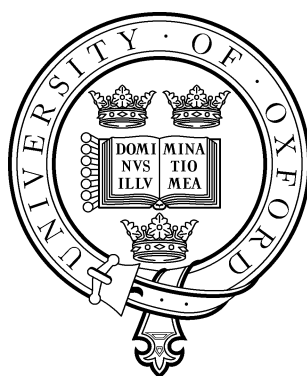


The Role of Two Pore Channels (TPCs) in Pancreatic Beta Cell Stimulus-Secretion Coupling



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New College

Oxford University

This thesis is submitted in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy

Trinity Term 2012

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Abstract

This thesis presents an investigation into the role of the recently identified two pore channels (TPCs) in β -cell stimulus-secretion coupling. TPCs are the receptors for calcium mobilising messenger nicotinic acid adenine dinucleotide phosphate (NAADP) located in the membrane of acidic intracellular calcium stores. It is proposed that they are responsible for the ATP-sensitive potassium channel (K_{ATP} channel) independent pathway of stimulus-secretion coupling; and that this pathway is not subordinate to the K_{ATP} channel dependent pathway; but an alternative explanation of stimulus-secretion coupling in its own right.

The first section of this thesis presents a characterisation of sub-membrane calcium signals observed in primary mouse β -cells in response to glucose and the membrane-permeable acetoxymethyl ester form of NAADP (NAADP-AM) using the non-ratiometric fluorescent calcium indicator fluo-4 and total internal reflection (TIRF) microscopy. These are compared to global cytosolic calcium changes observed with epifluorescence microscopy. Factors affecting the shape and time course of responses are investigated, and pharmacological tools used to provide evidence for the role of intracellular calcium release from acidic stores mediated by NAADP.

Having characterised the calcium responses of β -cells using TIRF; the second part of the thesis examines the effects of knocking out TPC2 (single KO), or both TPC1 and TPC2 (DKO) on these responses; after an initial assessment of pancreatic islet and β -cell morphology using electron microscopy. Gender differences in β -cell responses to glucose and NAADP are assessed in both wild type and knockout animals.

Finally, the third section presents the discovery of elementary calcium release events in pancreatic β -cells. The current project visualises what are likely the triggering events for the global calcium signals examined in sections one and two. They take the form of localised calcium release in response to NAADP-AM and glucose; akin to sparks and puffs observed by stimulation with cADPR and IP_3 . Optical quantal analysis demonstrates the quantal nature of the events and estimates the size of the unitary calcium release unit (CRU) for NAADP.

To Mama and Papa

*Jedes Naturgesetz, das sich dem Beobachter offenbart,
lässt auf ein höheres, noch unerkanntes schliessen.*

—Alexander von Humboldt

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Abbreviations

ACh	Acetylcholine
Actb	Beta-actin
ADP	Adenosine diphosphate
AM	Acetoxymethyl ester
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BF	Brightfield
BL	Baseline
BP	Base pair
BSA	Bovine serum albumin
C57BL/6	C57 black 6, inbred strain of <i>mus musculus</i>
cADPR	Cyclic ADP ribose
cAMP	Cyclic AMP
CCD	Charge coupled device
CD38	Cluster of differentiation 38 (also cADPR hydrolase)
CICR	Calcium-induced calcium release
cNed-19	cis-Ned-19
CRAC	Calcium release activated calcium

CRU	Calcium release unit
DAG	Diacylglycerol
dH_2O	distilled water
DKO	Double knockout
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGTA	Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'-tetraaceticacid
EM	Electron microscopy
ER	Endoplasmic reticulum
ES Cell	Embryonic stem cell
FBS	Foetal bovine serum
Fluo-4	2-([2-(2-(5-[Bis(carboxymethyl)amino]-2-methylphenoxy)ethoxy)-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl](carboxymethyl)amino)acetic acid
G	Glucose concentration [mM]
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GPN	Glycyl-L-phenylalanine-beta-naphthylamide
HEK	Human embryonic kidney 293 cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hank's balanced salt solution
IC ₅₀	Half maximal inhibitory concentration
IP_3	Inositol 1,4,5- trisphosphate
IP_3R	Inositol 1,4,5- trisphosphate receptor
K_{ATP}	ATP-sensitive potassium channel
KO	Knockout
LAMP	Lysosomal-associated membrane protein
MIN6	Transgenic C57BL/6 mouse insulinoma cell line
MRC	Medical Research Council (UK)
mRNA	Messenger ribonucleic acid
NAADP	Nicotinic acid adenine dinucleotide phosphate
NADP	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NCX	Sodium calcium exchanger
NIH	National Institutes of Health (US)
OCDEM	Oxford Centre for Diabetes, Endocrinology and Metabolism
ORAI1	Calcium release-activated calcium channel protein 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Penstrep	penicillin/streptomycin
PFS	Perfect Focus System
PH	Peak height
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PMCA	Plasma membrane calcium ATPase

RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic acid
rNed-19	Ned-19, racemic mixture
ROI	Area of interest
RPMI	Roswell Park Memorial Institute Medium
RT	Reaction time
RT-PCR	Reverse transcription polymerase chain reaction
RyR	Ryanodine receptor
SD	Standard deviation
SEM	Standard error of the mean
SERCA	Sarcoendoplasmic reticular calcium ATPase
SOCE	Store-operated calcium entry
SR	Sarcoplasmic reticulum
STIM1	Stromal interaction molecule 1
SUR	Sulfonyl urea receptor
TD	Time to ‘dip’
TIRF	Total internal reflection fluorescence
tNed-19	trans-Ned-19
TPC	Two-pore channel
TPCN	Two-pore channel gene
TR	Time to rise
TRP	Transient receptor potential
TRPC	Transient receptor potential (canonical)
TRPM	Transient receptor potential (melastatin)
TRPV	Transient receptor potential (vanniloid)
UV	Ultraviolet
VGCC	Voltage-gated calcium channel
V_m	Membrane potential
WT	Wild type

Chapter 1

Introduction

1.1 Calcium Homeostasis and Signalling

1.1.1 Calcium as a Messenger

Calcium is the most ubiquitous messenger known. This single ion controls processes as diverse as muscle contraction, hormone release, neural transmission and cell movement. Calcium is the key signal initiating fertilisation and the key signal leading to cell death. Calcium controls this vast array of processes through binding and unbinding from target proteins, through the electrical currents it generates by moving across cytosolic or intracellular membranes, and by the concentration gradients it creates within intracellular space, leading to calcium signals of different amplitudes and morphologies (Cheng and Lederer, 2008).

1.1.2 Calcium Homeostasis

In order to control and utilise this powerful cell signalling tool, the calcium concentration within a cell and its organelles needs to be tightly regulated.

Regulation of Intracellular Calcium Concentration

Cytosolic free calcium in mammalian cells is kept at ~ 100 nM even though in the extracellular fluid it can reach up to 2 mM. This is achieved by extrusion of calcium

across the cell membrane, sequestration into intracellular stores, and chelation by intracellular buffers; see Schwaller (2012) for review. The former two processes occur through the action of a variety of ATP-dependent calcium pumps and exchangers, and lead to the creation of concentration gradients across cellular and organellar membranes (see Clapham, 2007, for review). Fig. 1.1 illustrates the various ways in which calcium concentration is controlled within the cytosol. The main mechanisms by which this is achieved are the plasma membrane calcium ATPase (PMCA), which expends ATP to pump calcium against its concentration gradient across the cell membrane; the sarcoendoplasmic reticular ATPase (SERCA), which moves calcium into the endoplasmic reticulum (ER) by the same method (Carafoli, 1991); the calcium uniporter, which transports calcium into mitochondria, and the sodium-calcium exchanger, which exists in the cell membrane as well as the membranes of organelles and makes use of the sodium gradient across these membrane to pump calcium out of the cytosol in exchange for sodium's entry; see Carafoli et al. (2001) for review.

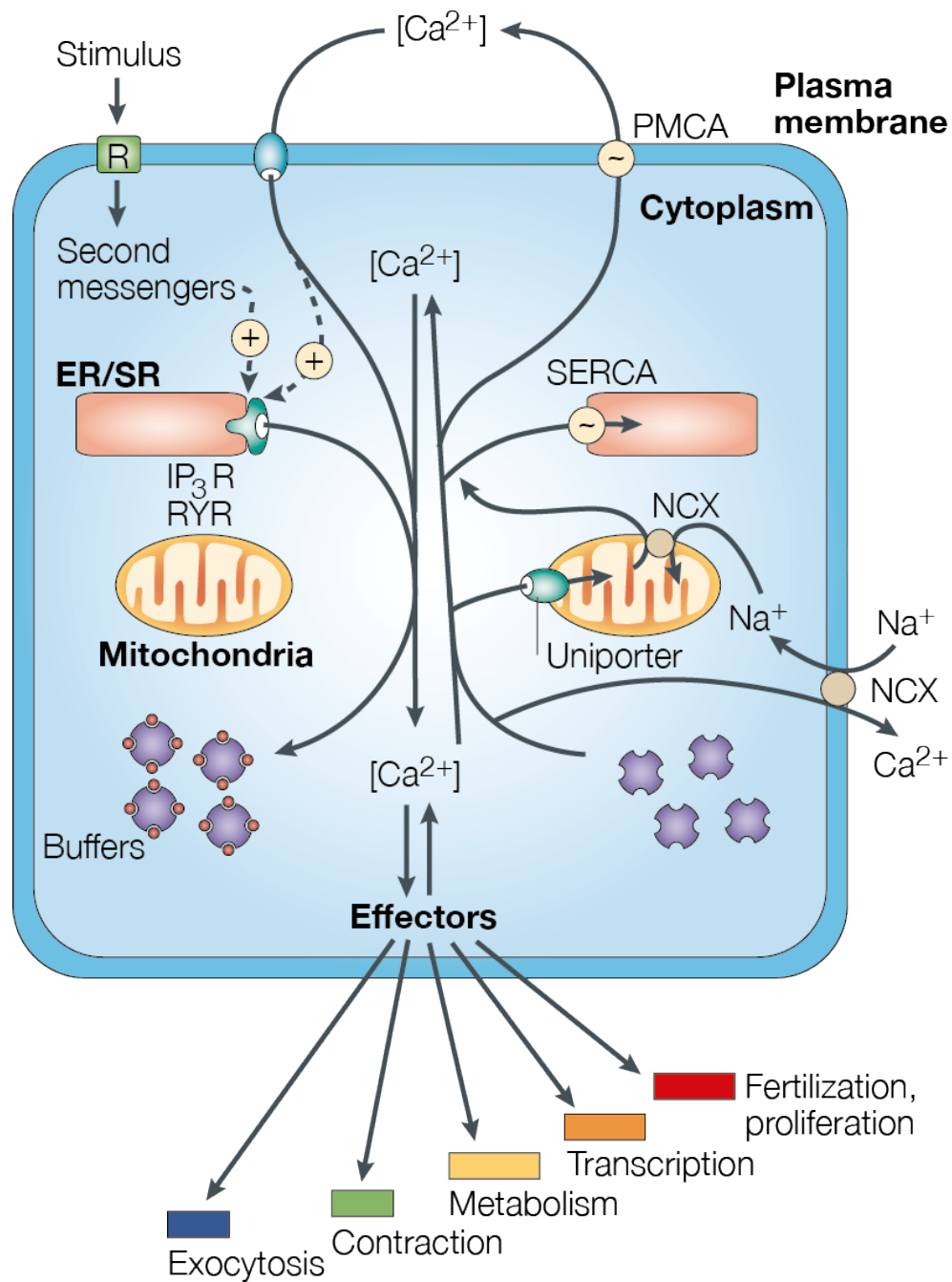


Figure 1.1: Calcium Homeostasis. Calcium Pumps (Plasma Membrane Calcium ATPase, PMCA; Sarco-Endoplasmic Reticular ATPase, SERCA; Sodium-Calcium Exchanger, NCX; and Calcium Uniporter are shown (Modified from Berridge et al., 2003)

