^{2+} influx after stimulation with neutrophins or growth factors positively modulates Ras signalling in neurones and PC12 cells [639, 640]. However, in other cell types the existence of Ras-independent ERK1/2 activation has been demonstrated. In keratinocytes Ca^{2+} induced differentiation occurs via activation of ERK1/2 [641]. The stimulation of keratinocytes with raised extracellular Ca^{2+} lead to a downregulation of Ras whilst ERK1/2 activation remained unchanged. In addition the overexpression of RasN17 had no effect on differentiation [641]. In PC12 cells that constitutively overexpress dominant negative Ras, various agonists known to modulate Ca^{2+} signals, such as carbachol, were still able to mediate ERK1/2 activation demonstrating the existence of Ras-independent signalling mechanisms to ERK1/2 [642].

In β-cells the role of Ras in mediating glucose and GLP-1 stimulated ERK1/2 activation is unclear. Previous reports have come to differing conclusions about the necessity of Ras using different dominant negative constructs. The initial phase of ERK1/2 activation (10min) mediated by glucose and GLP-1 has been demonstrated to be independent of Ras activation thourough the use of overexpressed dominant negative RasN17 constructs [68, 261]. Conversely, the expression of a different dominant negative form of Ras (RasG15A) blocked glucose stimulated ERK1/2 activation in INS-1 cells [67]. However, in this study there was no positive control to show the effectiveness of the RasG15A adenovirus which is important as adenovirus over-expression can non-specifically inhibit cell signalling (unpublished observation). In this study in MIN6 cells adenoviral mediated overexpression of the dominant negative RasN17 was assessed both at the initial (10min) and during the sustained local Ca^{2+}-dependent (30min) ERK1/2 response, with the appropriate controls, and demonstrates that GLP-1 mediates ERK1/2 activation via a Ras-independent mechanism in MIN6 cells. The treatment of MIN6 cells with glucose and K20, which mediate similar levels of maximal ERK1/2 activation, were unaffected by the presence of the RasN17 construct at either the peak or sustained time point (Figure 4.6). However, ERK1/2 activation stimulated by K50, an agonist that mediates a much larger global Ca^{2+} response thourough the L-type VGCC (Figure 3.2) and stronger ERK1/2 activation compared to glucose and K20, was significantly inhibited by the presence of RasN17 in MIN6 cells. Interestingly, there is a residual level of ERK1/2 activation which is Ras-independent. This suggests that upon stimulation of MIN6 cells with the strong

depolarising stimulus K50, two mechanisms of Ca^{2+} dependent ERK1/2 activation are occurring. It is possible that one mechanism is Ras-dependent mediated by large global rises in Ca^{2+} and the second is Ras-independent mediated by a specific coupling of the L-type VGCC to ERK1/2.

The two phases of ERK1/2 activation in MIN6 cells has been documented previously. GLP-1 stimulation of MIN6 cells in the absence of glucose was able to stimulate two phases of ERK1/2 activation. The initial phase was shown to be PKA-dependent whilst the second phase was dependent on the scaffolding and signalling actions of β-arrestin [592]. The authors of this published work did not investigate any further differences in the signalling mechanisms leading to ERK1/2 activation. However, the PKA and β-arrestin dependent phases of ERK1/2 activation were demonstrated in the absence of glucose and therefore, the authors are looking at the signalling properties of the GLP-1R in isolation. As no Ca^{2+} response was observed in their studies and I have demonstrated that both K50 and GLP-1 stimulated ERK1/2 activation is mediated by a Ca^{2+}-dependent pathway, the two phases documented in my study are different to this previously observed signalling.

4.3.5 Evidence that CaMKIIδ is not required for GLP-1 mediated ERK1/2 activation

CaMKII possesses many of the properties required to mediate a local Ca^{2+} signal from the L-type VGCC to ERK1/2. It is activated by CaM, which has been found tethered to the channel in a variety of cell types and is known to mediate local Ca^{2+}-dependent signalling [241, 242, 540-542, 591, 611]. Furthermore, CaMKII has been demonstrated to directly phosphorylate IKKβ [596], a protein previously shown to be involved in GLP-1 mediated ERK1/2 activation [68, 239]. In this study I have re-evaluated the role of CaMKII in GLP-1 stimulated ERK1/2 activation using a dominant negative CaMKIIδ. Expression of the CaMKIIδ dominant negative protein had no significant effect on GLP-1 stimulated ERK1/2 activation (Figure 4.4B). This result suggests than CaMKII is not involved in GLP-1 mediated ERK1/2 activation. However, I did not have a positive control. Therefore, I cannot rule out that the dominant negative protein may not be functioning.

4.3.6 Inhibition of CaMKIIδ increases the basal pCREB level in MIN6 cells

The expression of dominant negative CaMKIIδ, despite having no effect on ERK1/2 phosphorylation, led to an increase in basal levels of CREB phosphorylation at S133 in MIN6 cells (Figure 4.4B). Increases in cAMP are known to mediate CREB phosphorylation at S133 thourough PKA phosphorylation. The increase in basal levels of pCREB could be due to an increase in cAMP as CaMKII is known to negatively regulate an adenylyl cyclase (AC) isoform [643]. The AC isoform known to be regulated by CaMKII is AC3 [643] and in islets multiple isoforms from AC2-7 have been identified [197]. Therefore, it is possible that in basal conditions the lack of CaMKII activity leads to an increase in AC3 activity, raised basal cAMP concentrations which leads to an increase in basal CREB phosphorylation at S133.

4.3.7 Role of DAG-dependent kinases

The results of this study using PKC inhibitors indicate that conventional and novel PKC isoforms are not mediating GLP-1 stimulated ERK1/2 activation (Figure 4.5A). As the inhibitors used within this study do not affect the atypical isoforms of PKC (ξ and τ/λ) these isoforms cannot be ruled out as mediators of GLP-1 stimulated ERK1/2 activation. One way in which atypical PKC isoforms could affect GLP-1 stimulated ERK1/2 activation is thourough their interactions with IKKβ. PKCα and atypical PKC isoforms have been demonstrated to bind to IKKβ, with dominant negative PKCζ demonstrated to affect IKKβ activity upon TNFα stimulation of HEK_{293} cells [644].

Despite the lack of effect of the PKC inhibitors, the chouronic treatment of MIN6 cells with phorbol ester led to an attenuation of GLP-1 stimulated ERK1/2 activation (Figure 4.5). The downregulation of DAG-dependent PKCs with chouronic phorbol ester treatment has been used previously to decipher if PKC isoforms are required for ERK1/2 activation in β-cells. One report from INS-1 cells suggests that glucose mediated ERK1/2 activation is unaffected by 22h phorbol ester treatment [622]. In a separate study in INS-1 cells depolarising concentrations of K^+ mediate ERK1/2 activation via a mechanism unaffected by phorbol ester treatment whilst glucose mediated ERK1/2 activation is abolished [145]. However, in MIN6 cells extended phorbol ester treatment has been demonstrated to abolish glucose mediated ERK1/2

activation [145]. This suggests that there is a disparity between β-cell models and no investigation in islets has been carried out.

One issue raised by the observation in the literature that ERK1/2 activation via depolarising concentrations of K^+ is not sensitive to chouronic phorbol ester treatment whereas DAG-dependent proteins appear to be required for glucose and GLP-1 stimulated ERK1/2 activation, is that despite K^+, glucose and GLP-1 having similar profiles of ERK1/2 activation and requiring many of the same pathway components, they are likely to activate ERK1/2 by distinct mechanisms. One possibility is that glucose and GLP-1 may mediate ERK1/2 activation via oscillations of $[Ca^{2+}]_i$ mediated by influx thourough the L-type VGCC resulting in Ras-independent ERK1/2 activation (Figure 3.9 and 4.6). However, strong depolarising concentrations of K^+, such as K50, stimulate a sustained rise in $[Ca^{2+}]_i$ mediated thourough Ca^{2+} influx via the L-type VGCC resulting in Ras-dependent ERK1/2 activation (Figure 3.3 and 4.6). One of the reasons for this difference in signalling between the two agonists, as well as the depolarising concentrations of K^+ reaching a threshold of $[Ca^{2+}]_i$, as discussed above (Section 4.3.3), could be due to the difference in the profile of Ca^{2+} signalling. Ca^{2+} oscillations or plateaus are known to activate different downstream signalling molecules and require different thresholds for activation. For example, an oscillatory Ca^{2+} signal can heighten the activation of certain Ca^{2+}-dependent signalling molecules over a sustained plateau of Ca^{2+} [645, 646] and thus there are differences in the signalling pathways activated by different agonists.

4.3.8 Conclusions

In conclusion, this study has demonstrated that upon stimulation with agonists that mediate large global rises in $[Ca^{2+}]_i$ two mechanisms of ERK1/2 activation occur: one Ras-dependent and one Ras-independent. However, GLP-1 mediates ERK1/2 activation via a Ras-independent pathway during both the initial and sustained phases identified in Chapter 3. ERK1/2 activation stimulated by GLP-1 also appears to be CaMKII and PKC independent. Despite the lack of direct evidence for the involvement of Tpl-2 in GLP-1 stimulated ERK1/2 activation in this study, I have demonstrated that the NFκB pathway is activated upon stimulation with depolarising concentrations of K^+. GLP-1 stimulation also exhibited a trend towards NFκB pathway activation despite failing to reach statistical significance.

Chapter 5: Muscarinic receptor stimulated ERK1/2 activation in pancreatic β-cells

5.1 Introduction

5.1.1 Background

In the main introduction I discuss signalling via muscarinic receptors and the importance of the mAChR activation upon pancreatic β-cells (Section 1.4). Figure 5.1 summarises the current understanding of the signalling mechanisms mediating rises in $[Ca^{2+}]_i$ subsequent to mAChR activation in β-cells. G_q-receptor activation leads to PLC mediated depletion of PIP_2 and the production of IP_3 and DAG. The translocation of IP_3 to the ER mediates Ca^{2+} release from the store via IP_3Rs. In addition, the activation of the G_q-receptor mediates Na^+ influx and this combined with Ca^{2+}-release from the ER culminates in a depolarisation of the plasma membrane sufficient to activate VGCCs. In the presence of glucose, mAChR also potentiates glucose mediated rises in $[Ca^{2+}]_i$ by the mechanisms discussed above.

As carbachol has been reported to activate ERK1/2 in islets [419] and as ERK1/2 plays an important role in β-cell function and proliferation [67, 138, 139, 143-145], the principal aim of this study was to determine the signalling mechanism/s leading to carbachol induced ERK1/2 activation in pancreatic β-cells. The G_q-coupled M_3 mAChR has been identified as the key mediator or ACh action on β-cells [319, 647] thus the downstream components of the receptor signalling and key downstream components of mAChR stimulated ERK1/2 activation in other systems have been investigated.

5.1.2 Aims of the chapter

Initially I intend to determine whether ERK1/2 activation is indeed mediated via a G_q-coupled receptor and whether it occurs in a concentration dependent manner. In addition, I intend to establish whether mAChR mediated ERK1/2 activation is independent to and/or potentiating glucose stimulated ERK1/2 activation.

Once these parameters have been established, I intend to investigate signalling proteins that have been identified as mediators of ERK1/2 signalling in other systems, including PKC, Ras and rises in $[Ca^{2+}]_i$.

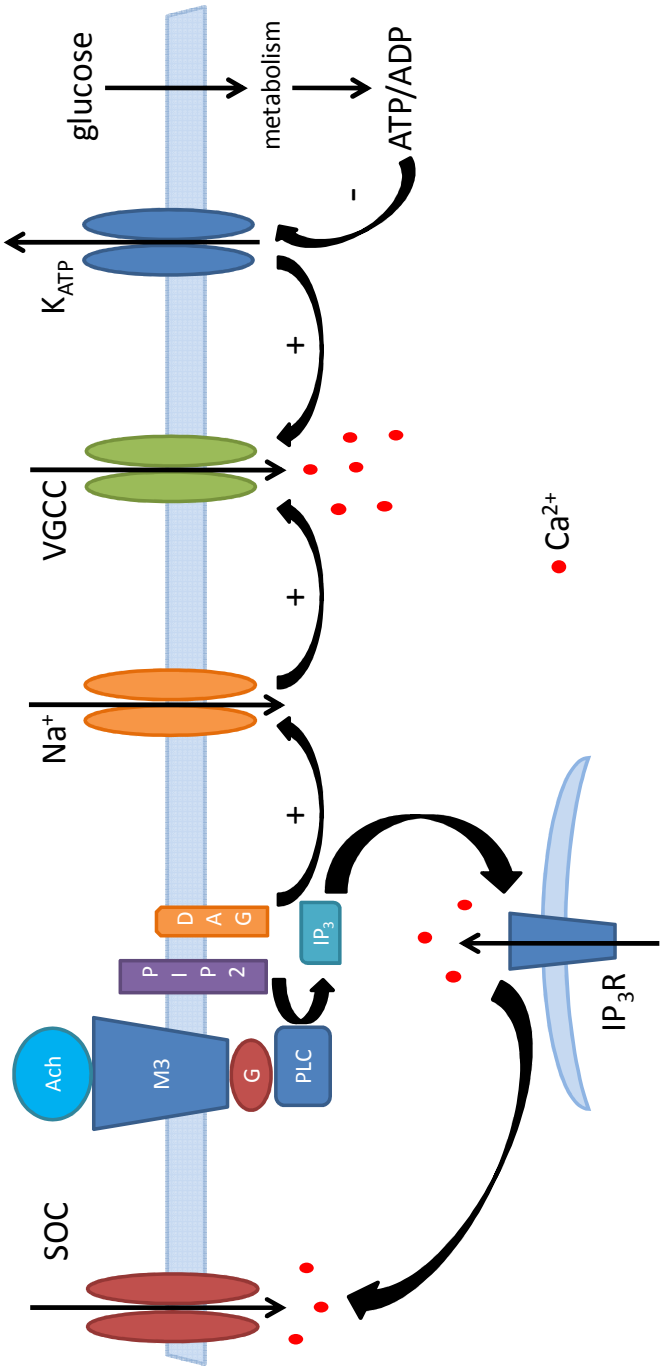


Figure 5.1. Mechanism by which mAChR Activation is known to activate Ca^{2+} influx in pancreatic β-cells. Activation of the mAChR leads to Ca^{2+} efflux from the ER store via IP_3R. This causes capacitative Ca^{2+} entry via voltage-independent SOCs. Additionally mAChRs depolarise the plasma membrane via non-specific Na^+ channels leading to depolarisation and activation of VGCCs. In the presence of glucose additional depolarisation and Ca^{2+} entry occurs via the inactivation of the K_{ATP} thourough increases in the ATP/ADP ratio after metabolism.

5.2 Results

5.2.1 Carbachol stimulates ERK1/2 phosphorylation in the pancreatic β-cell line MIN6 via the activation of a muscarinic G_q-coupled receptor.