Initiation and Termination of Calcium Signals

Calcium signals are initiated by the movement of calcium into the cytosol down its concentration gradient (either across the plasma membrane, or across the membrane of an organelle) by means of calcium-selective, or cation-selective ion channels. Calcium signalling is terminated by the inactivation of these channels and the action of the above-mentioned calcium pumps and exchangers that return calcium to intracellular stores or remove it from the cell (Carafoli et al., 2001). The period for which calcium remains elevated (locally or globally), and its spatial distribution depends primarily on the particular constellation and density of calcium release channels and pumps/exchangers within the cell (or area within the cell) in question. Different cell types express different sets of channels and pumps, and at different densities and subcellular distributions; allowing them to tailor the movement of calcium to their requirements of it as a messenger (Berridge et al., 2003).

1.1.3 Calcium Influx across the Cell Membrane

Voltage-dependent Calcium Influx

The main route by which cytosolic calcium is increased is by influx across the cell membrane. This most often occurs through voltage gated calcium channels (VGCCs) in response to membrane depolarisation. These voltage-gated channels can be of various subtypes (L, T, N, P, Q, and R); the most common of which in β -cells, the L-type channel, is thought to be responsible for the majority of calcium influx in response to glucose (see for example Braun et al., 2008). Although voltage gated calcium entry is the most common mode of influx; there are other channels in the cell membrane that allow the passage of calcium into the cell which are voltage independent. These include ligand gated channels, receptor operated channels, second-messenger

operated channels, thermosensor- and stretch-activated channels, and store-operated channels (discussed below) as described by Berridge et al. (2003).¹

Store-operated Calcium Entry (SOCE)

Store-operated, also known as capacitative, calcium entry occurs in response to ER calcium depletion. A sensor in the ER membrane, STIM1, reacts to the falling calcium concentration within the store and leads to influx of calcium across the plasma membrane through ORAI1, which, in turn, can be taken up by the ER. The SOCE current through ORAI1 is known as I_{CRAC} . The existence of this pathway was first suggested by Putney (1986); and was initially believed only to be present in non-excitable cells (Liu and Gylfe, 1997). It has been shown to exist in pancreatic β -cells; see Dyachok and Gylfe (2001); and may be relevant for the creation of complex calcium oscillations observed in response to glucose. For a review of SOCE, see Lewis (2011); Berna-Erro et al. (2012).

1.1.4 Calcium Sequestration from Intracellular Stores

Organelles as Calcium Stores

Apart from entering the cell by influx through cell membrane channels, cytosolic calcium can also be raised by release from intracellular stores. This is one mode of action of G-protein coupled receptors, which stimulate the production of second messengers that in turn release calcium by binding to their respective receptors on intracellular stores. The identity of two messengers releasing calcium in this way has been established and studied for a long time; IP_3 and cADPR; activating IP_3 receptors and ryanodine receptors, respectively.

¹Many of these channels belong to the transient receptor potential (TRP) family of channels (TRPV, TRPC, and TRPM).

IP₃-mediated Calcium Release

Since its discovery in 1983 (Streb et al., 1983), IP₃ has become established as the prototypical calcium-releasing messenger. It is produced in response to many agonists (often via G-proteins) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into the soluble IP₃ and the membrane-bound diacylglycerol (DAG) by phospholipase C (Berridge and Fain, 1979). IP₃ binds its receptor, which is mainly found on the ER (though there have been some reports of its occurrence on Golgi apparatus- and cell membranes; see for example Dellis et al., 2006). Calcium release via IP₃Rs has been established as originating in the ER; see for example (Berridge and Irvine, 1984). IP₃R activation is dependent on cytosolic calcium concentration. If this is at resting level (~ 100 - 200 nM), IP₃ stimulates calcium release in a dose dependent manner; if cytosolic calcium is in the micromolar range, it inhibits it; resulting in a bell shaped dose-response curve (see Iino (1987) for the first demonstration of IP₃R calcium sensitivity; and Bezprozvanny et al. (1991) for the bell-shaped dose response curve). This gives the signal a self-limiting capacity. For a comprehensive review of IP₃R function, see for example Taylor and Tovey (2010).

cADPR-mediated Calcium Release

A second calcium mobilising messenger, cyclic ADP ribose (cADPR), also targets the ER as a calcium store. cADPR acts on ryanodine receptors (RyRs) that are usually activated by cytosolic calcium. Like IP₃Rs, RyRs show a bell shaped concentration-response curve to cytosolic calcium (Meissner et al., 1997). Notably, the concentrations required to activate RyRs are higher than those that stimulate the IP₃Rs. This shifts their deactivation threshold toward higher concentrations, also, which makes them ideally placed for calcium induced calcium release (discussed below). cADPR sensitises RyRs to calcium (Galione et al., 1991), and might do so without binding directly to the receptor itself. Rather; it is assumed that an intermediary binding protein is involved (Walseth et al., 1993). Notably, in pancreatic β -cells, cADPR can lead to

calcium entry across the cell membrane. This has been proposed to be the result of an action on a member of the TRPM2 channel family (Togashi et al., 2006). For recent reviews of cADPR and RyR signalling; see Lee (2011) and Lanner (2012).

NAADP-mediated Calcium Release

More recently, a third calcium mobilising messenger was discovered in the form of Nicotinic acid adenine dinucleotide phosphate (NAADP). This was first described as an impurity in commercially available NADP preparations (Lee and Aarhus, 1995) and was found to release calcium in sea urchin eggs. Since then, its presence as a calcium-mobilising messenger has been demonstrated in many mammalian cells, including pancreatic β -cells (Johnson and Mislser, 2002; Masgrau et al., 2003). NAADP is the most potent calcium mobilising messenger known to date; releasing calcium at nanomolar concentrations (Lee, 1997; Cancela et al., 1999); compared to the micromolar concentrations of IP_3 and cADPR required to stimulate calcium release from the ER.

NAADP is thought to be synthesised through base-exchange catalysed by ADP-ribosyl cyclases (most likely CD38 and CD157; Lee and Aarhus, 1995). Fig. 1.2 shows CD38 catalysing the reactions producing NAADP (in the presence of acidic pH) and cADPR (in the presence of neutral pH) depending on the enzyme's surroundings. There is much debate about where CD38 is localised within (or indeed outside) the cell when it produces NAADP in response to stimulation, and how it arrives there (see Lee, 2012 for review). Since NAADP is not membrane permeable², it would have to be transported across the cell membrane if it were produced extracellularly; or the lysosomal membrane, if produced within these acidic stores.

NAADP does not release calcium from the ER, like IP_3 and cADPR; but from acidic organelles (see Churchill et al., 2002, for the first demonstration). For a comprehensive review of the many elegant studies that paved the way to this now established fact, see

²Although some groups have suggested that it might be (Shawl et al., 2012).

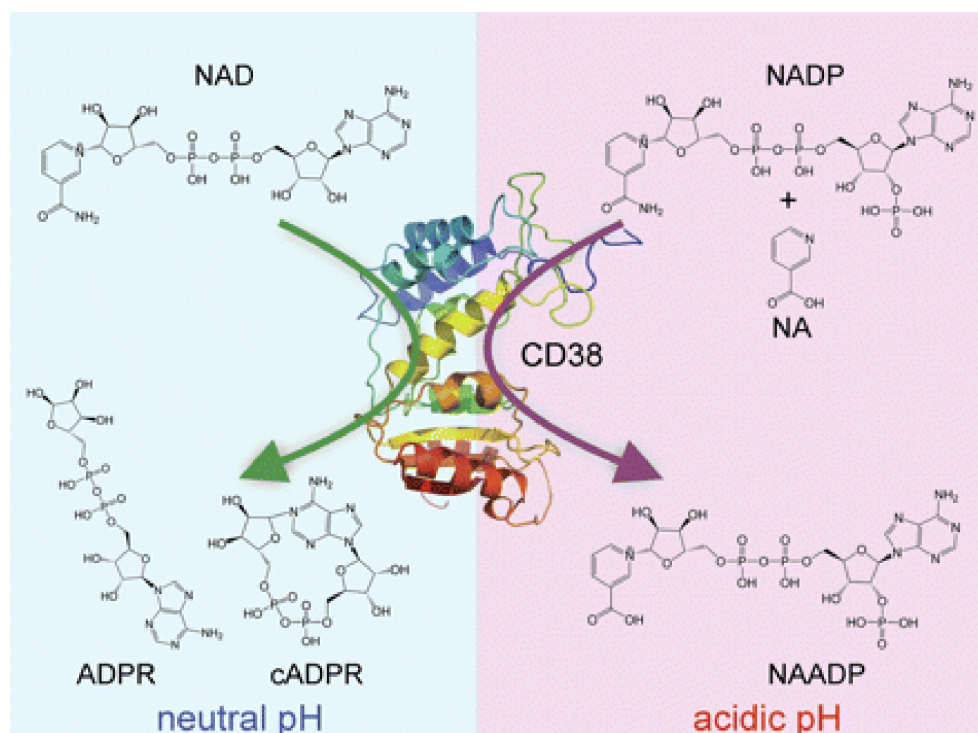


Figure 1.2: NAADP Synthesis by CD38 (Reproduced from Lee, 2012)

Morgan et al. (2011); Galione et al. (2010). In β -cells, NAADP may release calcium from insulin containing vesicles (Mitchell et al., 2003), which, in their ‘mature’ state, have a pH of ~ 5.5 (Orci et al., 1986).