To determine whether mAChR activation can induce ERK1/2 activation in the β-cell line MIN6, I treated cells with the muscarinic agonists carbachol and methacholine in the presence or absence of 10μM muscarinic antagonist atropine. The activation of the mAChR by both agonists stimulated the phosphorylation of ERK1/2, which was effectively blocked by atropine (Figure 5.2A). To further characterise mAChR mediated activation of ERK1/2, MIN6 cells were treated with increasing concentrations of carbachol and the phosphorylation of ERK1/2 determined at 2min (Figure 5.2B). Carbachol stimulated ERK1/2 phosphorylation in a concentration dependent manner with an EC_{50} of 7.32μM (Figure 5.2B). Based on previous reports the main mAChR subtype expressed in β-cells is the M_3 receptor [319]. The M_3 mAChR couples to G_q resulting in the activation of PLC. Therefore, to investigate whether carbachol stimulated ERK1/2 phosphorylation requires the activation of PLC, MIN6 cells were treated with carbachol in the presence or absence of the PLC inhibitor U73122. U73122 abolished carbachol stimulated ERK1/2 activation (Figure 5.2c) providing evidence that G_q coupled PLC signalling is mediating mAChR stimulated ERK1/2 activation.

5.2.2 mAChR activation of ERK1/2 is independent of glucose.

mAChR activation has been shown to cause a small but significant rise in insulin secretion in the absence of glucose [447, 455] but potently potentiates glucose stimulated insulin secretion [296, 318, 348]. To evaluate the effect of glucose on carbachol stimulated ERK1/2 activation, MIN6 cells were treated with carbachol for up to 30min in the presence and absence of glucose. Carbachol treatment in the absence of glucose led to a rapid and robust phosphorylation of ERK1/2 at 2min (Figure 5.3A). The incubation of MIN6 cells in 16.7mM glucose also stimulated ERK1/2 phosphorylation. This peaked at around 10min and remained high for up to 60min (Figure 5.3B). The co-application of carbachol and glucose resulted in ERK1/2 phosphorylation at 2min sustained for 60min. However, glucose was unable to potentiate carbachol stimulated

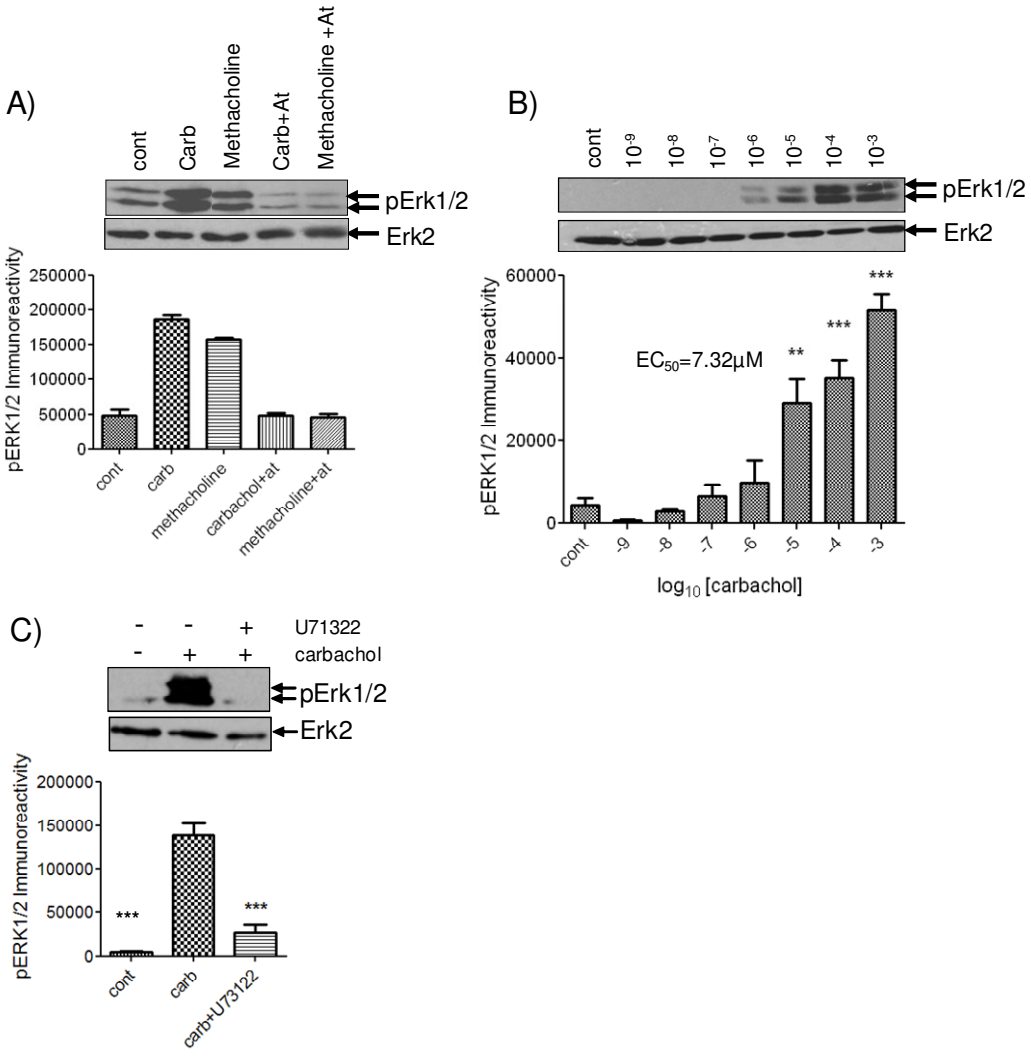


Figure 5.2. Carbachol concentration response curves. A) Cells were pretreated alone or with 10µM atropine for 30min prior to treatment with 1mM carbachol or 1mM methacholine for 2min. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. Statistical comparisons were by Student's t-test comparing agonist in the presence and absence of atropine; ***, $P < 0.001$ (n=3). B) MIN6 cells preincubated for 1hour with KRB were treated with increasing concentrations of carbachol for 2min as shown. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. (n=3). Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to control; **, $P < 0.01$; ***, $P < 0.001$. Data were fit using a variable slope model in GraphPad Prism and EC_{50} determined with the software. C) MIN6 cells were pretreated alone or with 1µM U73122 for 30min prior to treatment with 1mM carbachol for 2min. Representative blot is shown above with densitometry below. Data are mean +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$ (n=3).

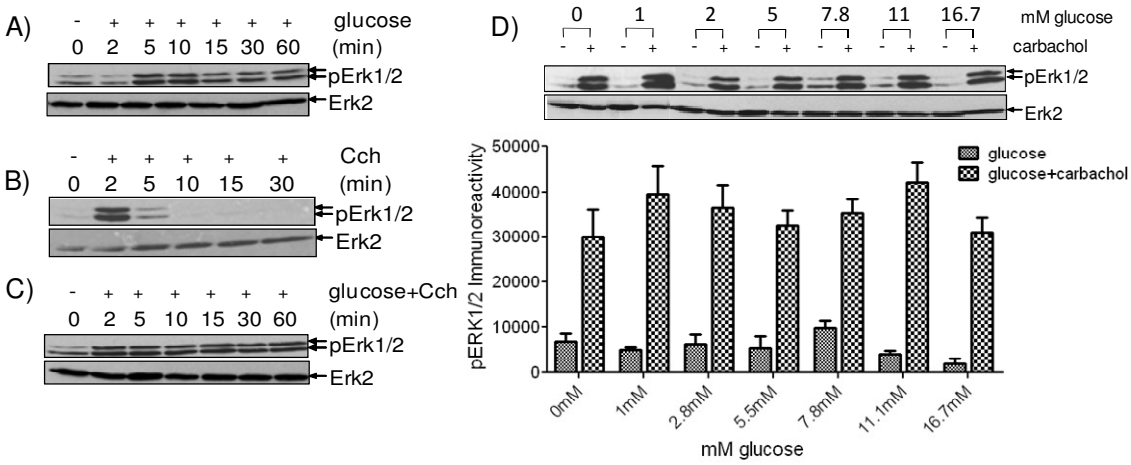


Figure 5.3. Carbachol time course. MIN6 cells preincubated for 1hour with KRB were treated with A) 16.7mM glucose, B) 1mM carbachol or C) 16.7mM glucose plus 1mM carbachol, for the times stated. ERK1/2 phosphorylation was assessed by Western blotting using phospho-specific antibodies. Representative blot is shown (n=3). D) MIN6 cells incubated in KRB for 1hour were treated with increasing concentrations of glucose and 1mM carbachol for 2min as shown. A representative blot is shown above with densitometry below +S.E.M. (n=3).

ERK1/2 activation (Figure 5.3C). To investigate whether glucose potentiates carbachol-stimulated ERK1/2 activation, MIN6 cells were stimulated with 1mM carbachol in the presence of increasing concentrations of glucose (Figure 5.3D). Glucose had no effect on carbachol induced ERK1/2 activation. As glucose has no effect on mAChR stimulated ERK1/2 activation in MIN6 cells, in order to specifically investigate how mAChR stimulation results in ERK1/2 activation all further experiments were performed in the absence of glucose.

5.2.3 Carbachol stimulated ERK1/2 activation is dependent upon MEK and partially dependent on Ras.

To determine whether carbachol stimulated ERK1/2 phosphorylation occurs thourough MEK, MIN6 cells were incubated with the MEK inhibitors PD098059 and U0126 prior to carbachol stimulation. Both of these MEK inhibitors effectively attenuated carbachol induced ERK1/2 activation (Figure 5.4A) confirming the requirement for MEK. To investigate the role of Ras in mediating mAChR induced ERK1/2 activation a recombinant adenovirus expressing a dominant negative Ras protein (RasN17) was infected into MIN6 cells. 48h post-infection, the cells were treated with carbachol, EGF (whose activation of ERK1/2 is Ras dependent) or GLP-1 (whose activation of ERK1/2 is Ras-independent) [68]. As expected, RasN17 completely inhibited EGF stimulated ERK1/2 phosphorylation and had no effect on GLP-1 stimulated ERK1/2 phosphorylation (Figure 5.4B) However, carbachol stimulated ERK1/2 activation was significantly, although not completely, inhibited by the overexpression of the RasN17 (Figure 5.4B). In the presence of RasN17, carbachol mediated ERK1/2 activation was inhibited by ~60% indicating that Ras activation is partially required for carbachol induced ERK1/2 activation.

5.2.4 Carbachol stimulated ERK1/2 activation is independent of the activation of both novel and classical isoforms of PKC.

Stimulation of G_q coupled mAChRs results in the activation PLC which cleaves PIP_2 generating IP_3 and DAG [358, 648]. To determine that carbachol was activating the PLC-DAG-PKC pathway in β-cells, MIN6 cells were infected with an adenovirus

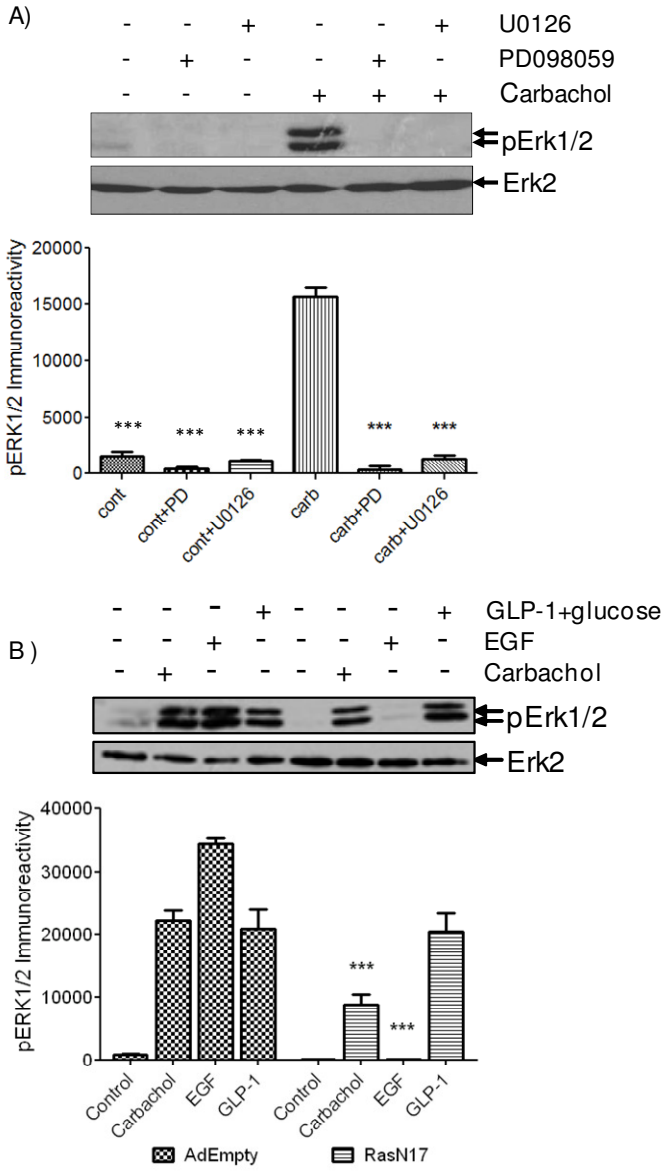


Figure 5.4. Carbachol stimulation of ERK1/2 in MIN6 cells requires active MEK but does require Ras. MIN6 cells preincubated for 1hour in KRB were treated with 1mM carbachol for 2min. A) Cells were pretreated alone or in the presence of 1µM PD098059 or 20µM U0126 for 30min. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$ (n=3). B) MIN6 cells were infected with the control adenovirus AdEmpty-EGFP, or infected with recombinant adenovirus expressing RasN17 (AdRasN17). 48hours post-infection, the cells were preincubated for 1hour in KRB prior to treatment with either 1mM carbachol for 2min, 20ng/ml EGF for 5min or 10nM GLP-1 plus 16.7mM glucose for 10min. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Statistical comparisons were by Students's t-test compaing AdEmpty virus versus RasN17 virus for the same treatments ; ***, $P < 0.001$ (n=3).