In marked contrast to IP_3 and cADPR, NAADP mediated calcium release is not affected by cytosolic calcium concentration (Chini and Dousa, 1996). NAADP is self-desensitising in its own right, however. As shown in Fig. 1.3; when applied intracellularly, the resultant calcium release in mammalian cells shows a characteristic bell-shaped concentration-response curve (Berg et al., 2000; Masgrau et al., 2003).³ Pharmacologically the NAADP response can be inhibited by agents that interfere with the function of acidic stores such as GPN and bafilomycin A1⁴, as well as drugs that

³This is potentially due to the fact that its receptor has two binding sites of different affinities (Rosen et al., 2009).

⁴GPN lyses acidic stores osmotically by two methods; as a substrate for cathepsin C, and in a protease-independent mode, likely to involve the organelles acidic pH (Duman et al., 2006). Bafilomycin A1 also blocks the organelle proton pump; leading to alkalisation of the organelle and its incapacity to store calcium (Drose and Altendorf, 1997).

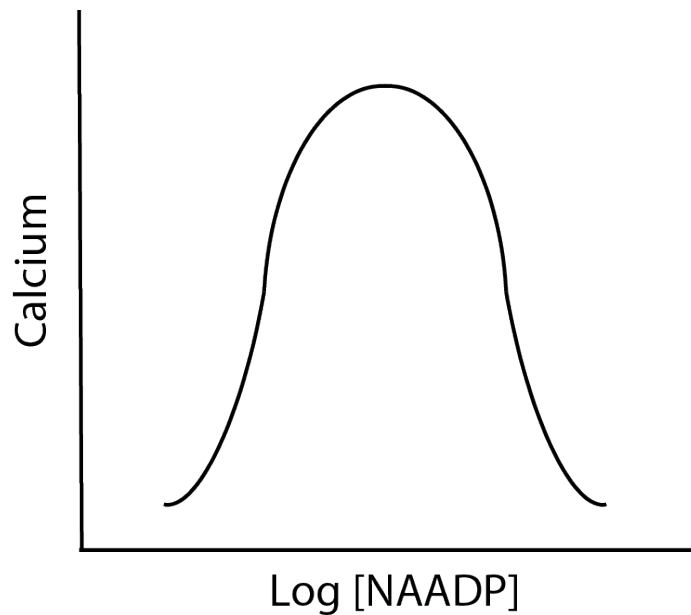


Figure 1.3: NAADP shows a Bell-shaped Dose-Response Curve

block the NAADP receptor. The latter includes the specifically synthesised NAADP antagonist Ned-19 (Naylor et al., 2009), as well as some known antagonists of L-type calcium channels (dihydropyridines; diltiazem), and the anti-diabetic drug and K_{ATP} channel antagonist glibenclamide at high concentrations (Genazzani et al., 1997).

Calcium-induced Calcium Release (CICR)

Calcium itself can have a regulated positive feedback effect on calcium release channels (including IP_3 Rs and RyRs); leading to regenerative calcium release. This phenomenon is known as calcium-induced calcium release (CICR) and can be regulated by IP_3 and cADPR. It is responsible for the augmentation of small, local signals into larger ones that can involve the entire cell; or get propagated across multiple cells, if these are connected by gap junctions. It is crucial in excitation-contraction coupling in heart muscle, where calcium is released from the sarcoplasmic reticulum (SR) in response to influx through L-type channels (Fabiato, 1983). CICR has been demonstrated to occur in pancreatic β -cells (Islam et al., 1992; Lemmens et al., 2001); and to amplify calcium-dependent exocytosis (Kang and Holz, 2003).

1.1.5 Elementary Calcium Events

In addition to the widespread or ‘global’ calcium signals mentioned above, local and ‘elementary’ signals have been recorded which act as trigger events for the former. When discussing ‘elementary’ calcium events; this does not necessarily refer to calcium release through an individual calcium channel (a true unitary calcium release), but simply a small local calcium release that acts as a trigger or building block for larger calcium signals that cause physiological action at the level of the whole cell. The first elementary calcium events were discovered in the form of IP₃R dependent calcium puffs in *Xenopus* oocytes (Parker and Ivorra, 1990). This was followed by the discovery of RyR dependent calcium sparks in cardiac myocytes (Cheng et al., 1993). Since then, equivalent elementary calcium events have been described in many cell types (including non-excitabile cells) through various calcium channels and in response to many agonists. When calcium is released through a single channel or a small number of channels, the resultant signal is known as a blip in the case of the IP₃ receptor (stimulated by IP₃), and a quark when mediated by the ryanodine receptor (stimulated by cADPR). Larger events, resulting from the opening of slightly bigger groups of channels, are known as puffs and sparks, respectively; see Cheng and Lederer (2008) for review. This is the tip of an iceberg of terminology associated with the field of ‘sparkology’, illustrated in abbreviated form in Table 1.1.

Elementary calcium signals can be measured in their amplitude, diameter in three dimensions and time course. These various parameters can be used to produce frequency histograms in analogy to those of classical quantal analysis in neuromuscular junction (see Del Castillo and Katz, 1954, for the original). The resultant histograms will often show a modal (Poly-Gaussian) distribution, illustrating the quantal nature of elementary calcium events (see Appendix A.8). ‘Quantal’ here refers to the fact that each recorded calcium signal’s amplitude (or other parameter) is a multiple of a particular smaller amplitude (or other parameter), known as the quantum or unitary event. The possible shapes of these frequency histograms for different calcium signals

Calcium Release Unit (CRU)	Cluster of calcium channels in the ER/SR membrane
Calcium Spark	Release from a single CRU
Calcium Syntilla	Calcium spark in a neuronal presynaptic terminal
Calcium Sparklet	Transient from a single calcium channel
Calcium Ember	Long-duration low amplitude calcium release event
Ghost Spark	Spark in a treated cell that normally lacks sparks
Calcium Glow	Long-duration spark
Calcium Burst	Long-duration compound spark
Calcium Quarx	Release through a single RyR
Calcium Quantum	Signal that confers to a single RyR or coupled subgroup in a CRU
Compound Calcium Spark	Also called macrospark- synchronous activation of multiple CRUs
Calcium Wave	Regenerative release that travels across the cell
Calcium Wavelet	Abortive calcium wave
Calcium Spike	Local calcium transient in the presence of excess calcium buffers
Calcium Puff	Local release of IP ₃ origin
Calcium Blip	A very small calcium puff
Calcium Mark	Calcium transient originating from the mitochondrion
Calcium Clock	Oscillatory calcium release coupled to the electrophysiological clock

Table 1.1: *Sparkology Terminology (Abbreviated from Cheng and Lederer, 2008).*

and their respective physiological implications are contested, and many parameters influence the variability in the observed results.

Quantitative and qualitative definitions of the identity of elementary calcium signals aside; their functional purpose is clear as a trigger for cellular processes of the utmost importance. Having defined the general pattern of IP₃- and cADPR-mediated calcium signals above; and observed that NAADP acts on acidic stores in a similar way as do the above messengers on the ER; it would seem likely that there exist equivalent elementary events to puffs and sparks mediated by NAADP.

1.2 Two Pore Channels (TPCs)

1.2.1 Discovery of TPCs

Two pore channels (TPCs), named such not because they have two ion conducting pores, but because they have two pore-forming domain repeats in their molecular structure, have been known for over a decade. They were cloned from rat (Ishibashi et al., 2000) and *Arabidopsis thaliana* (Furuichi et al., 2001) within the space of a

few months; and shown to mobilise calcium in response to sucrose in *Arabidopsis thaliana* shortly after (Furuichi et al., 2001). It took a further 8 years until they were considered in the context of NAADP signalling (Calcraft et al., 2009).

1.2.2 TPCs as NAADP Receptors

The first evidence for the identity of TPCs as NAADP receptors came from overexpression studies. HEK cells showed a potentiated response to NAADP following overexpression of sea urchin or mammalian TPC isoforms (Calcraft et al., 2009; Ruas et al., 2010). These studies were followed by a plethora of expression studies in various other systems; see Marchant et al. (2012), for review. If parts of the pore-forming region of the TPC gene were mutated, overexpression failed to elicit this amplified response to NAADP; see for example Brailoiu et al. (2010). In the same paper, the authors re-directed TPCs to the cell membrane and were consequently able to record NAADP dependent currents in whole cell patch-clamp. The arrival of TPC knockout mice allowed for the demonstration of impaired NAADP signalling in pancreatic β - and acinar cells in the absence of TPC1 or TPC2; see for example Calcraft et al. (2009); Chuang (2011); Arredouani et al. (submitted for publication).

More recently, patch clamp studies on individual purified lysosomes of HEK cells have confirmed that human TPC2 conducts an NAADP activated current in response to stimulation at 60 nM, but not 5 μ M NAADP. This current is non-rectifying and is sensitive to intralysosomal pH. Furthermore it was shown that human TPC2 is highly selective for calcium (Schieder et al., 2010; Schieder, 2011). Pitt and colleagues have shown by insertion of TPC2 into lipid bilayers that these are regulated by luminal pH as well as luminal calcium (Pitt et al., 2010). It has also been shown that human TPC1 is activated by NAADP, high intralysosomal calcium concentrations, as well as being voltage-gated. TPC1 mediated currents could be recorded in the absence of NAADP (Rybalchenko et al., 2012).

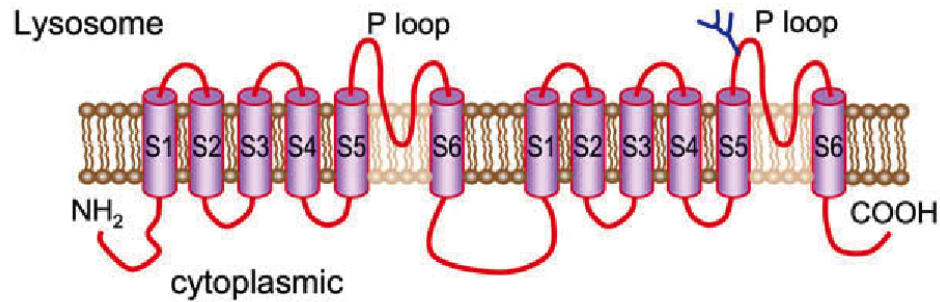


Figure 1.4: Structure of the Two Pore Channel. The Schematic illustrates the two Repeats with Pore Loops on the lysosomal side and the N-terminus and C-terminus on the cytoplasmic Side (Modified from Zhu et al., 2010c)

1.2.3 TPC Structure

TPCs are related in their structure to voltage-gated sodium and calcium channels. However, while the latter comprise four pore-forming repeats of six transmembrane α -helices and a pore-loop each; TPCs possess only two. The structure of TPC with its two pore-forming repeats is shown in Fig. 1.4. The other ion channel subfamily that is structurally related to TPCs is the TRP channel family, which only possesses a single repeat of six α -helices. It was thus suggested that TPCs might be an evolutionary intermediary between those two groups of channels (Ishibashi et al., 2000).⁵ Fig. 1.5 shows the phylogenetic tree for TPCs.