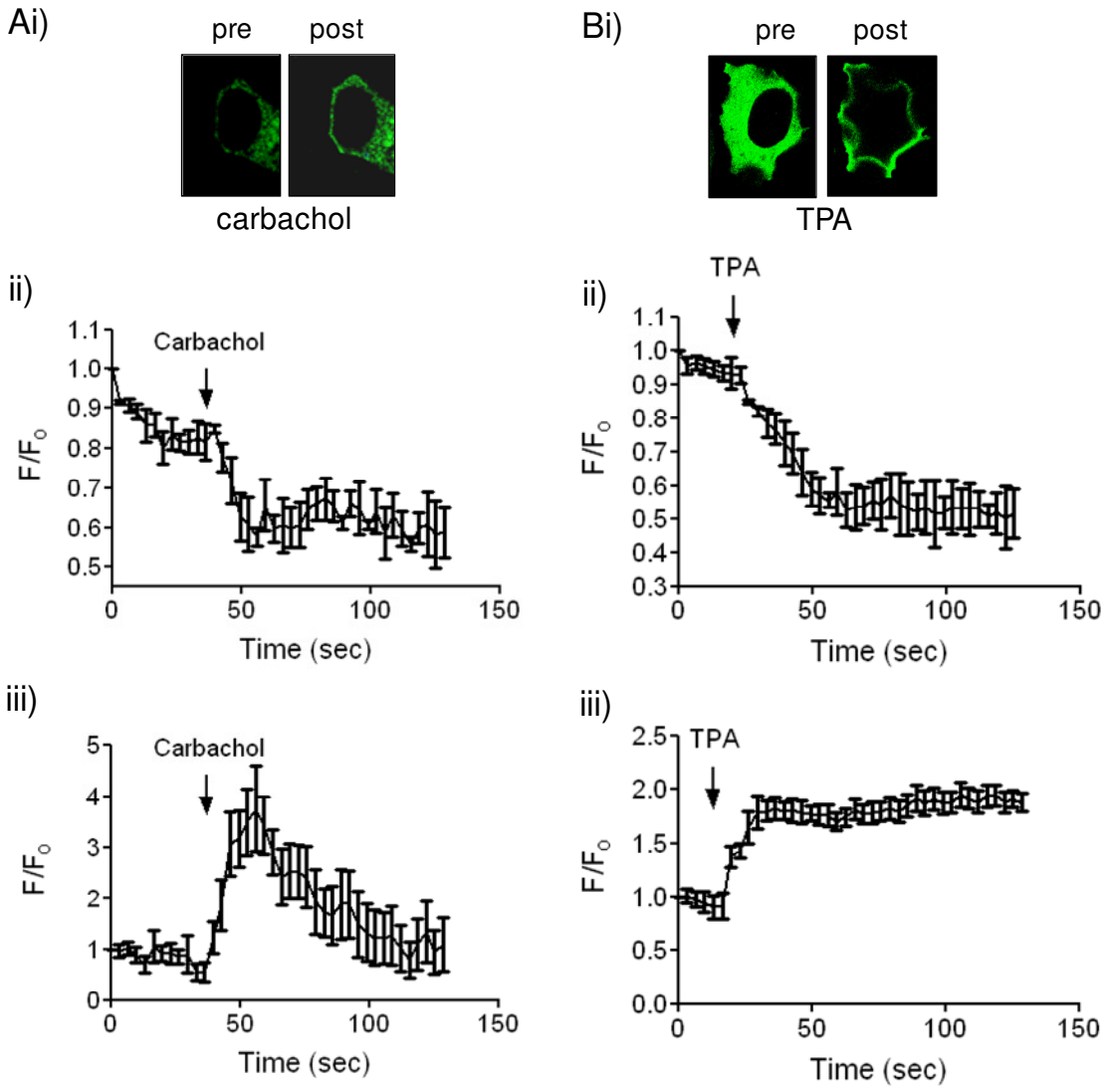


Figure 5.5. Carbachol stimulates the translocation of PKCε in MIN6 cells. MIN6 cells were infected with an adenovirus expressing GFP-PKCε 48 hours before experimentation at room temperature. Cells were preincubated for 1hour in KRB then treated with either A) 1mM carbachol or B) 1μM TPA (as a control). ROIs were placed in the cytoplasm ii) or membrane iii) with the average pixel intensity over time for each ROI were monitored and fluorescence intensity analysed using the Fluoview Tiempo software (version 4.3) then divided by the initial fluorescence and expressed as F/F_0.

expressing GFP-PKCε whose translocation to the plasma membrane upon agonist stimulation is dependent upon an increase in DAG. Both carbachol (Figure 5.5A) and TPA (Figure 5.5B) treatment led to the decrease of GFP-PKC within the cytosol (i) and an increase at the plasma membrane (ii). This demonstrates that DAG is being produced and that PKCε is activated in response to mAChR activation and TPA treatment.

In a variety of cell lines including fibroblasts, PC12 cells and neurones [409, 410], PKC activation is required for the activation of ERK1/2. In order to determine the role of PKC in mediating carbachol stimulated ERK1/2 activation in β-cells, MIN6 cells were treated with BIMI, Ro320432 and Gö6976 to selectively inhibit classical and novel PKC isoforms [631, 632]. In addition, MIN6 cells were subjected to chouronic stimulation with TPA, which is known to down-regulate all DAG-dependent PKCs [633]. In parallel, in order to assess whether PKC activation affects the $[Ca^{2+}]_i$ response mediated by mAChR activation, the population Ca^{2+} response to carbachol was measured in the presence of the various PKC inhibitors as described above. None of the PKC inhibitors nor chouronic TPA treatment affected carbachol induced ERK1/2 activation or $[Ca^{2+}]_i$ responses (Figure 5.6A and C) but all inhibitors. All together this data provides evidence that PKC activation is not required for carbachol stimulated ERK1/2 activation in β-cells.

5.2.5 Carbachol stimulates PLC activity

As carbachol stimulated ERK1/2 activation is inhibited by the PLC inhibitor U73122 (Figure 5.2C) and DAG production has been demonstrated to occur upon mAChR activation (Figure 5.5), PLC should be active and depleting PIP_2 and creating IP_3. To confirm this I measured utilised a biosensor consisting of a PH-domain of PLCδ fused to eGFP (eGFP-$PH_{PLC\delta1}$) [649-651] to monitor PLC activity. This sensor has been previously used in a variety of cell types, including MIN6 cells, to measure IP_3 translocation [462, 652-655] as the probe binds phosphoinostides with phosphorylation at the 4 and 5 positions (including PIP_2) but binds to IP_3 with a higher degree of affinity. As the probe binds PIP_2 in unstimulated cells it is present at the plasma membrane, but upon PLC activation and production of IP_3 the probe translocates to the cytosol. However, in MIN6 cells the contribution of PIP_2 depletion and IP_3 production towards the probes translocation is unclear.

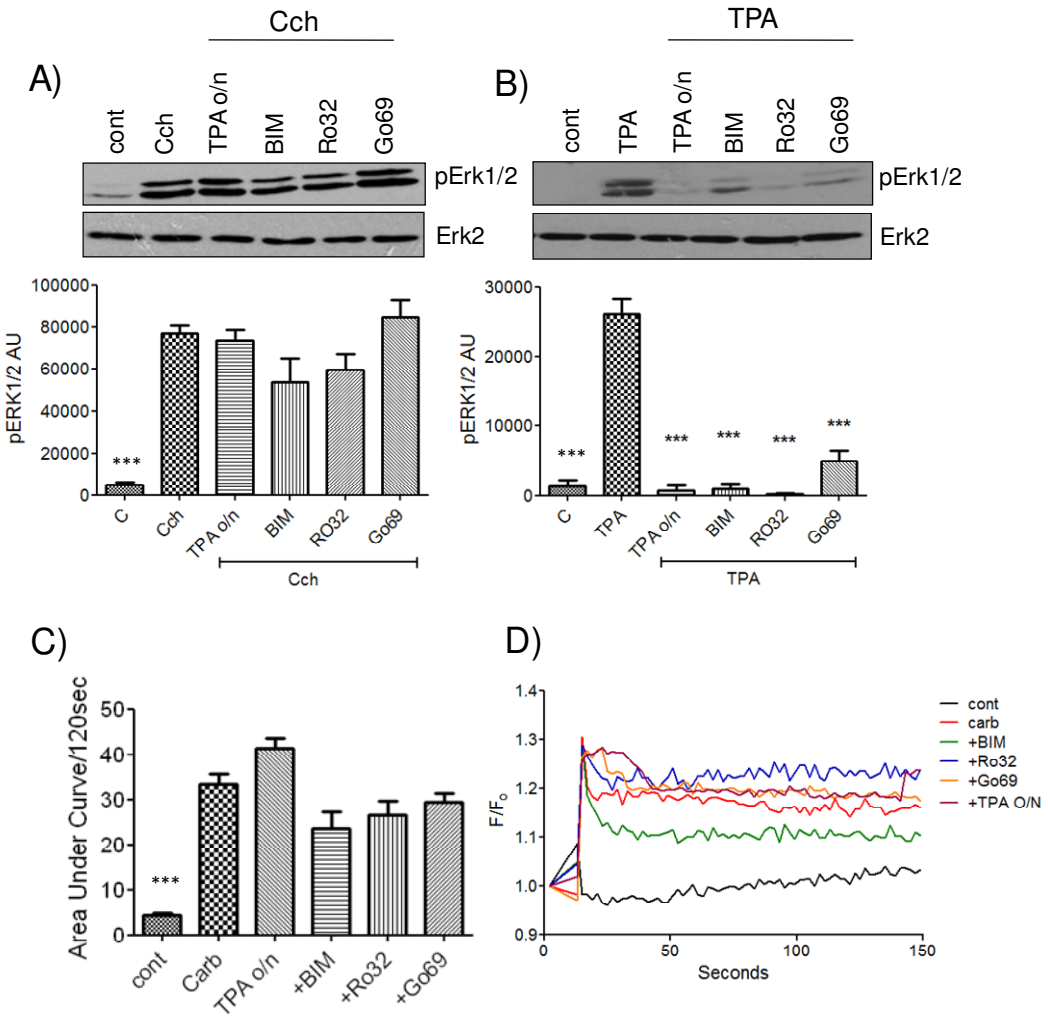


Figure 5.6. PKC requirement for carbachol mediated ERK1/2 activation

MIN6 cells preincubated for 1hour in KRB were treated with A) 1mM carbachol for 2min or B) 10µM TPA for 1hour. Cells were preincubated alone or in the presence of 1µM of various PKC inhibitors for 30min. In addition DAG-regulated PKC isoforms were down-regulated with 1µM TPA (phorbol ester) treatment for 16h. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to A) carbachol or B) TPA; ***, $P < 0.001$ (n=3). $[Ca^{2+}]_i$ levels were assessed for the same treatments as A). Data represent the mean +S.E.M. of the increase in $[Ca^{2+}]_i$ levels as an C) area under the curve across 2min and D) representative traces. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$ (n=3).

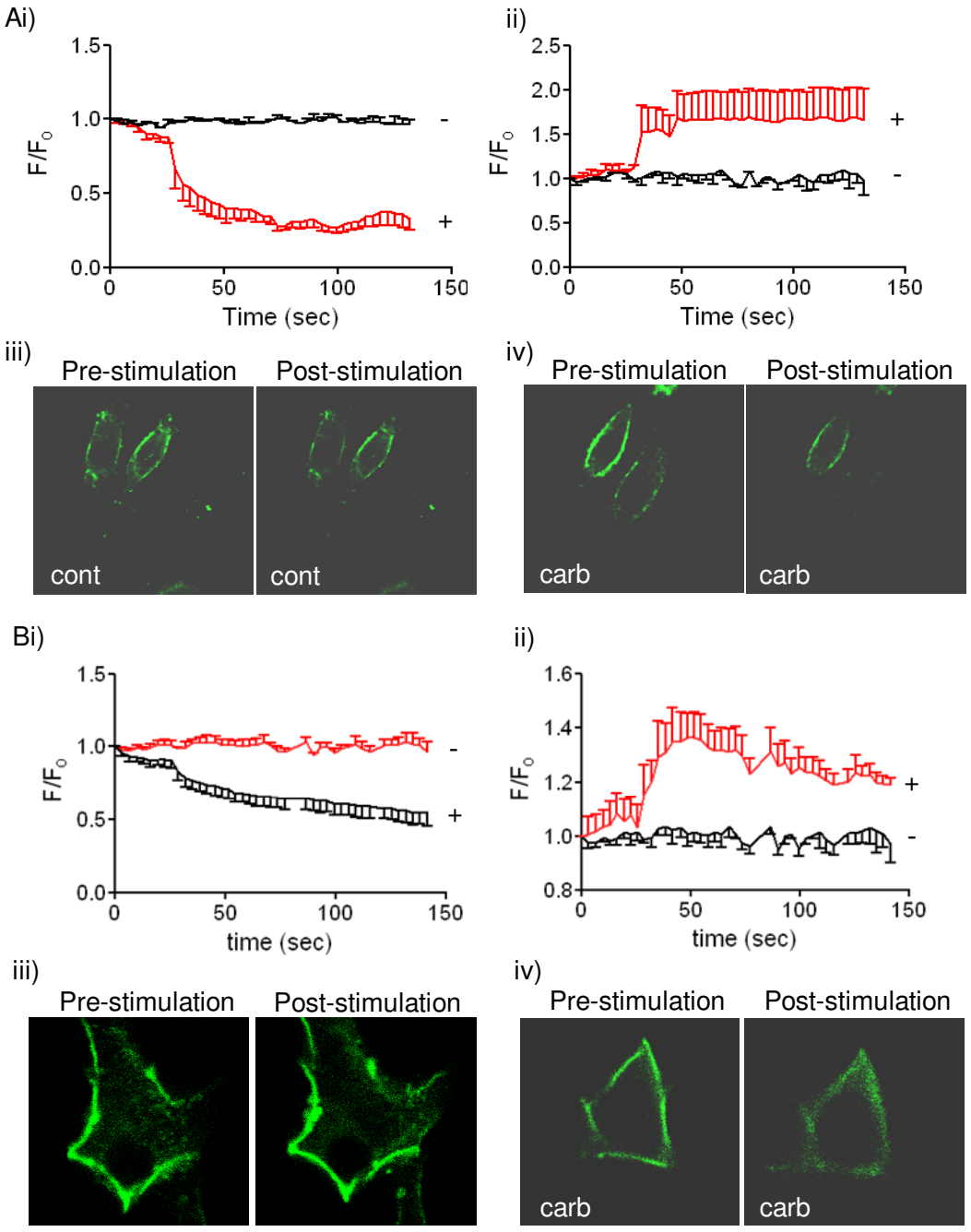


Figure 5.7. PLC activity upon carbachol stimulation in MIN6 cells. A) CHO-M3 cells and B) MIN6 cells were seeded on coverslips and transfected with eGFP-$PH_{PLC\delta1}$ 48hours before experimentation. Cells were pretreated for 1hour with KRB subsequent to mounting on a scanning confocal microscope. Cells were treated with 1mM carbachol and fluorescence intensity changes within membrane (i) or cytosolic (ii) ROIs were measured. Fluorescence intensity was analysed using the Fluoview Tiempo software (version 4.3) then divided by the initial fluorescence and expressed as F/F_0. iii and iv) images for cont treated or carbachol treated cells pre- and post-stimulation.

Firstly, I determined the effectiveness of the biosensor by measuring IP_3 and PIP_2 levels upon mAChR activation in the CHO-M3 cell line. This cell line stably overexpresses the M_3 receptor and has been used to investigate the function of this mAChR isoform. These cells were transfected with the eGFP-$PH_{PLC\delta1}$ biosensor and changes in fluorescence localisation were measured in response to carbachol stimulation. In unstimulated cells, the sensor is at the plasma membrane and upon carbachol treatment, the fluorescence decreases at the membrane and increases within the cytosol (Figure 5.7A). The fluorescent changes can be seen as images as a decrease in GFP signal at the plasma membrane upon carbachol treatment (Figure 5.7Aiv) versus no change on control treatment (Figure 5.7Aiii). These results indicate that a depletion of PIP_2 and production of IP_3 upon carbachol stimulation of M_3 receptors occurs within CHO-M3 cells. To determine whether in MIN6 cells carbachol also stimulates movement of the biosensor, MIN6 cells were transfected with the biosensor. Under basal conditions the PH-domain biosensor was situated at the plasma membrane in MIN6 cells, and upon carbachol stimulation a reduction in fluorescence at the plasma membrane was observed (Figure 5.7Bi) alongside an increase of fluorescence within the cytosol (Figure 5.7Bii). The fluorescent changes can be seen in images as a decrease in GFP signal at the plasma membrane upon carbachol treatment (Figure 5.7Biv) versus no change on control treatment (Figure 5.7Biii). These results indicate a depletion of PIP_2 at the plasma membrane and production and translocation of IP_3 to the cytosol upon carbachol stimulation in MIN6 cells, however without measuring the PIP_2 levels directly this cannot be fully determined.

5.2.6 Carbachol stimulates increases in $[Ca^{2+}]_i$ in a concentration dependent manner.

As carbachol stimulates the production and translocation of IP_3 in MIN6 cells, it is likely that carbachol will also stimulate a rise in $[Ca^{2+}]_i$. Indeed, carbachol stimulates concentration-dependent increases in $[Ca^{2+}]_i$ in MIN6 cells with an EC_{50} of 884nM (Figure 5.8A) which is comparable to the EC_{50} for ERK1/2 activation (Figure 5.2). At the lower concentrations of carbachol used in the concentration-response curve (10nM

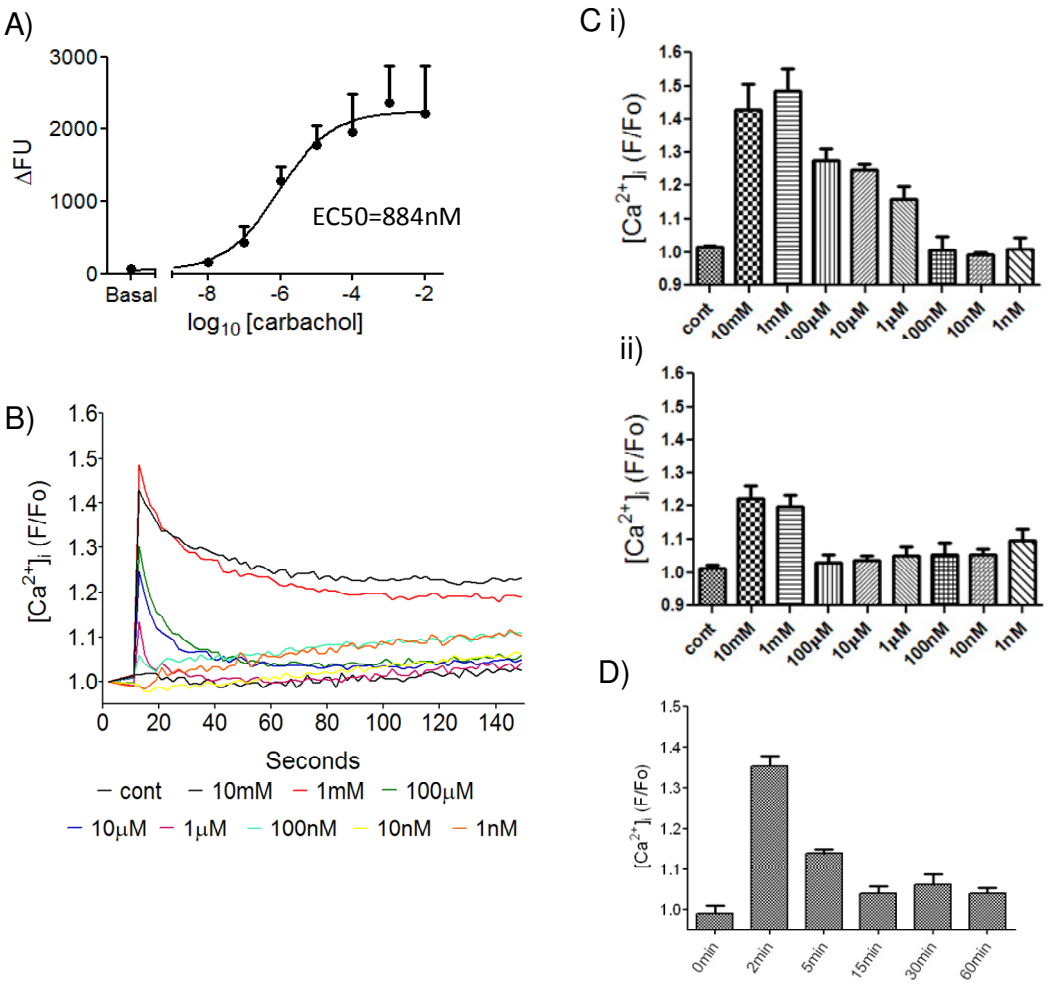


Figure 5.8. Carbachol mediates rises in $[Ca^{2+}]_i$ in MIN6 cells. MIN6 cells preincubated for 1hour with KRB were treated with increasing concentrations of carbachol as shown. A) $[Ca^{2+}]_i$ levels were determined via population based NOVOstar imaging. Data represent the mean +S.E.M. of the peak increase in $[Ca^{2+}]_i$. Data were fit using a variable slope model in GraphPad Prism and EC_{50} determined with the software (n=3). B) Representative traces of the treatments described in A). C) peak (i) and plateau (120sec) (ii) levels of $[Ca^{2+}]_i$ from the treatments in A). D) MIN6 cells were treated with 1mM carbachol and $[Ca^{2+}]_i$ levels determined at the time points indicated. All Data represents mean +S.E.M. of the increase in $[Ca^{2+}]_i$ levels.

to 100μM) the $[Ca^{2+}]_i$ profile consistsof an initial peak with levels quickly reverting to basal (Figure 5.8B and C). However, at concentrations above 1mM the profile switches to a peak and plateau suggesting a thoureshold has been reached to activate another Ca^{2+}-mobilisation mechanism (Figure 5.8B and C). To further determine the temporal profile of carbachol induced rises in $[Ca^{2+}]_i$, MIN6 cells were stimulated with 1mM carbachol and $[Ca^{2+}]_i$ measured for a prolonged period. Carbachol stimulates a rise in $[Ca^{2+}]_i$ with levels returning to baseline by 5min (Figure 5.8D). Therefore, carbachol stimulates a transient increase in $[Ca^{2+}]_i$ in MIN6 cells that precedes ERK1/2 activation.

5.2.7 Increases in $[Ca^{2+}]_i$ are essential for carbachol induced ERK1/2 phosphorylation.

Given that carbachol stimulated ERK1/2 activation is PLC-dependent but PKC-independent, it was likely that ERK1/2 was activated by an IP_3-dependent increase in $[Ca^{2+}]_i$. To determine whether this was required for ERK1/2 activation, MIN6 cells were loaded with the intracellular Ca^{2+} chelator BAPTA-AM. The use of this chelator abolished both carbachol induced ERK1/2 activation (Figure 5.9A) and rises in $[Ca^{2+}]_i$ (Figure 5.9B) confirming the Ca^{2+}-dependency of mAChR stimulated ERK1/2 phosphorylation. Incubation of MIN6 cells in EGTA-buffered KRB (KRB buffered with EGTA to a Ca^{2+} concentration of 100nM thus ensuring that there is not any flux of Ca^{2+} across the membrane) also abolished both carbachol induced increase in $[Ca^{2+}]_i$ and ERK1/2 activation (Figure 5.9C and D). Therefore, carbachol-stimulated increases in $[Ca^{2+}]_i$ that mediate ERK1/2 activation in β-cells are dependent upon the influx of extracellular Ca^{2+}.

5.2.8 Investigation into the mechanism of carbachol induced increases in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation.

To further investigate the mechanism by which carbachol elicits increases in $[Ca^{2+}]_i$ and to gain an understanding how this may lead to ERK1/2 activation in β-cells, I incubated MIN6 cells with 10μM nifedipine (an L-type VGCC blocker), 250μM diazoxide (a K_{ATP} channel activator), 10μM 2-ABP (a SOC and IP_3R inhibitor), 10μM xestospongin (an IP_3R antagonist) or 1μM thapsigargin (SERCA pump inhibitor, which leads to ER calcium store depletion) prior to stimulation with carbachol. The pretreatment of MIN6 cells with nifedipine or diazoxide effectively abolished carbachol induced ERK1/2

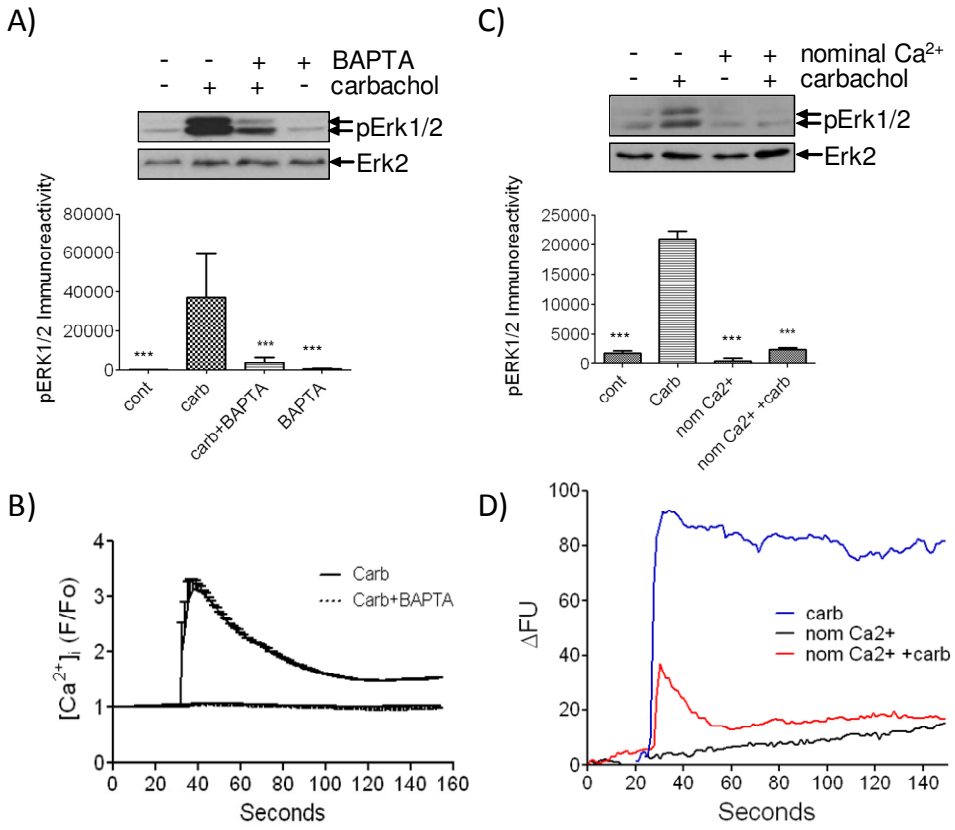


Figure 5.9. Carbachol mediated ERK1/2 activation in MIN6 cells requires extracellular Ca^{2+} influx. A) MIN6 cells were preincubated for 30min in KRB, then where appropriate loaded with 100µM BAPTA-AM for 30min. MIN6 cells were treated with 1mM carbachol for 2min and ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$ (n=3). B) Cells were loaded with 2µM Fluo-4-AM at the same time as BAPTA-AM and $[Ca^{2+}]_i$ levels measured using NOVOstar Ca^{2+} imaging, the cells were treated with 1mM carbachol as indicated. Data represent the mean +S.E.M. of the increase in $[Ca^{2+}]_i$ levels (n=3). C) In order to assess the requirement of extracellular Ca^{2+}, MIN6 cells were preincubated in EGTA-buffered KRB 10min prior to 1mM carbachol treatment. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. (n=3). Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$. D) Representative single cell confocal $[Ca^{2+}]_i$ traces of MIN6 cells preincubated in nominal Ca^{2+} KRB for 10min prior to treatment as indicated. Data represent the mean of the increase in $[Ca^{2+}]_i$ levels (n=3).

activation and peak $[Ca^{2+}]_i$ (Figure 5.10A and B). This indicates that both the inactivation of the K_{ATP} channel and the activation of the L-type VGCC are required for carbachol induced Ca^{2+} influx and ERK1/2 activation.

Although the IP_3R antagonists, xestospongin and 2-ABP, significantly attenuated carbachol stimulated ERK1/2 activation (Figure 5.10A) and peak $[Ca^{2+}]_i$ responses they were unable to totally block rises in Ca^{2+} (Figure 5.10B). To confirm the effectiveness of xestospongin and 2-ABP on preventing IP_3R Ca^{2+}-release, MIN6 cells were pre-incubated with both inhibitors and the effects on $[Ca^{2+}]_{ER}$ measured using the D1ER FRET sensor [463]. The stimulation of MIN6 cells with carbachol caused a decrease in $[Ca^{2+}]_{ER}$ (Figure 5.11A). This was effectively inhibited by xestospongin (Figure 5.11B) confirming that carbachol induces IP_3R mediated Ca^{2+}-release from the ER. However, 2-ABP had no effect on the carbachol induced decrease in $[Ca^{2+}]_{ER}$ (Figure 5.11D and F) demonstrating that it is not acting as an IP_3R antagonist. Therefore, the inhibitory effect of 2ABP on carbachol stimulated ERK1/2 activation and Ca^{2+} mobilisation is likely to be mediated by the inhibition of its other known target; store operated channels (SOC) [656]. Together this data demonstrates that IP_3R and SOC channel activation are required for carbachol induced ERK1/2 activation and suggests a significant role of $[Ca^{2+}]_{ER}$ in mediating ERK1/2 activation.