TPCs are encoded by two non-allelic genes (TPCN1 and TPCN2) in primates and some rodents (including mice), three in other mammals (TPCN1, TPCN2, and TPCN3), and up to 6 in some other species (see Zhu et al., 2010b, for review).

⁵Four-pore channels function as monomers, while one-pore channels form tetramers. TPCs have been suggested to form dimers (Ishibashi et al., 2000), and recently shown to be capable of forming heterodimers as well as homodimers (Funnell, 2011; Rietdorf et al., 2011).

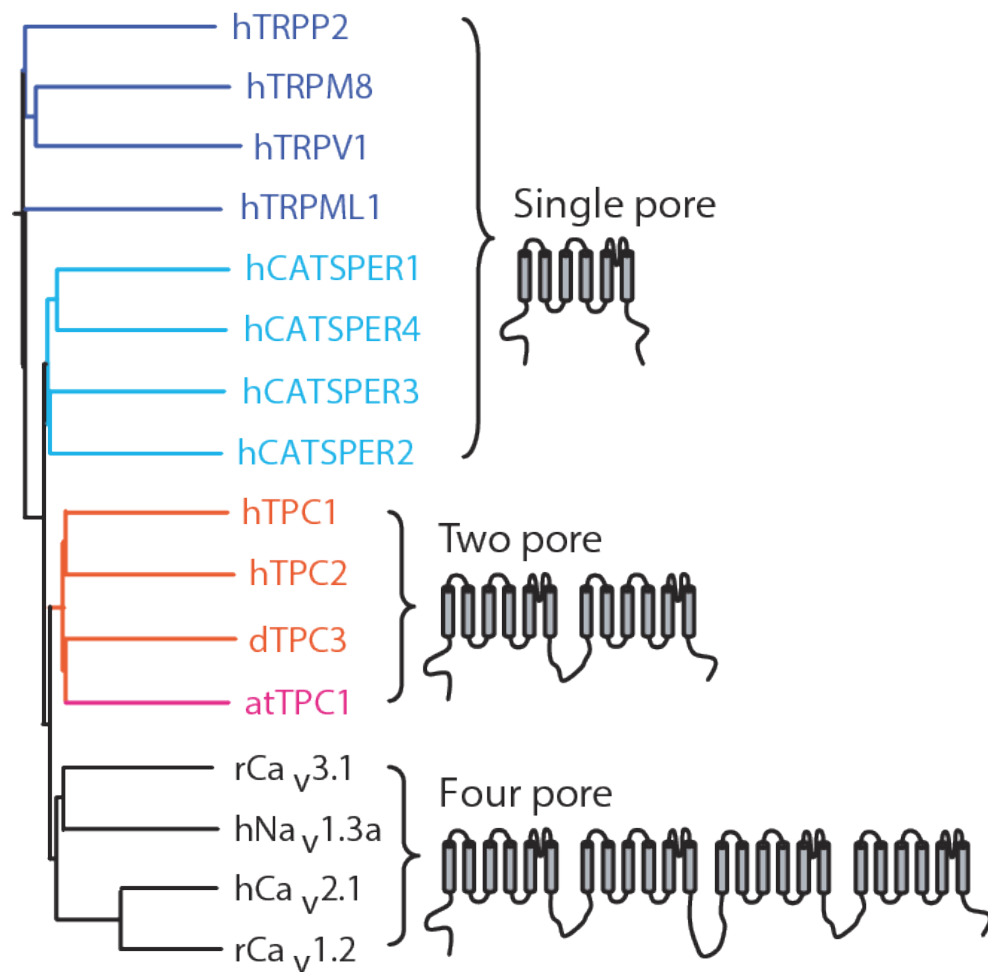


Figure 1.5: Phylogenetic Tree of TPCs and Related Four-pore voltage-gated Sodium and Calcium Channels and One-pore Potassium Channels. *h*, human; *d*, dog; *r*, rat; *at*, *Arabidopsis thaliana* (Reproduced from Calcraet et al., 2009)

1.2.4 Localisation of TPCs

Expression of TPCs in Tissues

TPCs in general seem to be widely expressed. In humans, for example, TPC2 is expressed across many different tissues (Calcraft et al., 2009); as shown in Fig. 1.6. In mouse, also, TPC1 and TPC2 are expressed across a large range of tissues; and give the impression that TPC1 expression is higher than TPC2 expression (Zong et al., 2009; Schieder, 2011). The expression of TPC1 mRNA in mouse whole pancreas has been demonstrated (Chuang, 2011); and more recently the expression of both TPC1 and TPC2 mRNA specifically in mouse islets (Arredouani et al., submitted for publication). Also, the expression of mCherry-tagged TPC1 and TPC2 has been demonstrated in MIN6 cells and human pancreatic β -cells, respectively (Arredouani et al., submitted for publication). TPC2 staining co-localised with LAMP1, a lysosomal marker, in primary human β -cells.

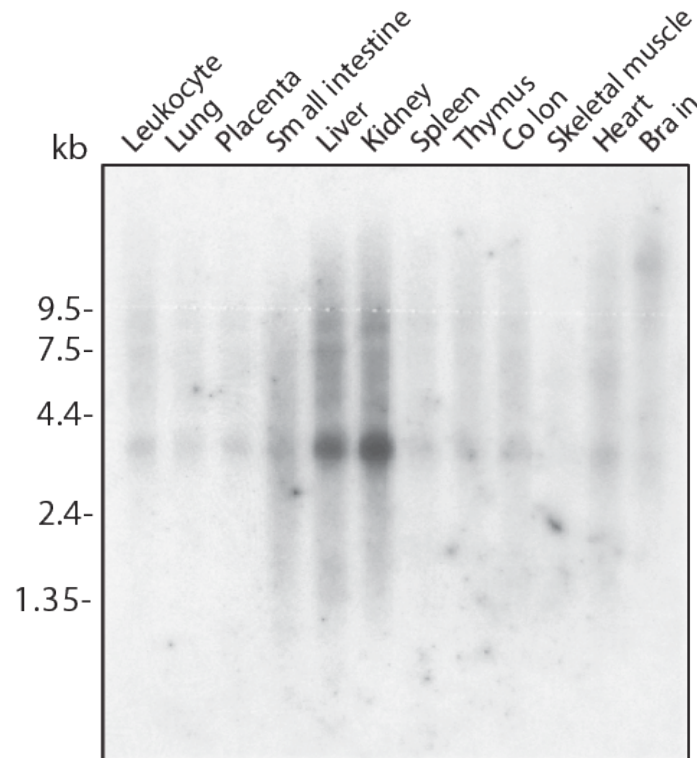


Figure 1.6: Northern Blot Analysis of TPC2 Expression in Human Tissues. TPC2 is denoted by the 3.2kbp Band which is present in all Tissues (Reproduced from Calcraft et al., 2009)

Localisation of TPCs within the Cell

Within the cell, TPCs localise to acidic stores; see for example Calcraft et al. (2009); Schieder (2011); Ruas et al. (2010). While TPC2 clearly co-localised with lysosomal marker LAMP1, TPC1 showed much less overlap with this. Instead, it seemed to co-localise to some extent with markers of early and late endosomes. Fig. 1.7 shows TPC in the context of the lysosome and its calcium regulation. The various channels and transporters that are expressed in the lysosome and interact with TPC to allow for NAADP to function as a calcium releasing messenger, are depicted.

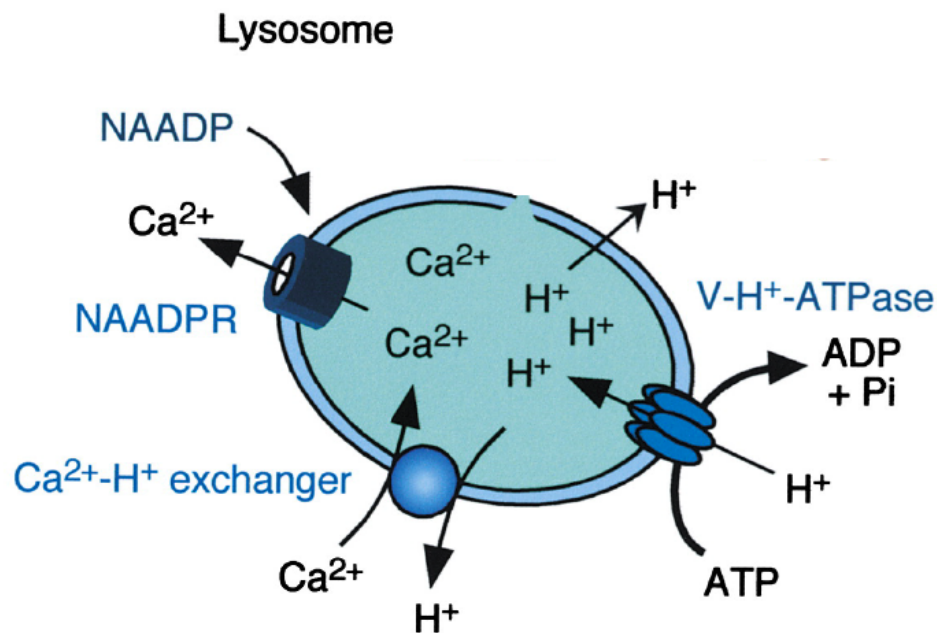


Figure 1.7: Calcium Homeostasis in the Lysosome is Controlled by multiple Channels and Transporters (Modified from Churchill et al., 2002)

1.2.5 Physiological Role of TPCs

There have been few studies on the physiological significance of endogenous TPCs in humans. One study, which predated the association of TPCs with NAADP signalling, found that TPCN2 was associated with hair-colour determination in European populations (Sulem et al., 2008). Beyond this, there have been some links with certain forms of hearing impairment (Kalay et al., 2007) and various carcinomas; see Hooper and Patel (2012) for review. From animal studies, there is evidence for their role in smooth muscle contraction (Aley et al., 2010), the secretion of von Willebrand factor in endothelial cells (Esposito et al., 2011), as well as ventricular myocyte contraction (Elson et al., 2011). In order to be able to better investigate the physiological role of TPCs, knockout mice lacking TPC1, TPC2, or both TPC1 and TPC2 were generated. The effect of the different knockouts on the functions of various cell types, and the phenotypes of the animals as a whole are currently being investigated. It has so far been suggested that pancreatic β -cell stimulus-secretion coupling is disrupted in TPC2

KO mice (Arredouani et al., submitted for publication); and that TPC KO interferes with glucose tolerance (Tunn, personal communication).

1.3 Pancreatic β -Cells and their Role in Glucose Homeostasis

1.3.1 Islets of Langerhans

The Islets of Langerhans make up the endocrine part of the pancreas.⁶ They consist of cell clusters of four types; α , β , δ , and PP cells. The large majority of cells in the endocrine pancreas are β -cells (50-80 %; In't Veld and Marichal in Islam, 2010). Although discovered in 1869 (Langerhans, 1869), the precise function of the islets remained unknown until Banting and Best first isolated insulin from them (Banting and Best, 1922); though it had already been suggested by Laguesse (1893) that they might be endocrine in nature. The cells within the islet responsible for the production of insulin when blood glucose is raised are the β -cells.

1.3.2 β -Cell Function in Health and Disease- Insulin Secretion and Diabetes

In response to an increase in glucose, insulin is released from β -cells by exocytosis of insulin containing vesicles. This release occurs in two phases as illustrated in Fig. 1.8. In Diabetes Mellitus this secretion is compromised. In very simplified terms this occurs either by the absence of sufficient numbers of β -cells ('reduced β -cell mass'; usually caused by their autoimmune destruction) in Diabetes Type 1; or by insufficient release of insulin from existing β -cells in Diabetes Type 2⁷. Characteristic for Type 2

⁶The majority of the pancreas consists of exocrine tissue.

⁷There is debate over whether reduced β -cell mass is also a contributing factor in Type 2 Diabetes; or whether it occurs as a consequence of the disease; see Ashcroft and Rorsman (2012), for review.

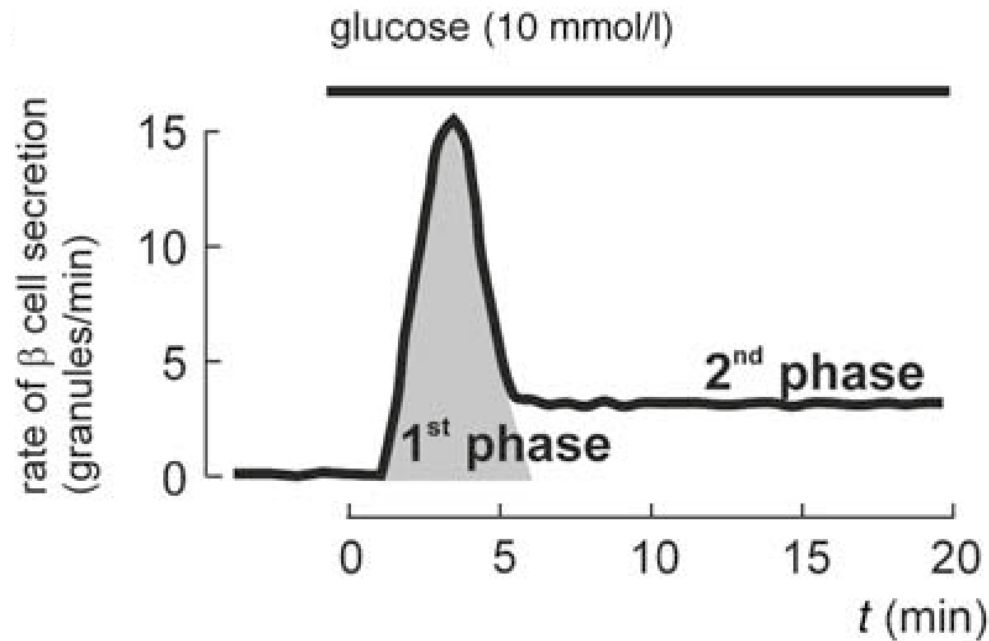


Figure 1.8: *Two-Phase Insulin Secretion from an Individual β -Cell (Reproduced from Rorsman and Renstrom, 2003)*

Diabetes is the lack of phase one insulin release (Hosker et al., 1989). For a review of insulin exocytosis, see Rorsman and Renstrom, 2003.⁸

1.3.3 Stimulus-Secretion Coupling

The K_{ATP} Channel Hypothesis

The above-mentioned exocytosis of insulin requires influx of calcium through L-type calcium channels in the β -cell membrane. For these to be activated, however, it is necessary that the membrane potential move from its resting value of ~ -70 mV to that for L-type channel activation (~ -30 mV). The electrical excitability of β -cells in response to glucose was first discovered and characterised in 1968 (Dean and Matthews, 1968); but the precise nature of the channels mediating depolarisation,

⁸It should be noted that biphasic insulin secretion is a phenomenon observed at the level of individual β -cells and islets. As insulin secretion from the pancreas into the portal vein is not biphasic, but pulsatile with a period of around 5 min, it may be the case that complex synchronisation between islets is needed to move from a biphasic to an oscillatory pattern; or that biphasic release is an *in vitro* phenomenon, caused by experimental conditions.

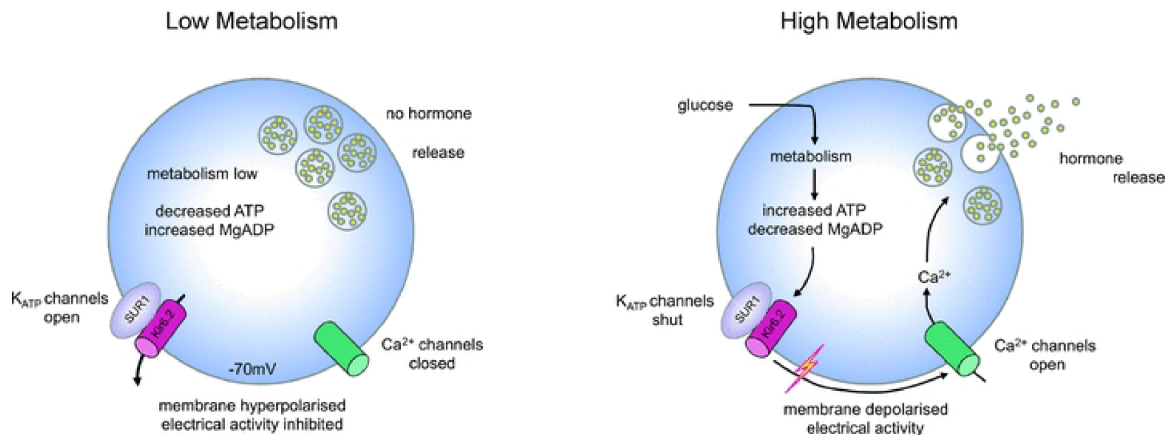


Figure 1.9: *The K_{ATP} Channel Hypothesis of Stimulus-Secretion Coupling (Modified from McTaggart et al., 2010)*

and, importantly, linking metabolism with electrical activity, remained unknown. The breakthrough arrived with the discovery of the K_{ATP} channel in heart (Noma, 1983), which was shown to be sensitive to intracellular ATP concentration (Cook and Hales, 1984), and glucose (Ashcroft et al., 1984) in β -cells.

These results led to the ‘consensus model’ of stimulus-secretion coupling: the K_{ATP} Channel Hypothesis. According to this; an increase in blood glucose leads to increased glucose uptake via GLUT transporters⁹ in the β -cell membrane and its metabolism to ATP. The cytoplasmic increase in ATP leads to the closure of K_{ATP} channels, which in turn leads to membrane depolarisation, triggering L-type calcium channel opening, thus allowing the influx of calcium and a consequent exocytosis of insulin containing vesicles. This is illustrated in Fig. 1.9. Current anti-diabetic drugs of the sulfonylurea class make use of this. They lead to insulin secretion by blocking the K_{ATP} channel. There are also some rare forms of juvenile diabetes that are specifically the result of a genetically determined malfunction of the K_{ATP} channel and respond to oral hypoglycaemic agents of the sulphonylurea class (for a comprehensive review, see McTaggart et al., 2010).

⁹GLUT2 in rodents; GLUT1 and GLUT3 in humans.

The Triggering and Amplifying Pathway Hypothesis

While the capacity of the K_{ATP} channel to lead to depolarisation of the membrane when closed by ATP is clear (although it appears that an additional unidentified current is necessary for this depolarisation to reach that required for L-type channel activation); many experiments have suggested that glucose must also act on other cellular targets to cause insulin release. In essence, these experiments can be divided into two groups: Those that demonstrate an action of glucose when all K_{ATP} channels are already closed (pharmacologically blocked by saturating sulfonylurea concentrations or genetically removed by knocking out K_{ATP} receptor subunits Kir6.2 (Miki et al., 1998) or Sur1 (Seghers et al., 2000)); and those that demonstrate the same when all K_{ATP} channels are incapable of closing ('propped open' pharmacologically by drugs like diazoxide); for a review of these studies see for example Henquin et al., 2003. Additionally, the importance of actually metabolising glucose has been stressed, as anti-diabetic drugs are not capable of mimicking glucose's actions fully unless there is some glucose present, also (Henquin, 1998).

The idea that targets other than the K_{ATP} channel are stimulated by glucose has become known as the ' K_{ATP} channel independent' hypothesis of stimulus-secretion coupling; or, as advocated by Henquin, the 'amplifying pathway' hypothesis. The difference between these two terms lies in the relative significance that is placed on the pathway in question with respect to the K_{ATP} channel dependent pathway. To Henquin there is '*a clear hierarchy between the two pathways: the amplifying pathway remains functionally silent as long as [the K_{ATP} channel dependent pathway] has not depolarised the membrane [...]*' (Henquin, 2000)¹⁰, while others have argued that K_{ATP} channel independent actions are crucial in their own right; and independent of glucose's actions on the K_{ATP} channel (see for example Islam, 2010).

¹⁰He even goes on to warn of the use of anti-diabetic drugs not targeting the K_{ATP} channel, were we ever to discover such: '*the natural hierarchy between the two pathways is a safeguard and should not be reversed pharmacologically*'.