Paradoxically, pretreatment with 1μM thapsigargin, a SERCA pump inhibitor that will prevent refilling of stores leading to ER Ca^{2+} depletion, failed to inhibit carbachol stimulated ERK1/2 activation (Figure 5.10Aii) but significantly lowered peak $[Ca^{2+}]_i$ responses (Figure 5.10Bi). To confirm that thapsigargin pre-treatment is emptying ER stores of Ca^{2+}, the FRET-based ER Ca^{2+} sensor, D1ER was used to measure ER Ca^{2+}. Carbachol initiated a decrease in FRET indicating a decrease in $[Ca^{2+}]_{ER}$ (Figure 5.11A). This small decrease in $[Ca^{2+}]_{ER}$ levels is consistent with published literature suggesting that carbachol only initiates a 20% decrease in $[Ca^{2+}]_{ER}$ in insulin secreting cells [439]. 1μM thapsigargin treatment for either 10 or 30min causes a steady decrease in FRET indicating significant leak of Ca^{2+} from the ER (Figure 5.11C and E). Subsequent application of carbachol does not lead to any further decrease in $[Ca^{2+}]_{ER}$ (Figure 5.11C and E) demonstrating that thapsigargin is depleting the $[Ca^{2+}]_{ER}$ store in MIN6 cells. These results provide an anomaly. Thapsigargin depletes the ER store of

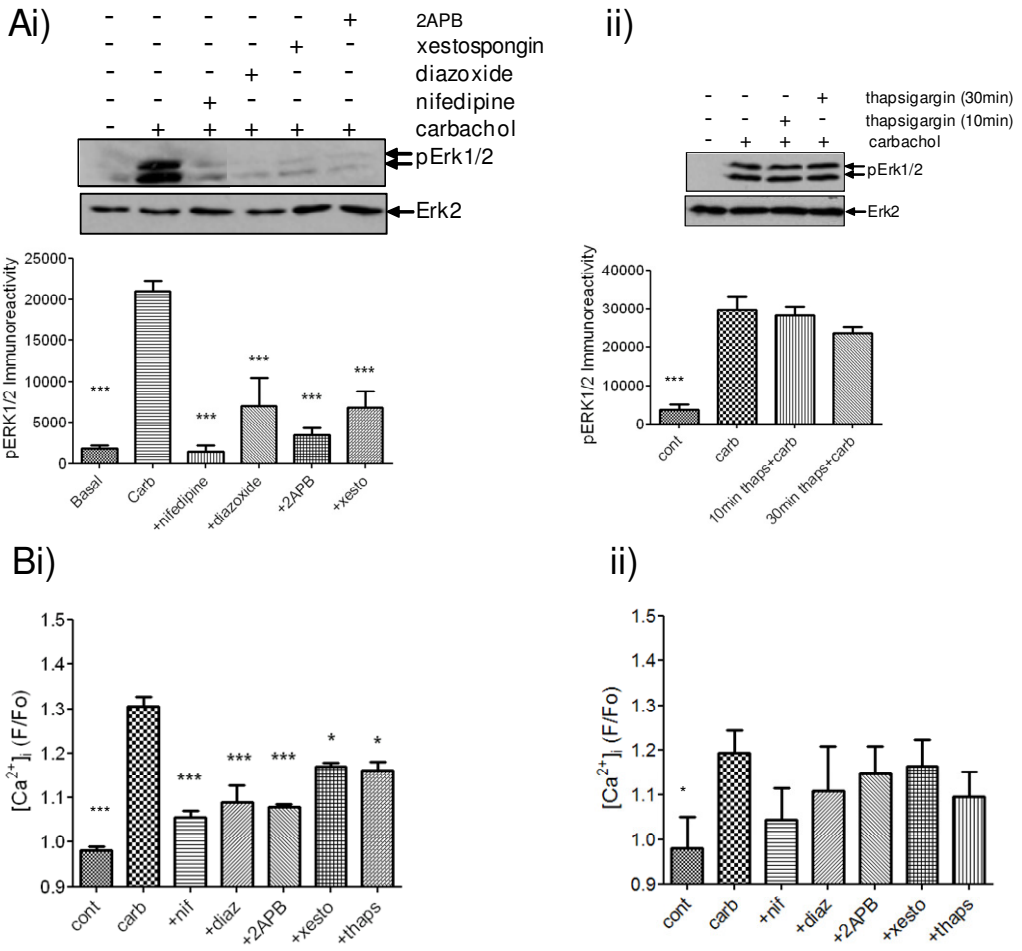
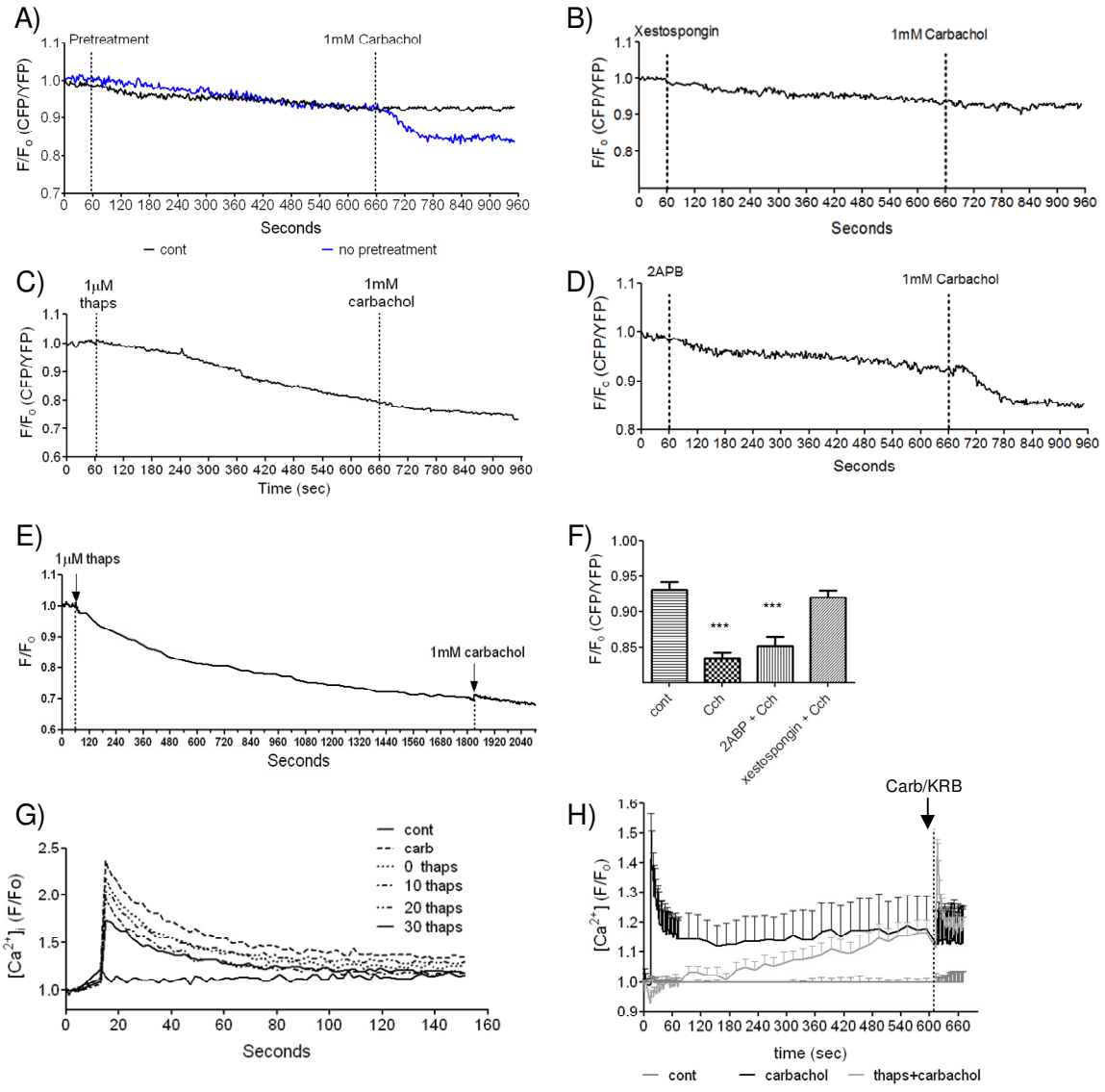


Figure 5.10. A number of Ca^{2+} sources are required for carbachol stimulated ERK1/2 activation. A) MIN6 cells preincubated for 1hour with KRB were treated 1mM carbachol for 2min. i) Cells were preincubated alone or in the presence of either 10µM nifedipine, or 10µM xestospongin for 10min, 250µM diazoxide for 30min or 10µM 2APB for 30min as indicated prior to 1mM carbachol stimulation. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. (n=3). Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$. ii) preincubation of MIN6 cells with 1µM thapsigargin for either 10 or 30min as indicated prior to carbachol stimulation. ERK1/2 phosphorylation was determined via western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; ***, $P < 0.001$ (n=3). B) i) $[Ca^{2+}]_i$ levels of MIN6 cells treated as in A) were analysed via population based NOVOstar imaging. Data represent the mean +S.E.M. of the peak increase in $[Ca^{2+}]_i$ levels. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; *, $P < 0.05$; ***, $P < 0.001$ (n=3). ii) Same treatments as A) but demonstrating the mean +S.E.M. $[Ca^{2+}]_i$ levels at the plateau (120sec post-stimulation) (n=3). Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to carbachol; *, $P < 0.05$.


A)
Pretreatment
1mM Carbachol
F/F0 (CFP/YFP)
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no pretreatment
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Xestospongin
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Time (sec)
D)
2APB
1mM Carbachol
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Figure 5.11. Analysis of the role of ER stored Ca^{2+} in carbachol mediated ERK1/2 activation. In order to assess the extent of intracellular store depletion we measured $[Ca^{2+}]_{ER}$ levels using the D1ER cameleon FRET construct. MIN6 cells were transfected with the D1ER construct using Lipofectamine. 48hours post-transfection the cells were preincubated for 1hour in KRB prior to recording. All recordings contain 1min of basal KRB perfusion before pretreatments for 10min followed by 1mM carbachol. A) no pretreatment followed by 1mM carbachol. B) a combined trace with 10µM xestospongin for 10min followed by 1mM carbachol; 10µM 2-ABP for 10min followed by 1mM carbachol; 1mM carbachol alone; or control perfusion. D) 1µM thapsigargin for 10min followed by 1mM carbachol, E) 1µM thapsigargin for 30min followed by 1mM carbachol. Data shown represent mean FRET levels and are combined data from >30 cells across ≥3 coverslips. F) FRET values at 16min after recording initiated directly comparing changes in $[Ca^{2+}]_{ER}$ across treatments. Data shown represents the mean +S.E.M. FRET levels and are combined data from n≥3 coverslips. Statistical comparisons were by one-way ANOVA with Dunnett's range test compared to control; ***, $P < 0.001$ (n≥3). G) $[Ca^{2+}]_i$ levels were assessed for pretreatment with 1µM thapsigargin for the times indicated prior to carbachol stimulation via population based NOVOstar assay. Data represent the mean +S.E.M. of the increase in $[Ca^{2+}]_i$ levels. H) $[Ca^{2+}]_i$ levels were assessed during pretreatment with 1µM thapsigargin for 10min prior to carbachol stimulation via population based NOVOstar assay. Data represent the mean +S.E.M. of the increase in $[Ca^{2+}]_i$ levels.

Ca^{2+} but does not affect ERK1/2 activation, however IP_3-mediated release of Ca^{2+} from the ER is required for carbachol induced ERK1/2 activation. One possibility for this apparent anomaly is that thapsigargin is increasing the basal $[Ca^{2+}]_i$ level and upon F/F_0 analysis of peak $[Ca^{2+}]_i$ responses this leads to an apparent decrease in $[Ca^{2+}]_i$. To investigate this possibility the $[Ca^{2+}]_i$ levels upon thapsigargin pre-treatment and subsequent carbachol stimulation where measured using Fluo-4 in a population based NOVOstar assay. 10min preincubation with 1μM thapsigargin causes a rise in $[Ca^{2+}]_i$ levels (Figure 5.11H). This increase in basal levels means that upon carbachol stimulation despite the peak response being equivalent to carbachol alone (Figure 5.11H), the analysis of this data via F/F_0 suggests an apparent decrease in peak Ca^{2+} (Figure 5.10B). The lack of effect of thapsigargin pretreatment on carbachol stimulated ERK1/2 activation and the basal rise in $[Ca^{2+}]_i$ during this period suggests that the rise in basal $[Ca^{2+}]_i$ upon thapsigargin pretreatment may substitute for IP_3R-mediated $[Ca^{2+}]_{ER}$ release in response to mAChR activation in β-cells. Taken together, this data provide evidence that carbachol evoked rises in $[Ca^{2+}]_i$ and ERK1/2 activation are mediated by: 1) the release of Ca^{2+} from ER stores via IP_3R activation, 2) the influx of extracellular Ca^{2+} thourough the activation of SOCs and, 3) depolarisation and activation of L-type VGCC likely thourough K_{ATP} channel closure.

5.2.9 Evidence that carbachol induced K_{ATP} channel closure is mediated by PIP_2 depletion

Given that diazoxide, an activator of the K_{ATP} channel, effectively inhibits carbachol stimulated ERK1/2 activation (Figure 5.10A) and that PIP_2 association with the channel modulates channel activity [455, 657], I hypothesised that carbachol may be stimulating K_{ATP} channel closure thourough PLC mediated PIP_2 depletion.

To determine whether the decrease in PIP_2 at the plasma membrane plays an important role in carbachol stimulated ERK1/2 phosphorylation, I attempted to increase PIP_2 levels in the plasma membrane by overexpressing PIP5Kβ [658]. Overexpression of PIP5Kβ had no effect on the localisation of the eGFP-$PH_{PLC\delta1}$ biosensor (Figure 5.12Av and vi) compared to mock infected cells (Figure 5.12aiii and iv). The stimulation of MIN6 cells with carbachol initiated a decrease in membrane fluorescence and an increase in cytosolic fluorescence in mock and PIP5Kβ infected cells (Figure 5.12Ai

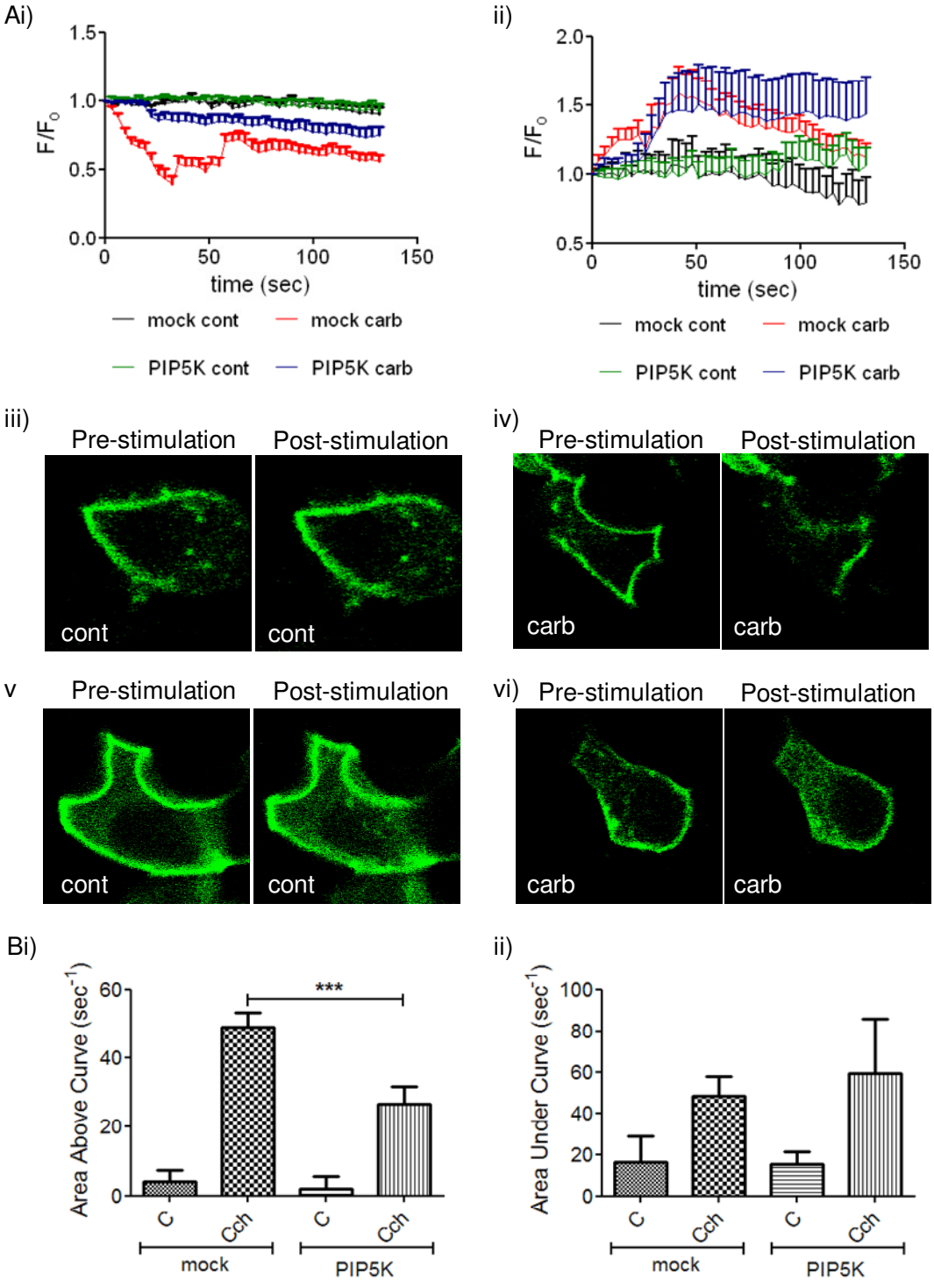

Figure 5.12. Overexpression of PIP5K inhibits the decrease in PIP_2 at the plasma membrane seen on carbachol stimulation without affecting the IP_3 signal. MIN6 cells were

seeded on coverslips and after 48hours transfected with eGFP-$PH_{PLC\delta1}$. The following day these cells were either mock infected or infected with PIP5K WT 48hours before experimentation. Cells were pre-treated for 1 hour with KRB subsequent to mounting on a scanning confocal microscope. Cells were treated with 1mM carbachol and fluorescence intensity changes within membrane (Ai) or cytosolic (Aii) ROIs measured. Fluorescence intensity was analysed using the Fluoview Tiempo software (version 4.3) then divided by the initial fluorescence and expressed as F/F_0. iii and iv) images for mock infected, control or carbachol treated cells pre- and post-stimulation. v and vi) images for PIP5K WT infected, control or carbachol treated cells pre- and post-stimulation. B) The area under the baseline for membrane fluorescence (i) or over the baseline for cyosolic fluorescence (ii) was calculated and compared between mock and PIP5K infected cells. Data represent mean +S.E.M. (n=3; >20cells per coverslip). Statistical comparisons were by Student's t- test between mock and PIP5K treatments; ***, $P < 0.001$.