1.3.4 Calcium Signalling in the Pancreatic β -Cell

Intracellular Calcium Stores

An alternative hypothesis to K_{ATP} channel closure that has been proposed to trigger stimulus-secretion coupling, is the local release of calcium from intracellular stores. The intracellular stores that are capable of storing and releasing calcium in β -cells are the endoplasmic reticulum, mitochondria, lysosomes, late endosomes, and insulin containing vesicles. The last three can be grouped under the umbrella term acidic stores, as their luminal pH ranges around 5.5 (Orci et al., 1986). Early endosomes and ‘immature’ vesicles have a more alkaline luminal pH (Lloyd-Evans and Platt, 2011). It was discovered in the late 1970s by studying calcium pyroantimonate precipitation that incubation with high glucose led to a calcium increase immediately beneath the cell membrane and in the vicinity of insulin containing vesicles; suggesting their functioning as calcium stores (see Wollheim’s description in his 1981 review; Wollheim and Sharp, 1981).

Calcium Oscillations

One of the ways in which intracellular stores might affect secretion of insulin in β -cells is their role in the generation of calcium oscillations. These are highly variable and complex, and form a field of research in themselves. Calcium oscillations are divided into at least three types depending on their frequency (Grapengiesser et al., 1991). The slowest of these has a period of ~ 5 min; possibly reflecting pulsatile insulin release as seen *in vivo*. Oscillations between different β -cells within the same cluster can be synchronised or unsynchronised; and it is currently under debate whether certain factors can act as pacemakers for the oscillations of groups of cells. Every calcium store, ion channel, and transporter has at some point been implicated in the generation of calcium oscillations; and to date their origin is still poorly understood.¹¹

¹¹Rorsman and colleagues claim that the reason for our lack of understanding of the origins of β -cell calcium oscillations lies with the preparation that is the dispersed β -cell culture. β -cells, so

Since their first description, computational modelling of calcium oscillations has been a useful tool to delineate the different components of such oscillations and interpreting experimental data with respect to their possible origins (see for example Sherman, 2011; Cha et al., 2011). It will be fascinating to see the results of ever more complex systems suggesting explanations for this phenomenon.

1.3.5 Evidence for TPC Involvement in Stimulus-Secretion Coupling

TPCs are thought to have a role in the K_{ATP} channel independent pathway of β -cell stimulus-secretion coupling for several reasons. Firstly, it seems that their agonist, NAADP, is critically involved in the process (see Zhao et al., 2012, for a recent review). Secondly, interference with TPC function seems to impair glucose dependent calcium and insulin release (Arredouani et al., submitted for publication).

The putative NAADP-producing enzyme, CD38, has been implied in stimulus-secretion coupling in primary mouse β -cells; amongst other experiments because in CD38 knockout mice, insulin signalling was impaired (Okamoto and Takasawa, 2002; Kim et al., 2008).¹² Apart from a potential role for CD38, it has also been shown that increases in glucose and glucagon like peptide 1 (GLP1) lead to NAADP production and calcium release from acidic stores in the β -cell line MIN6 Masgrau et al. (2003). The microinjection of NAADP and the uncaging of NAADP inside clonal pancreatic β -cells was shown to cause calcium elevation that was sensitive to bafilomycin but not thapsigargin Yamasaki et al. (2004). GLP1 induced NAADP production and calcium release were also demonstrated in primary β -cells (Kim et al., 2008). Rutter and colleagues demonstrated that NAADP leads to a decrease in intra-luminal calcium of insulin containing vesicles in permeabilised cells of the MIN6 cell line (Mitchell et

Rorsman claims, can only reasonably reflect their *in vivo* behaviour *in vitro*, if they are studied as part of the whole islet (Rorsman et al., 2011).

¹²It has been argued, however, that the impairment is only with respect to the autocrine actions of insulin; not the stimulation with glucose itself (Johnson et al., 2006).

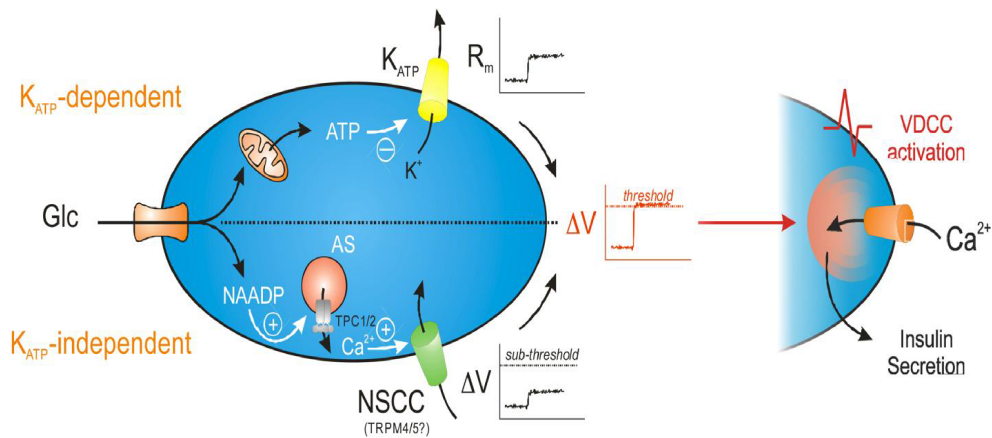


Figure 1.10: Stimulus-Secretion Pathways: K_{ATP} Channel Dependent Pathway (top) and a Possible Role for NAADP and TPCs as the K_{ATP} Channel Independent Pathway (bottom) (Reproduced from Arredouani et al., 2010)

al., 2003); suggesting that the target for NAADP in β -cells are the insulin containing vesicles. NAADP at nanomolar concentrations was shown by the same group to mobilise calcium when microinjected into single human β -cells (Johnson and Misler, 2002). At micromolar concentrations, it was shown to abolish the autocrine calcium response to insulin. Thus it appears that NAADP is involved in the transduction of all three major stimuli on β -cells: Glucose, GLP1, and Insulin.

Studies investigating the role of the NAADP receptor, TPC, have also suggested it play a role in stimulus-secretion coupling. The membrane-permeable TPC antagonist rNed-19 was shown to inhibit glucose induced calcium release in a concentration dependent manner (Naylor et al., 2009). The compound also inhibits insulin secretion from whole islets, and diminishes the effect of tolbutamide on insulin release (Arredouani et al., submitted for publication). Pilot experiments suggest that β -cells of TPC2^{-/-} mice lack the calcium response to glucose (Calcraft et al., 2009), the observed current in response to intracellular NAADP (Arredouani et al., 2010), and demonstrate impaired insulin secretion (Arredouani et al., submitted for publication).

The existence of NAADP- and/or TPC-dependent calcium micro-domains in β -cells in response to glucose elevation could explain why calcium signals are still observed in experiments where K_{ATP} channels do not function. Local calcium release might trigger the additional depolarisation required to reach threshold for L-type channel activation (in addition to the depolarisation caused by K_{ATP} channel closure). TPC activation could also be the cause of elementary calcium events; which may or may not lead to a global calcium signal via CICR (followed by cell membrane calcium-sensitive cation channel opening, calcium influx, and exocytosis). This possibility is illustrated in Fig. 1.10. Exocytosis could, however, also be caused directly by local calcium release without a global signal via cell membrane channels of any kind. Whether or not a local signal is amplified to a global one would depend on the precise location of the calcium stores and the expression patterns of TPCs and other channels. The proximity between acidic stores and ER, and the proximity of these to the cell membrane would determine the level of coupling that could potentially take place.

Evidence for Sub-membrane Calcium Release in β -Cells

Two studies have specifically investigated the role of sub-membrane calcium release from intracellular stores in stimulus-secretion coupling. Ravier and colleagues (Ravier et al., 2010) used TIRF microscopy to image sub-membrane calcium in response to glucose (with a ratiometric dye; D3cpv), and to KCl depolarisation (with fluo-4) in the presence of intracellular EGTA (often used to increase the probability of discerning sparks). They observed some short-lived calcium hotspots in response to depolarisation; but not in response to glucose.¹³ Because of the difficulty of observing these effects and their short-lived nature and amplitude, they concluded that *‘it is not plausible that such tiny changes are sufficient to double the rate of insulin secretion’* and that *‘glucose metabolism amplifies insulin secretion by an action that is [...] independent of a further [compared to cytosolic] increase in [sub-membrane calcium]’*. Previously,

¹³This is likely the result of their choice of experimental parameters, the importance of which for the detection of calcium spikes in β -cells will be discussed in Chapter 5.

Rutter and colleagues (Rutter et al., 2006) also touched upon the subject of localised calcium release in β -cells. They used a membrane-targeted pericam to compare sub-membrane and cytoplasmic calcium. They showed that glucose led to a more heterogenous calcium distribution below the membrane compared to the cytoplasm as a whole. They concluded that sub-membrane calcium hotspots in response to glucose stimulation could occur as a result of calcium release from secretory vesicles, but did not follow this up.

1.4 Total Internal Reflection Microscopy

1.4.1 Principle of Total Internal Reflection Fluorescence

The use of total internal reflection in microscopy was pioneered by Daniel Axelrod in the early 1980s (see Axelrod, 1981, for the first TIRF images of fluorescently labelled mammalian cells). It exploits the phenomenon that when a light beam is totally reflected off a glass-liquid interface, an exponentially decaying evanescent field¹⁴ is propagated across the interface. This evanescent field can be used to excite fluorophores in the immediate vicinity of the coverslip of a microscope without illuminating anything beyond a few 100 nm of the coverslip.

By Snell's Law (rearranged for total reflection; i.e. angle of transmission = 90°), the critical angle, α , at which the entirety of the light-beam is reflected off a glass-water interface, is calculated as follows:

$$\alpha \geq \sin^{-1} \left(\frac{n_2}{n_1} \right), n_1 > n_2 \quad (1.2)$$

¹⁴The decay length of the evanescent field, d (which is the length after which the field has decayed 37% in strength), can be calculated with the following equation:

$$d = \frac{\lambda}{\left(4\pi \sqrt{n_2^2 \sin^2 \theta - n_1^2} \right)} \quad (1.1)$$

where λ is the incident wavelength, n_1 is the refractive index of the cover-glass, n_2 the refractive index of water, and θ the angle of incidence.