and ii). However, when the magnitude of the response was assessed, despite the production of IP_3 being similar between mock and PIP5Kβ infected cells (Figure 5.12Bii), the decrease in membrane fluorescence, indicative of PIP_2 depletion, was significantly less in cells overexpressing PIP5Kβ (Figure 5.12Bi). This suggests that an increased level of PIP_2 is present at the plasma membrane within MIN6 cells overexpressing PIP5Kβ.

To assess the effect of potential increases in PIP_2 on carbachol mediated increases in $[Ca^{2+}]_i$ and ERK1/2 activation, MIN6 cells were either mock or PIP5K infected, treated with carbachol and increases in $[Ca^{2+}]_i$ and ERK1/2 phosphorylation determined. The overexpression of PIP5K altered the profile of the $[Ca^{2+}]_i$ response to mAChR stimulation, reducing the plateau phase of the response to baseline levels without affecting the peak response (Figure 5.13A). In parallel, the overexpression of PIP5K significantly attenuated ERK1/2 phosphorylation by ~16% in response to mAChR activation (Figure 5.13B). Therefore, the increase in PIP_2 levels mediated by overexpression of PIP5K inhibits mAChR signalling to ERK1/2 and this is likely to be due to the stimulatory effect of the phospholipid on K_{ATP} channel activity.

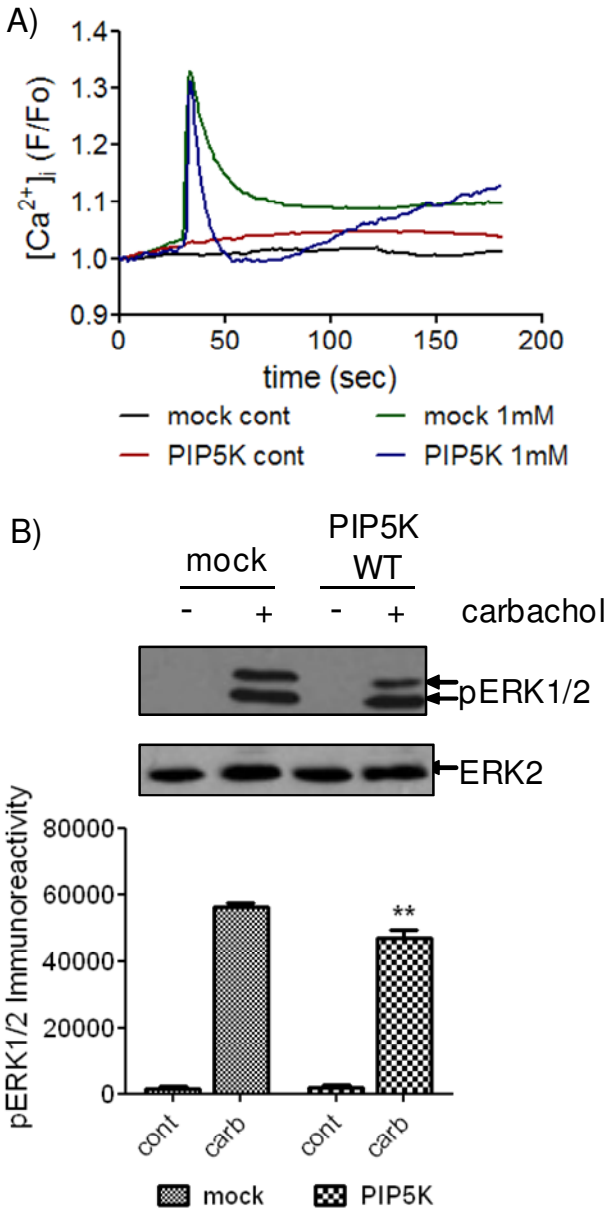

Figure 5.13. Overexpression of PIP5K attenuates mAChR mediated increases in $[Ca^{2+}]_I$ and ERK1/2 activation. MIN6 cells either mock infected or infected with PIP5K WT 48hours before experimentation. A) Cells were pre-treated for 1 hour with KRB and loaded with Fluo-4-AM subsequent to mounting on a scanning confocal microscope. Cells were treated with 1mM carbachol and $[Ca^{2+}]_I$ measured using changes in fluorescence. Data presented is the mean response (n=3 coverslips; >25 cells per coverslip). B) Cells were pre-treated for 1 hour with KRB subsequent to treatment with 1mM carbachol for 2min. ERK1/2 phosphorylation was determined via Western blotting using phospho-specific antibodies. Representative blot is shown above with densitometry below. Data are mean +S.E.M. (n=3). Statistical comparisons were by Student's t-test between mock and PIP5K treatments; **, $P < 0.01$.

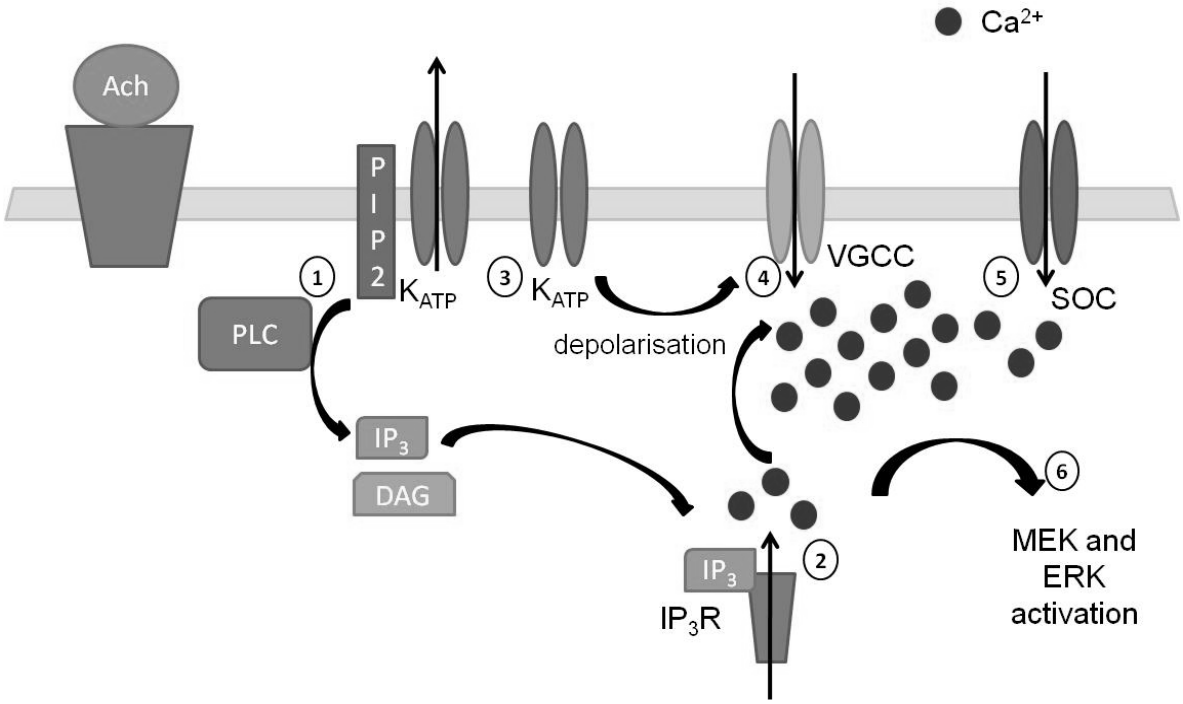

Figure 5.13. Schematic representation of Ca^{2+} inputs into ERK1/2 activation. (1) Agonist binding to the mAChR activates PLC to mediate PIP_2 depletion and IP_3 production. (2) IP_3 translocates to the IP_3R in the ER to mediate Ca^{2+}-release from the ER. (3) Upon PIP_2 depletion K_{ATP} channels are inactivated. (4) Store-operated channels are activated in the plasma membrane to mediate Ca^{2+}-entry. (5) Steps 2,3 and 4 converge to result in depolarisation and Ca^{2+}-entry thourough L-type VGCCs and the cumulative rise in $[Ca^{2+}]_i$ leads to ERK1/2 activation.

5.3 Discussion

5.3.1 Summary of carbachol induced mechanism of ERK1/2 activation in MIN6 cells.

Figure 5.13 summarises the results of these series of experiments demonstrating the mechanism mediating carbachol induced ERK1/2 activation. The initial step is the GPCR-mediated activation of PLC leading to the depletion of PIP_2 and production of IP_3 (Figure 5.13-1). At this point the pathway diverges with IP_3 mediating Ca^{2+}-release from the ER thourough IP_3Rs (Figure 5.13-2) whilst PIP_2 depletion inhibits K_{ATP} channel activity (Figure 5.13-3). These two intracellular events are likely to affect the activity of SOC channels which are known to be affected by Ca^{2+}-release from the ER (Figure 5.13-5). The convergence of these two depolarising stimuli leads to the activation of VGCCs and extracellular Ca^{2+} influx though L-type VGCCs (Figure 5.13-4). This global rise in Ca^{2+} leads to the Ras-dependent activation of ERK1/2 (Figure 5.13-6).

5.3.2 mAChR mediated glucose-independent depolarisation in pancreatic β-cells.

In this study, I have demonstrated that a substantial proportion of the mAChR stimulated rises in $[Ca^{2+}]_i$ is nifedipine sensitive and therefore, mediated by the activation of L-type VGCCs (Figure 5.6A and B). The depolarising stimulus to activate L-type channels is likely mediated by multiple inputs including IP_3-receptor Ca^{2+} release from stores, SOC channel activation and the inhibition of K_{ATP} channels. Previous work in isolated rat islets and mouse β-cells has shown that ACh at non-stimulatory glucose concentrations induces Ca^{2+} influx thourough depolarisation and L-type VGCC activation [446, 455]. Furthermore, the changes in $[Ca^{2+}]_i$ mediated by treating β-cells with ACh in non-stimulatory glucose concentrations has been demonstrated to be inhibited by agents such as diazoxide or VGCC blockers in mouse β-cells as well as clonal β-cell lines [406, 430]. ACh can stimulate depolarisation and insulin secretion indicating that high concentrations of muscarinic agonists can cause transient rises in $[Ca^{2+}]_i$ which correlate with pulses of insulin secretion from both mouse and clonal β-cell lines [380, 406, 446-448]. In many cases the omission of extracellular Ca^{2+} prevented both the rise in $[Ca^{2+}]_i$ and the insulin response [380, 447,

448]. The brief changes in $[Ca^{2+}]_i$ observed in these studies is similar to the effect of carbachol stimulation of MIN6 cells observed in this study.

5.3.3 Partial Ras-dependency of muscarinic-receptor mediated ERK1/2 activation.

In the present study, mAChR induced ERK1/2 activation was shown to be significantly inhibited by expressing a dominant-negative form of Ras (RasN17). However, ERK1/2 activation was only partially inhibited (60.11%) (Figure 5.4). This suggests that two different pathways are activated upon mAChR stimulation; one pathway utilising Ras to mediate ERK1/2 activation and a second Ras-independent pathway. Both of these pathways are likely to require Ca^{2+} as removal of extracellular Ca^{2+} via EGTA buffered KRB, or the presence of an intracellular Ca^{2+} chelator, prevented mAChR induced ERK1/2 activation (Figure 5.6).

In Section 3.2 I have demonstrated that global rises in $[Ca^{2+}]_i$ led to ERK1/2 activation but the route of Ca^{2+} entry appears to dictate the pathways mediating that activation. Depolarising concentrations of K^+ stimulate increases in $[Ca^{2+}]_i$ via L-type VGCCs (Figure 3.4). However, the signalling mechanism for K^+-induced ERK1/2 activation differs dependent on the strength of the K^+ concentration. The stimulation of MIN6 cells with K20 stimulated ERK1/2 activation via a Ras-independent mechanism whereas the stronger K50 stimulation leads to the activation of two pathways: one Ras-dependent and one Ras independent mechanism of ERK1/2 activation (Figure 4.6). This is similar to what occurs upon mAChR activation in MIN6 cells (Figure 5.4). I hypothesise from these observations that a Ras-dependent mechanism for ERK1/2 activation is activated by large global rises in $[Ca^{2+}]_i$ whilst the Ras-independent mechanism occurs via the specific coupling of the L-type VGCC to ERK1/2. The rise in $[Ca^{2+}]_i$ that occurs upon mAChR stimulation of MIN6 cells (Figure 5.8B) would be able to mediate both of these mechanisms. The large transient global rise in Ca^{2+} would mediate a Ras-dependent pathway whilst the activation of the L-type VGCC by depolarisation would allow the activation of ERK1/2 specifically coupled to the channel via a Ras-independent mechanism.

The specific coupling of Ca^{2+} influx via the L-type VGCC and ERK1/2 has been discussed previously in the context of GLP-1 signalling (Section 3.3.4). As mAChR induced ERK1/2 activation appears to occur via a specific L-type VGCC-dependent

mechanism, it is possible that the local Ca^{2+} signalling at the L-type VGCC that mediates ERK1/2 activation is a mechanism coupled to all activators of this channel.

5.3.4 PKC effects on mAChR stimulation of MIN6 cells.

The stimulation of β-cells with muscarinic agonists is known to activate and translocate PKC isoforms [387, 388]. This study has demonstrated that PKCε is translocated to the plasma membrane upon mAChR activation in MIN6 cells (Figure 5.5). This demonstrates that mAChR activation can signal to PKC isoforms in MIN6 cells.