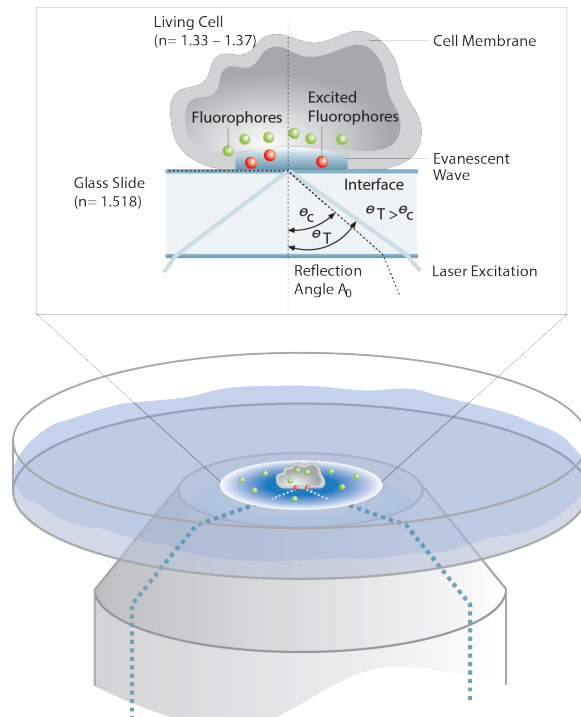


Figure 1.11: *TIRF Microscopy allows for the Exclusive Excitation of Fluorophores just beneath the Plasma Membrane of Intact Living Cells (Reproduced from Leica Microsystems GmbH, 2010)*

where n_1 is the refractive index of the cover-glass, and n_2 the refractive index of water.

This critical angle is used to direct a laser beam of a desired wavelength (for excitation of a chosen fluorophore) at the coverslip. Cells that are attached to this will be illuminated in the z axis only within the nanometer depth of the evanescent field- in practice this means only just beneath their respective cell membranes. This is illustrated in Fig. 1.11.

In a TIRF microscope, this laser can be aimed at the specimen either through the objective (objective-type TIRF) or through a prism in a location other than that of the objective (prism-type TIRF). Objective-type TIRF is slightly less ‘clean’ than prism-type TIRF, as a fraction of the excitation light is scattered in the objective; but this is rarely so confounding as to opt for the prism-type, which usually restricts

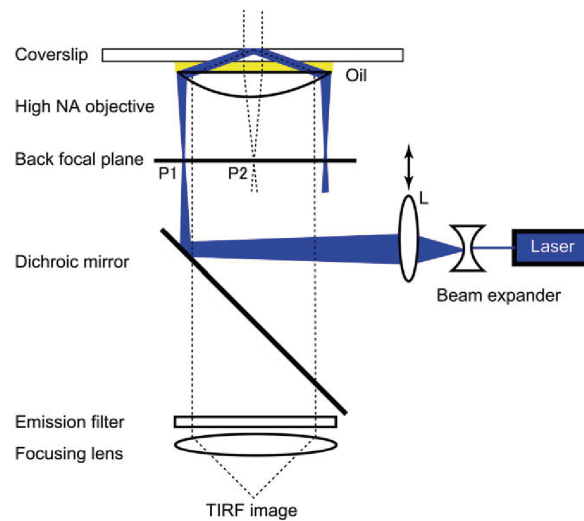


Figure 1.12: *TIRF Lightpath in the Inverted Microscope*
(Reproduced from Ravier et al., 2008)

access to samples (see Axelrod, 2001, for a review of different TIRF setups). Fig 1.12 illustrates the light-path in a TIRF microscope.

The advantages of TIRF for sub-membrane imaging over other forms of microscopy, notably confocal microscopy, lie in the nature of the evanescent wave. This is the key to creating an optical section that can be below 100 nm in depth. Confocal microscopy and two-photon microscopy can image thin sections; but do usually not resolve a depth below 600 nm (in the context of the β -cell, this would mean imaging two or more rows of secretory vesicles as opposed to one). Thus TIRF allows for immensely localised imaging with very little out of focus information affecting the sharpness of the image. Epifluorescence, on the other hand, has much less spatial resolution due to its illuminating the entire specimen and collecting all emitted light. Confocal microscopy also records less out of focus fluorescence than epifluorescence microscopy, but as this is excluded at the time of emitted light collection rather than excitation; the entire specimen is still illuminated. For cells that are markedly photo-sensitive, this is unsuitable. Two-photon microscopy does not have this problem, as only the optical section in question is illuminated, as in TIRF. However, the optical sections of this type of microscope are still relatively thick. Fig. TIRF3 illustrates epifluorescence-,

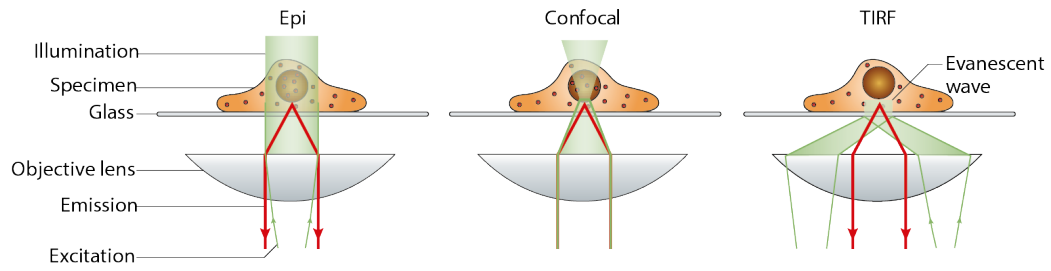


Figure 1.13: Comparison of Epifluorescence, Confocal and TIRF Microscopy
(Reproduced from Brandenburg and Zhuang, 2007)

confocal- and TIRF microscope excitation and emission light-paths. For a review of microscopy of sub-membrane events, see for example Steyer and Almers (2001).

1.4.2 TIRF Microscopy in the Study of β -Cells

As it is in the nature of TIRF to visualise specifically the area of and beneath the cell membrane, it lends itself to the study of exocytosis. Many aspects of insulin exocytosis in β -cells have been investigated in this way (Rutter et al., 2004; Ravier et al., 2008; Aoyagi et al., 2009; Hoppa et al., 2009; Ohara-Imaizumi et al., 2009). Sub-membrane calcium rise in the context of mediating exocytosis has been investigated; as well as calcium gradients between membrane and cell interior; but discrete triggering calcium signals in their own right have largely been omitted. Calcium signals observed in response to glucose have invariably been attributed solely to L-type channel influx; and often depolarisation or sulfonylureas were used as stimuli directly instead of glucose. This may be because the K_{ATP} channel hypothesis of stimulus-secretion coupling is so engrained that it seems a futile exercise to suggest other trigger sources for the required ‘exocytosis-calcium’, or it may be because experiments in calcium free solution consistently seemed to eliminate all responses (the reasons for this will be discussed in Chapter 3).

1.4.3 Imaging Sub-membrane Calcium using TIRF

In other cell types, localised sub-membrane calcium release from intracellular stores has been studied most successfully with TIRF; as exemplified by Parker and colleagues' imaging of IP₃R calcium puffs in SH-SY5Y cells (Smith and Parker, 2009; Smith et al., 2009). TIRF is excellent for detecting elementary calcium release because optical blurring by out-of-focus fluorescence is minimised; the point spread function¹⁵ of TIRF is very sharp (Cheng and Lederer, 2008). If combined with a fluorescent marker of the right calcium binding kinetics and longevity, (such as fluo-4¹⁶), TIRF is ideally placed for imaging localised sub-membrane calcium events.

1.5 Aims of the Present Investigation

1.5.1 Characterisation of β -Cell Calcium Responses using TIRF Microscopy

The present study sought to characterise the sub-membrane calcium changes observed in β -cells in response to glucose and NAADP using TIRF microscopy; and to compare these to global cytosolic calcium changes observed with epifluorescence microscopy. Factors affecting the shape and time course of these responses were investigated by varying experimental parameters and utilising pharmacological agents.

1.5.2 Investigation of the Effect of TPC Knockout on Pancreatic β -Cell Structure and Function

Having established the behaviour of wild type β -cells when imaged with TIRF under various conditions, the effects of knocking out TPC2 (single KO), or both TPC1

¹⁵The point spread function of an imaging system determines the quality of the image; it measures the amount of spread (blurring) observed when imaging an individual point source.

¹⁶This shows a 100-fold increase in fluorescence upon binding of calcium and has fast on and off kinetics; allowing for a good signal to noise ratio (Haugland, 2002).

and TPC2 (double KO, DKO) on these responses were investigated. Furthermore, the effects of this knockout on gross histology of the pancreatic islet and β -cell were examined using electron microscopy. Gender differences in responses to glucose were assessed in both wild type and knockout animals.

1.5.3 Visualisation of Elementary Events in Pancreatic β -Cells

While investigating the calcium responses discussed above; an experimental protocol was designed which most unexpectedly enabled the visualisation of the trigger events for these in the form of localised calcium signals in response to both glucose and NAADP; akin to the sparks and puffs observed with cADPR and IP_3 .

1.6 Outline of Thesis

After a brief summary of the methods used in the present investigation (Chapter 2), the characterisation of β -cell responses is discussed in Chapter 3. This is followed by the examination of the knockout animals in Chapter 4, and elementary glucose- and NAADP-dependent calcium responses in Chapter 5. The significance of the presented results for further research and possible drug discovery is outlined briefly in Chapter 6.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Aldrich, unless otherwise stated. NAADP-AM and rNed-19 were synthesised in house.

2.1.2 Culture Media and Recording Buffer

HANKS balanced salt solution was made up from tablets and supplemented with NaHCO_3 (0.35 g/l). For HANKS containing BSA (fraction V), 1 mg/ml was added.

Collagenase type V was used for all procedures (1.9 U/mg). Collagenase was made up as a $\times 10$ stock solution in HANKS (+ NaHCO_3 , -phenol red), sterile filtered and kept in 1 ml aliquots at -20° until use. It was diluted in HANKS immediately prior to procedure.

Cell culture medium consisted of RPMI 1640 (-glucose +glutamine, Gibco), supplemented with penstrep (10,000 U/ml penicillin / 10,000 μg /ml streptomycin, Gibco) and 10 % fetal bovine serum (FBS, Gibco). When needed, glucose was added to this from a 1 M stock solution in $d\text{H}_2\text{O}$.

All imaging experiments were conducted in modified Krebs-Ringer solution (pH: 7.4, 280-340 mOsm / kg) of the following composition:

NaCl	130 mM
KCl	5.2 mM
MgCl ₂	1 mM
HEPES	10 mM
Glucose	0-16.5 mM
CaCl ₂	0-1.7 mM
EGTA	0-5 mM
SrCl ₂	0-1.7 mM

Table 2.1: *Composition of Krebs-Ringer Solution for Imaging Experiments. Glucose, Calcium, Strontium and EGTA were varied depending on the Experiment*

2.1.3 Pharmacological Agents

All pharmacological agents used were dissolved in DMSO and stored at -20°C until use. NAADP and NAADP-AM were stored under Argon at -80°C until use. A list of drugs used and their concentrations is shown in Table 2.2.¹ The preincubation time for drugs added before the start of imaging varied depending on the particular experiment and is stated where relevant.