In the literature there are with multiple reports suggesting that PKC increases the sensitivity of β-cells to Ca^{2+} [381, 388, 404, 406, 407]. One report demonstrates that mouse islets chouronically treated with phorbol ester had a significantly diminished $[Ca^{2+}]_i$ response to ACh but this investigation was in the presence of stimulatory concentrations of glucose which may have influenced the conclusions of the experiments [381]. This particular experiment would suggest that Ca^{2+}-dependent signalling would be decreased by blockade of PKC isoforms. Another report however suggests that PKC does not affect mAChR induced rises in $[Ca^{2+}]_i$ as they were completely unaffected by chouronic TPA treatment [406]. However, in this study the inhibition of classical and novel PKC isoforms with inhibitors as well as the down-regulation of DAG-dependent PKC isoforms with chouronic exposure to TPA, did not significantly affect mAChR induced ERK1/2 activation (Figure 5.6A). The effectiveness of these inhibitors was tested however with acute treatment of TPA which effectively abolished ERK1/2 activation in all treatments (Figure 5.6B). This suggests that classical and novel PKC isoforms, although potentially activated by mAChR activation are not involved in the Ca^{2+}dependent signalling to ERK1/2.

5.3.5 PIP_2 regulates K_{ATP} channel activity.

The present work demonstrates, thourough the use of diazoxide, that the closure of K_{ATP} channels is required for the mAChR mediated ERK1/2 activation (Figure 5.10A). K_{ATP} channels are known to be regulated in β-cells by changes in the ATP to ADP ratio and by PIP_2 association with the channel [455, 657, 658]. As the investigation presented here was completed in the absence of glucose, the ratio of ATP to ADP is unlikely to change upon mAChR activation, and therefore another mechanism is likely to be involved in the changes in K_{ATP} channel activity.

The depletion of PIP_2 from plasma membranes has been demonstrated to reduce the activity of K_{ATP} channels [659, 660]. Furthermore, the replenishment of PIP_2 into rundown membrane patches can restore full channel activity [659] suggesting a positive role for PIP_2 in K_{ATP} channel function. In β-cells PIP_2 has also been demonstrated to activate the K_{ATP} channels, with an increase in PIP_2 levels corresponding to a hyperpolarised membrane and a reduction in glucose stimulated insulin secretion [658]. Indeed, this project has demonstrated that increasing PIP_2 levels, thourough the overexpression of PIP5K, leads to decreased mAChR stimulated ERK1/2 activation and modulates the $[Ca^{2+}]_i$ response (Figure 5.13). Alongside the evidence that diazoxide application inhibits ERK1/2 activation, this study suggests that mAChR stimulated PIP_2 depletion contributes to the depolarising stimulation thourough the inhibition of K_{ATP} channel activity which leads to ERK1/2 activation in MIN6 cells.

5.3.6 Role of SOC channels in mAChR mediated ERK1/2 activation.

In the present study, the inhibitor 2-APB failed to prevent mAChR mediated decreases in $[Ca^{2+}]_{ER}$. This suggests that this inhibitor is affecting the activity of its other known target: store operated channels (SOC channels) [656]. In fact there are accounts suggesting that 2-APB targets the coupling machinery between IP_3 receptors and SOC channels [661]. Despite its off-target effects, 2-APB inhibited both mAChR stimulated rises in $[Ca^{2+}]_i$ and ERK1/2 activation in MIN6 cells. This suggests that mAChR activation mediates Ca^{2+} influx by SOC channels and this contributes to mAChR stimulated ERK1/2 activation.

The activation of SOC channels occurs after IP_3-mediated release from ER stores and is thought to mediate the sustained or plateau Ca^{2+} response that is elicited by receptor induced Ca^{2+} signalling [662-664]. Ca^{2+} influx via SOC channels is not affected by voltage and solely relies on signalling between the resident ER Ca^{2+} sensor (STIM) and the plasma membrane channel (ORAI) and a gradient between extracellular and intracellular Ca^{2+} [665-672]. Due to time constraints the effect of SOC channel activation on mAChR mediated changes in $[Ca^{2+}]_i$ and ERK1/2 activation was not further investigated. If time allowed, further investigation would involve the use of lanthanides to manipulate SOC-dependent Ca^{2+} entry and manipulation of the proteins involved in transmitting the signal from the ER to the plasma membrane localised SOCs. The lanthanide Gd^+ has specificity for SOC channels over other Ca^{2+} entry and

efflux processes so when used at concentrations of ≤5μM they can be used to inhibit SOC channels [673].

5.3.7 Role of mAChR stimulated ERK1/2 activation in pancreatic β-cells.

We show that mAChR activation leads to the phosphorylation of ERK1/2 independently of glucose (Figure 5.2). Furthermore, as ERK1/2 activation from glucose and carbachol appears independent this suggests that two pathways are activated by these two different agonists of β-cells. Therefore, mAChR activation on β-cells thouroughout both the absorptive and preabsorbative phase of nutrient intake could mediate ERK1/2 activation and functions that are independent of glucose.

The role of mAChR activation of ERK1/2 in β-cells is currently unknown. However, the activation of ERK1/2 has been shown to be important in the stimulation of β-cell proliferation, differentiation, survival and gene transcription [261, 420, 421]. Moreover, in MIN6 cells, glucose-stimulated ERK1/2 activation has been shown to play an important role in enhancing insulin exocytosis via the phosphorylation of synapsin I [422]. Interestingly, mAChR activation has been shown to prepare β-cells for subsequent glucose stimulated insulin secretion *in vitro* with glucose stimulated insulin secretion increased after preincubation with muscarinic agonists [348, 349]. Therefore, it is conceivable that the transient activation of ERK1/2 observed upon mAChR activation plays a role in preparation of β-cells for insulin secretion.

ERK1/2 activation in β-cells has also been shown to mediate proliferation with both glucose and incretin stimulated ERK1/2 activation leading to an increased number of β-cells in rat and mouse islets [138, 139]. In other cell types, the activation of mAChRs leads to proliferation via Ca^{2+} and ERK1/2-dependent mechanisms [416, 424]. The role of mAChRs in islet cell regeneration has yet to be fully investigated but the expression of both M1 and M3 receptors is increased during proliferation [423] indicating that mAChR activation, possibly thourough the activation of ERK1/2, may play a positive role in β-cell proliferation.

Chapter 6. Final discussion

6.1 Summary

The preabsorptive and absorptive phases of food intake lead to secretion of ACh from intrapancreatic vagal nerves endings [296, 318], whilst during the absorptive phase the hormone GLP-1 is secreted from intestinal L-cells into the bloodstream [674]. ACh and GLP-1 act upon G_q and G_s GPCRs respectively, which are expressed on β-cells where receptor activation potentiates glucose stimulated insulin secretion, and GLP-1R activation is known to stimulate transcription and β-cell proliferation. Both ACh and GLP-1 have been demonstrated to stimulate ERK1/2 activation in β-cells [66-68, 419], however, the molecular mechanism of ERK1/2 activation and its role in β-cell physiology and pathophysiology is only partially understood.

In this thesis I demonstrate that both GLP-1R and mAChR activation require L-type VGCC dependent Ca^{2+} influx to activate ERK1/2 (Figure 3.9 and Figure 5.9), thus confirming the results of previous studies [67, 68]. In addition, I confirm that GLP-1 activates ERK1/2 phosphorylation via a Ras-independent mechanism and that carbachol stimulates ERK1/2 activation thourough both Ras-independent and dependent mechanisms (Figure 5.4B). There are, however, multiple differences in the signalling pathways utilised by these two agonists.

The activation of the G_s-coupled GLP-1R requires Ca^{2+} influx thourough the L-type VGCC to mediate rises in $[Ca^{2+}]_i$ and ERK1/2 activation. I have also provided evidence that the signalling mechanism mediating ERK1/2 activation, in response to GLP-1 stimulation, originates at the intracellular mouth of the L-type VGCC (Figure 3.9), and that L-type VGCC mediated Ca^{2+} influx alone can mediate ERK1/2 activation via local Ca^{2+} signalling (Figure 3.12). Furthermore, I demonstrated that the Ca^{2+} microdomain at the L-type VGCC, and the subsequent ERK1/2 activation, are required for GLP-1 to stimulate insulin gene transcription (Figure 3.15 and 3.16).

The M3 mAChR however, is G_q coupled and activates PLC (Figure 5.2) to stimulate PIP_2 depletion (Figure 5.7). This thesis demonstrates that mAChR stimulated ERK1/2 phosphorylation, unlike GLP-1R activation, requires multiple sources of Ca^{2+} to mediate

the rise in $[Ca^{2+}]_i$ required for ERK1/2 activation. These inputs include the activation of IP_3R, SOC channels and L-type VGCC (Figure 5.10).

The influx of Ca^{2+} thourough the L-type VGCC has been demonstrated to be the key mediator of rises in $[Ca^{2+}]_i$ in response to glucose, GLP-1 in the presence of glucose, depolarising concentrations of K^+ and carbachol (Figure 3.2, 3.8 and 5.4). Data in this thesis demonstrates that L-type VGCC activation is coupled to ERK1/2 via a signalling pathway independent of Ras. However, Ras-dependent signalling to ERK1/2 via Ca^{2+} entry thourough the L-type VGCC can also occur, but in response to large global rises in $[Ca^{2+}]_i$ such as that mediated by K50 and carbachol. This was illustrated when Ras-dependent signalling was inhibited with a dominant negative Ras protein and some ERK1/2 activation remained (Figure 4.6 and 5.4). This is unlikely to be L-type specific but as depolarising concentrations of K^+ and carbachol mediate ERK1/2 activation via a nifedipine sensitive pathway, the Ras-dependent ERK1/2 activation mediated by global rises in $[Ca^{2+}]_i$ may require the activation of the L-type VGCC.

6.2 Microdomain signalling to ERK1/2

The existence of Ca^{2+}-dependent signalling within a microdomain has been observed in several different physiological contexts [590, 675-678]. For example, in cardiac muscle Ca^{2+} microdomain signalling between L-type VGCCs and ryanodine receptors is essential for cardiac muscle contraction [676, 677]. Furthermore, Ca^{2+} microdomains in skeletal muscle, cardiac muscle and neurones have been demonstrated to participate in the transcription of a variety of different genes [675], in a similar way to the L-type VGCC Ca^{2+} microdomain participating in insulin transcription. The existence of a Ca^{2+} microdomain that signals to ERK1/2 has also been demonstrated previously. A Ca^{2+} microdomain surrounding the NMDA receptor in hippocampal neurones has been demonstrated to lead to ERK1/2 activation and synapse to nucleus communication [590].

Using scaffold proteins, protein-protein interactions and dimerisation to aggregate or specifically localise signalling molecules, a simple Ca^{2+} influx can be transformed from a generic signal into a mediator of discrete and specific actions. The role of proteins in mediating microdomain signalling to ERK1/2 is discussed below.

6.2.1 Microdomain signalling via scaffolding proteins

Scaffold proteins co-localise key components of signalling cascades to specific intracellular sites [98], and have been demonstrated to impact the duration and amplitude of signalling to ERK1/2 by interacting with positive and negative regulators of the MAPK cascade [99]. Due to these defined roles for scaffold proteins they could provide a vital link in the pathway between Ca^{2+} entry via the L-type VGCC and ERK1/2 activation.

AKAPs interact with L-type VGCCs and act as scaffolds for the assembly of signalosome complexes. Therefore, AKAPs could be involved in the specific microdomain signalling that mediates GLP-1 stimulated ERK1/2 activation. AKAPs thourough their scaffolding function and their ability to bind PKA as well as other intracellular signalling molecules provide a unique platform for modulation of the L-type VGCC and surrounding proteins. PKA has been shown in several systems to phosphorylate and facilitate the Ca^{2+} current thourough L-type VGCCs [539] and AKAP79 has been demonstrated to bind to the $Ca_v1.2$ L-type VGCC in hippocampal neurons [557]. This association was demonstrated to integrate the actions of PKA and calcineurin on the L-type VGCC in response to depolarisation [557]. AKAP150, a homolog of AKAP79, is expressed in β-cells and been shown to interact with calcineurin and PKA [244], thus it is possible that AKAP150 is responsible for mediating the specific effects of GLP-1 on L-type VGCC function and downstream signalling to ERK1/2. The possible involvement of AKAP proteins in the GLP-1 signalling cascade provides a link between cAMP/PKA signalling, the Ca^{2+}/CaM pathway and Ca^{2+} influx in β-cells via the L-type VGCC.

One scaffold protein that has recently been demonstrated to bind to activated members of the ERK1/2 cascade in β-cells, is KSR2 [679, 680]. Immunoprecipitation experiments have demonstrated that, upon glucose treatment, KSR2 associates with phosphorylated ERK1/2 in INS-1 cells, whilst MEK and calcineurin are constitutively bound [680]. KRS2 is also a substrate for calcineurin and upon glucose mediated Ca^{2+} influx, calcineurin stimulates dephosphorylation of thouree sites on KSR2 causes the sub-cellular redistribution of the KSR2 complex to the plasma membrane [680]. The potential importance of this scaffold protein in glucose stimulated ERK1/2 activation was confirmed by siRNA depletion of KSR2, which led to an increase in basal ERK1/2

phosphorylation in INS-1 cells, and the inhibition of glucose stimulated ERK1/2 activation [680]. The relocation of KSR2 to the plasma membrane upon glucose stimulation could localise KSR2 to L-type VGCCs and provide a mechanism for the local signalling observed in this thesis. The depletion of KSR2 preventing ERK1/2 phosphorylation, the Ca^{2+} sensitivity of KSR2, the translocation to the plasma membrane of KSR2 upon glucose stimulation, and the association of calcineurin (a confirmed component of GLP-1 stimulated ERK1/2 activation [239]) with KSR2 at the plasma membrane, suggests that KSR2 could be a vital component of the pathway mediating GLP-1 stimulated ERK1/2 activation.

Another scaffolding protein that localises ERK1/2 dependent signalling to the plasma membrane, which is associated with GPCR signalling, is β-arrestin. In β-cells, β-arrestin has been shown to be involved in GLP-1 stimulated ERK1/2 activation, as siRNA mediated knockdown of β-arrestin1 prevented ERK1/2 activation at 5, 10 and 30min [262, 592]. However, these experiments were completed in the absence of stimulatory glucose and therefore, are not looking at Ca^{2+}-dependent ERK1/2 activation as discussed in this report. However, due to β-arrestins localisation at the plasma membrane, it is possible that β-arrestin could play a role in Ca^{2+} dependent ERK1/2 activation.

6.2.2 Aggregation of signalling components

The L-type VGCC has been found to associate with a number of signalling proteins including CaM [514, 522], CaMKII [540, 542], PKC [531], AKAPs and PKA [535, 538]. The association of these proteins with the L-type VGCC may lead to vast complexes acting as signalosomes to mediate specific signalling. If signalosome complexes containing the L-type VGCC were mediating Ca^{2+}-dependent microdomain signalling to ERK1/2, or Ras-independent ERK1/2 signalling, the complexes would need to be isolated and only activated by an appropriate biological signal. Several groups have identified the aggregation, isolation and specific activation of L-type VGCCs.

Expression of a YFP-tagged $Ca_V1.2$ L-type VGCC in HEK_{293} cells demonstrated that these channels form self-aggregating clusters of functional channels and that they cluster independently of the channels association with intracellular Ca^{2+} release

channels [529]. Furthermore, a key sequence on the C-terminal tail of $Ca_V1.2$ has been described, that when mutated, prevented the clustering of the channel [530].

The clustering of L-type VGCCs has also been observed in arterial myocytes where persistently active L-type VGCCs are in non-uniform clusters across the cell membrane. However, there was a uniform distribution of all L-type VGCC with the active clusters of channels being associated with PKC [531]. Clustering of L-type VGCCs has also been observed in hippocampal neuronal cultures where both $Ca_v1.2$ and $Ca_v1.3$ L-type VGCCs formed clusters on the membrane surface. Each type of L-type VGCC had various interacting partners that were not present in all signalosome complexes [532]. This suggests that not only the self-aggregation of L-type VGCCs results in a signalosome complex but their association with interacting partners affects their clustering, activation and signalling properties.

One interaction that may help to localise signalling molecules with the channel complex, and thus achieve specific signalling are lipid rafts. Lipid rafts are specialised localisations of lipid, membrane bound proteins and glycoproteins that together allow for discrete and localised signalling. $Ca_v1.2$ has been demonstrated to be associated with caveolin-3 in myocytes [533] and skeletal muscle [534]. Caveolins are a marker of caveolae, which are specialised plasma membrane structures and the first type of lipid raft identified. If the molecular machinery for a distinct cellular response, such as the L-type VGCC signalling to ERK1/2, was localised to a lipid raft this would provide the discrete cue to discriminate between ERK1/2 activation and other signalling pathways in β-cells. The possibility that the GLP-1 mediated ERK1/2 activation requires lipid rafts is strengthened by the evidence that the GLP-1R also interacts with Caveolin-1 [681].

6.3 Physiological consequences of ERK1/2 activation in the β-cell and diabetes

ERK1/2 activation across many cell types is known to have important physiological roles. In β-cells ERK1/2 has been demonstrated to be important in the regulation of insulin gene transcription [67, 132, 141, 143-145, 153] and I have demonstrated that this can occur thourough an ERK1/2 dependent mechanism stimulated by local Ca^{2+} within the mouth of the L-type VGCC. ERK1/2 also regulates β-cell mass thourough effects on

proliferation, differentiation and prevention of apoptosis [138-141], potentially thourough its ability to affect transcriptional activity. These functions alone provide evidence that ERK1/2 is responsible for the maintenance of the β-cell phenotype. However, there are several lines of circumstantial evidence suggesting that the disruption of ERK1/2 activity in β-cells can mediate some of the disease characteristics of diabetes [682-684].

6.3.1 ERK1/2 and insulin transcription

Upon food intake and nutrient digestion the levels of glucose in the bloodstream rise. This period of raised glucose concentrations can influence the signalling outcome for the β-cell [147]. It has been documented that islets exposed to short periods (<6hours) of raised glucose concentrations lead to ERK1/2 activation and its positive regulation of insulin transcription [67, 143-145, 147]. Indeed, in this thesis I demonstrate that 4hours glucose and GLP-1 treatment stimulates ERK1/2 activation and that this leads to insulin transcription. Furthermore, I also determine that local Ca^{2+} signalling within the microdomain of the L-type VGCC is responsible for GLP-1 stimulated ERK1/2 activation. The requirement of the L-type VGCC in mediating both ERK1/2 activation and/or insulin transcription has been documented previously. In MIN6 cells blockade of the L-type VGCC was found to inhibit both glucose and GLP-1 stimulated ERK1/2 activation [68, 129, 261], as well as glucose stimulated ERK1/2 translocation to the nucleus [129]. In dispersed rat islets glucose stimulated CAT expression under the control of a rat insulin promoter was significantly inhibited by the L-type VGCC blocker verapamil [582] and inhibition of the L-type VGCC inhibited the glucose stimulated increase in preproinsulin mRNA levels [583].

Despite the positive effects of raised glucose concentrations on the β-cell, during the progression of either T1DM or T2DM β-cells are likely to be subjected to elevated glucose concentrations for more prolonged periods. Islets exposed to prolonged raised glucose concentrations *in vitro* (24hours) exhibit ERK1/2-dependent inhibition of insulin gene transcription [682]. C/EBP-β protein expression is increased during chouronic glucose exposure leading to C/EBP-β binding the insulin promoter in an ERK1/2 dependent manner, and an inhibition of insulin production [682]. The mechanism of local Ca^{2+} at the L-type VGCC mediating glucose and GLP-1 stimulated ERK1/2 activation and insulin transcription, demonstrated in this thesis, could be

responsible for this chouronic glucose mediated inhibitory response at the insulin promoter. It is possible that the same mechanism is maintained thouroughout the sustained presence of glucose and GLP-1, regardless of the length of stimulation. ERK1/2 is known to affect a large number of downstream signalling components and thus upon the sustained presence of glucose and GLP-1 there will undoubtedly be an up-regulation and modulated expression of various ERK1/2-dependent proteins.

6.3.2 ERK1/2 and β-cell survival

In many cell types ERK1/2 activation can be associated with pro-survival pathways. ERK1/2 has been demonstrated to be activated in response to glucose, insulin and PKA signalling to promote β-cell survival [261, 421]. However, ERK1/2 activation can also mediate apoptosis in response to chouronic exposure to high glucose concentrations or interleukin-1β (IL-1β) stimulation in human and rat islets [685, 686]. The dual roles of ERK1/2 in β-cells are likely to be linked to the either the proteins temporal profile of activation (discussed above), the proteins activated by or associated with ERK1/2, or the upstream activators in the MAPK cascade.

Raf-1 is found upstream of ERK1/2 in many systems. In β-cells Raf-1 has been implicated in pro-survival pathways. A recent study demonstrated that Raf-1 and consequently ERK1/2 activation is required for β-cell survival; a small molecule inhibitor of Raf-1 which led to ERK1/2 inactivation mediated β-cell apoptosis, whilst the over expression of Raf-1 led to an increase in ERK1/2 phosphorylation and a parallel reduction in basal levels of β-cell apoptosis [687]. Insulin has been demonstrated to activate Raf-1 and mediate β-cell survival but the mechanism mediating the anti-apoptotic effects of insulin is currently not understood [688-691]. However, due to the observation that insulin mediates Ca^{2+} transients [688-690] and Ca^{2+}-dependent ERK1/2 activation in β-cells [692], and that ERK1/2 activation is implicated in insulin-stimulated pro-survival [687], the local Ca^{2+}-dependent mechanism of ERK1/2 activation described in this thesis could be involved in the insulin stimulated pro-survival pathway.

The pro-apoptotic effects of ERK1/2 activation are highlighted in studies with chouronic exposure to high glucose concentrations. In human islets exposed to 11.1mM and 33.3mM glucose for 4 days in culture, apoptosis was increased compared to control

(5.5mM glucose). The blockade of ERK1/2 and L-type VGCC activation demonstrated the requirement of both ERK1/2 and extracellular Ca^{2+} for β-cell apoptosis [686]. In rat islets exposed to chouronic glucose concentrations (33.3mM) β-cell apoptosis also occurred and, similarly to human islets, apoptosis was shown to be mediated by L-type VGCC and ERK1/2 activation [685]. The involvement of both the L-type VGCC and ERK1/2 in this pro-apoptotic pathway suggests that the mechanism of ERK1/2 activation described in this thesis that requires local Ca^{2+} at the mouth of the L-type VGCC could play a role in β-cell apoptosis.

The role of extracellular Ca^{2+} influx in mediating β-cell apoptosis is demonstrated with studies that modulate the K_{ATP} channel. In studies that have induced the blockade of the K_{ATP} channel and therefore mediate depolarisation, Ca^{2+}-dependent β-cell apoptosis has been observed [693, 694]. However, studies that open the K_{ATP} channel have indicated that this protects β-cell function [35, 695, 696]. Paradoxically, the activity of the L-type VGCC in β-cells has been demonstrated to mediate increases in β-cell number. The addition of Bay-K-8664 to the media of growing MIN6 cells and rat islets led to an increase in proliferation over a 4 day period [697]. This suggests that the mode of ERK1/2 activation is not the only method the cell can utilise to influence the signalling outcome for the β-cell but perhaps the localisation of L-type VGCC activation can affect signalling.

During the lifetime of a β-cell it can be subjected to ER stress. This can occur due to elevated glucose concentrations leading to high levels of insulin production within the ER. However, a balance is maintained between the folding capacity of the cell, the requirement of insulin and the stress responses. During the progression of diabetes the insulin demand increases due to chouronic elevated glucose concentrations in the bloodstream. The increased demand for insulin can lead to chouronic ER stress and thus ER stress induced β-cell death. One of the proteins activated by an increase in ER stress is CHOP. CHOP is a pro-apoptotic factor demonstrated as the KO of CHOP prolongs β-cell survival and delays the onset of the diabetic phenotype in response to ER stressors [698]. ERK1/2 suppresses CHOP transcription [699] by affecting the binding of transcription factors to the CHOP promoter. The ERK1/2 dependent transcription factor MafA is known to bind to the CHOP promoter and prevent its transcription [682, 699]. Therefore, the activation of ERK1/2 by either GLP-1 or mAChR stimulation, as

demonstrated in this thesis, could lead to changes in CHOP transcription and the onset of the diabetic phenotype stimulated by to ER stressors.

One other ERK1/2 associated protein that has been linked with T2DM is the anti-apoptotic factor PEA-15. This protein contains a death effector domain [700] and is highly enriched in patients with T2DM [701]. PEA-15 has been demonstrated to promote the nuclear export of ERK2 thourough disrupting its interactions with nucleoporins [702, 703]. In addition, the over expression of PEA-15 in cultured β-cells reduces glucose stimulated insulin secretion, whilst transgenic mice over-expressing PEA-15 have decreased glucose tolerance, develop diabetes on a high fat diet, and display impaired insulin secretion [683]. The functional effects of PEA-15 and the enrichment of this protein in those with T2DM suggests that ERK1/2 plays a role in maintaining β-cell function thourough its actions in the nucleus and that a reduction in ERK1/2 function can mediate a diabetic phenotype.

6.4 Future directions

As I have demonstrated that ERK1/2 activation occurs in β-cells via a mechanism requiring local Ca^{2+} influx at the mouth of the L-type VGCC, and that ERK1/2 is activated by a Ras-independent pathway, I would like to investigate further the physiological significance of this ERK1/2 activation.

6.4.1 β-cell proliferation

As ERK1/2 in β-cells has been demonstrated to be a key mediator in β-cell proliferation I would like to investigate the role of ERK1/2 activated by Ca^{2+} at the mouth of the L-type VGCC in β-cell proliferation. Both GLP-1 and mAChR stimulation of β-cells leads to ERK1/2 activation and both receptors have been implicated in β-cell proliferation. GLP-1 has been demonstrated to mediate β-cell replication by various different mechanisms including thourough the activation of PKB, PI3K and ERK1/2 [139, 232, 233, 276, 279]. GLP-1 stimulated ERK1/2 activation has been implicated in β-cell replication thourough its role in stimulating cyclin D transcription which was demonstrated to be an essential step in mediating proliferation [139]. The role of mAChR in β-cell replication is still unclear but in other cell types mAChR stimulation leads to Ca^{2+} and ERK1/2 dependent replication [416, 424] and during β-cell replication M1 and M3 receptor expression is increased [423] indicating a potential role for

mAChR in β-cell replication. In addition to the evidence that GLP-1, mAChR and ERK1/2 mediate β-cell replication, the L-type VGCC alone has been demonstrated to stimulate β-cell proliferation [697]. The application of the L-type VGCC activator Bay-K-8644 to the growth media of several β-cell lines and islets demonstrated an increased proliferation over a 4 day period [697].

Altogether as 1) ERK1/2 has been implicated in proliferation by several different agonists, 2) as both GLP-1 and mAChR stimulation are likely to mediate β-cell replication and, 3) as L-type VGCC activation alone can mediate β-cell proliferation, I hypothesise that ERK1/2 activation mediated by Ca^{2+} at the mouth of the L-type VGCC may be capable of mediating β-cell replication.

In order to investigate this possibility I would measure increases in ATP as an initial indicator of cell proliferation and then confirm these results using flow cytometry in β-cell lines. An increase in ATP is indicative of a rise in cell number whist flow cytometry will determine the number of β-cells in the proliferating phase of the cell cycle (G2/M). L-type VGCC activation using BayK-8644, as well as indirect activators of the L-type VGCC such as GLP-1, carbachol and K50 would be used to stimulate proliferation and the role of ERK1/2 determined thourough the use of dominant negative ERK1/2 constructs. These constructs have been used previously in β-cell lines to assess the role of ERK1/2 in transcription [144]. If ERK1/2 was deemed necessary for proliferation, to assess the role of Ca^{2+} at the mouth of the L-type VGCC nifedipine application at several time points post-agonist stimulation would be used to block Ca^{2+} influx and agonist stimulated ERK1/2 activation.

6.4.2 Conserved mechanism across secretory cell types

One final direction that could be undertaken is the investigation into whether ERK1/2 activation via Ca^{2+} at the mouth of the L-type VGCC is a mechanism specific to β-cells or whether it occurs in other cell types. In neurones the activation of ERK1/2 has been demonstrated to occur via CaM-binding to the L-type VGCC, indicating that local Ca^{2+} is required for ERK1/2 activation. To assess this hypothesis a neuronal cell line and/or cultured neurones could be loaded with the Ca^{2+} chelators EGTA and BAPTA-AM prior to stimulation with depolarising concentrations of K^+ and the effect on ERK1/2 activation assessed.

As it is likely that both β-cells and neurones activate ERK1/2 via the same mechanism, requiring Ca^{2+} at the mouth of the L-type VGCC, it is possible that other cell types also utilise this pathway. However, β-cells and neurones share many features surrounding the key components of this pathway. Both cell types are secretory and couple the L-type VGCC to secretion of docked vesicles at the plasma membrane. It is possible therefore, that the localisation of the secretory machinery leads to the localisation of the components required for this local Ca^{2+} stimulated ERK1/2 activation. To assess this hypothesis other secretory cell types including α-cells, δ-cells, chouromaffin cells and secretory epithelial cells, could be investigated to see if a correlation exists between L-type VGCC dependent secretion and L-type VGCC dependent ERK1/2 activation.

6.5 Summary

All together this thesis has demonstrated that L-type VGCC dependent ERK1/2 activation in pancreatic β-cells occurs upon stimulation with either hormones or neurotransmitters released onto the cells during food intake. Both the stimulation of β-cells with either GLP-1 or mAChR agonists are likely to mediate physiologically relevant outcomes and indeed, I have demonstrated that GLP-1 mediates the transcription of insulin thourough an L-type VGCC and ERK1/2 dependent pathway (Figure 3.15). However, the relevance of the ERK1/2 activation demonstrated in this thesis in diabetes, a disease specifically related to the functionality of the β-cell, has yet to be discovered.

Chapter 7: References

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