Nifedipine	1-10 μ M
Diazoxide	100-400 μ M
Bafilomycin A1	1-3 μ M
Thapsigargin	1 μ M
GPN	50 μ M
rNed-19	100 μ M
tNed-19	10 nM-100 μ M
NAADP	60-100 nM
NAADP-AM	60-100 nM

Table 2.2: *Concentrations of Pharmacological Agents used for Imaging Experiments*

¹tNed-19 was obtained from Tocris and Enzo Life Sciences, Nifedipine from Research Biochemicals International.

2.1.4 Animals

Experimental animals were kept at the Oxford Biomedical Sciences Building. Mice were housed in clean air at constant optimal temperature and humidity with a 12 h light-dark cycle. Water and standard dry pellet nutrition were *ad libitum*. Animals were all of the strain C57BL/6. Four different genotypes on two different genetic backgrounds were used: TPCN2^{-/-} and TPCN2 WT on one, and TPCN1^{-/-}TPCN2^{-/-} (DKO) and DKO WT on another. Mice were used at 10-14 weeks of age exclusively. When knockout animals were used, age-, sex- and background-matched wild type control animals were always tested in tandem.

2.2 Isolation of Pancreatic Islets and Preparation of Primary β -Cell Cultures

2.2.1 Surgery and Pancreas Digestion

Animals were transported to the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM) in the morning on the day of culturing and used within one hour of their arrival. Mice were terminated by cervical dislocation and pinned out for procedure. Surgery was conducted under a dissecting microscope (Leica). The abdomen was opened (lateral incision for skin, medial incision for muscle/peritoneum), the liver positioned upwards against the thorax and the small intestine shifted to expose the duodenum and common bile duct. The ampulla of Vater was located on the duodenum, and clamped using a curved haemostat. The pancreas was back-injected by inserting a 30 G needle rostrocaudally into the common bile duct (see Fig. 2.1) and gently infusing 2-5 ml of collagenase; inflating the pancreas (see Fig. 2.2).

Upon complete inflation, the pancreas was excised from the abdominal cavity and placed into a 15 ml falcon tube in a 37°C water-bath for 11-13 minutes (the precise time depended on the batch of collagenase in question). Subsequently, 12 ml

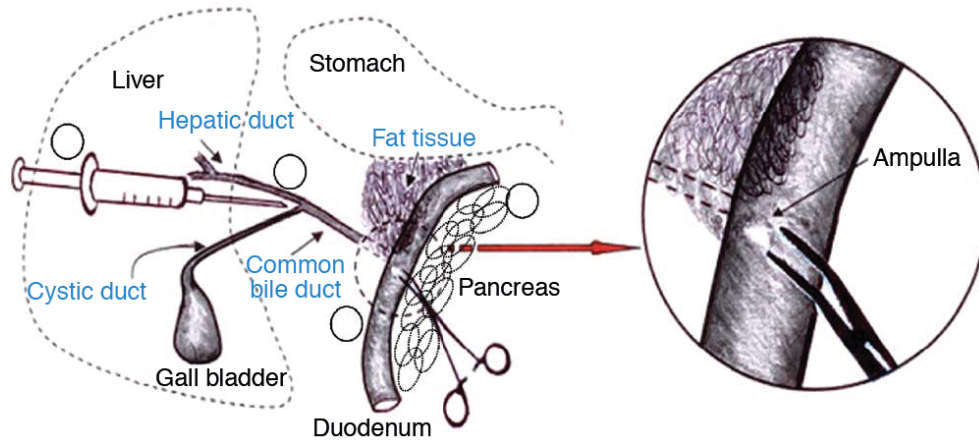


Figure 2.1: *In-situ Pancreas Perfusion with Collagenase*
(Modified from Li et al., 2009)

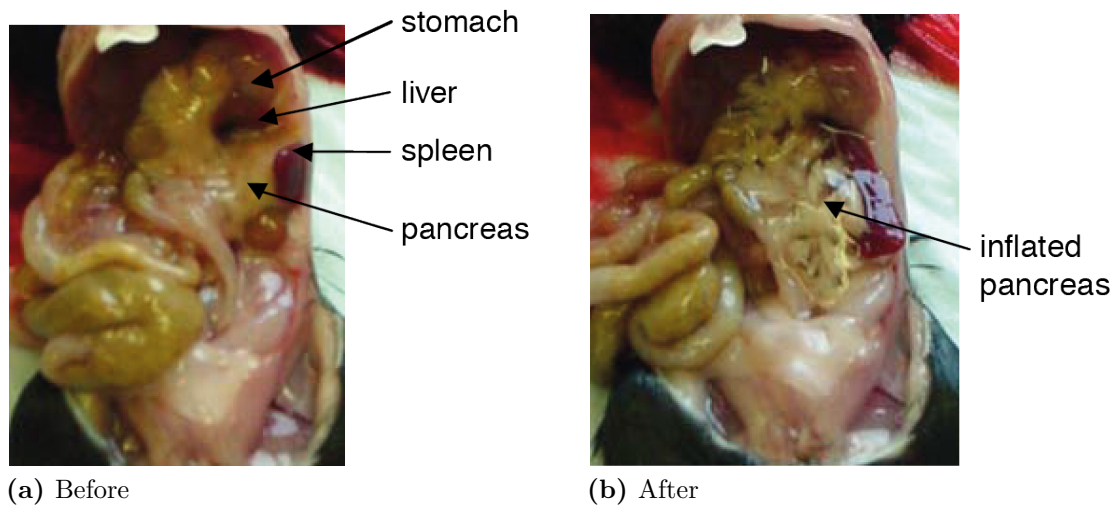


Figure 2.2: *Abdominal Cavity before (a) and after (b) Inflation of Pancreas with Collagenase*
(Modified from Ravier and Rutter in Ward and Tosh, 2010)

of HANKS containing 0.2% BSA (fraction V) were added and the mixture shaken vigorously. It was then left to settle on ice for 5 min. The supernatant was removed; and 7 ml of HANKS were added to the remaining 3 ml of fluid. The tubes were turned upside-down several times, and then left on ice for a further 3 min. This step was repeated once more before the tissue's final suspension in 10 ml HANKS (BSA-free).

2.2.2 Isolation of Islets of Langerhans

To isolate the pancreatic islets from the exocrine tissue, the suspension was poured into a 'suspension cell' petri dish (around 2 ml at a time), and placed under a stereo dissecting microscope in a sterile hood. Islets were handpicked using a 10 μ l Gilson pipette (tip coated in RPMI) and transferred to a second cell suspension dish filled with HANKS (BSA free). From this, a clean pick was made into a further dish filled with RPMI containing 10 mM glucose.²

2.2.3 Dispersion of Islets into Single Cells and Cell Clusters

All islets were manually spun to the centre of the dish and added in one action to a 15 ml falcon tube containing 1 ml enzyme-free cell dissociation HANKS (Gibco). The tube was immediately placed in a 37°C water-bath for 8.5 min. After this, the mixture was pipetted up and down ~ 10 times using a 1 ml Gilson pipette. The vigour and amount of pipetting determines whether more or less of the resultant cell suspension conclude as single cells or as clusters. The more mixing is done, the more cell clusters break up into individual cells. Fig. 2.3 shows single β -cells (2.3a) and a mixture of clusters and single cells (2.3b) at 100x magnification. 9 ml of RPMI were added to this, and the cells spun down using a centrifuge at 89 rpm for 5 min (~ 300 g). Finally the supernatant was removed to leave 1 ml, which was again gently pipetted up and down ~ 20 times with a 1 ml Gilson pipette.

²Islets can be automatically separated by size filtering or density gradient, but these methods are more crude and traumatic for the islets (Ward and Tosh, 2010).

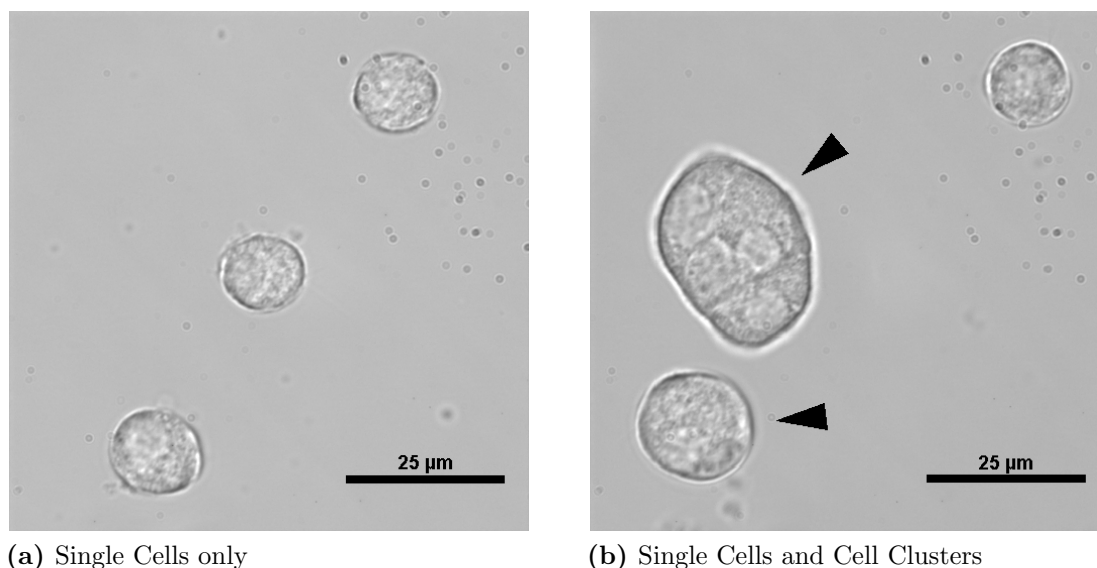


Figure 2.3: Isolated β -cells (a) and a Mixture of Single β -cells and β -cell Clusters (b) at 100x Magnification after Dispersion of Pancreatic Islets (Clusters are marked with arrows).

2.2.4 Preparation and Culture of Primary β -Cells

Cells were plated onto poly-L-lysine coated glass coverslips of 25 mm diameter (Menzel), which were placed in cell* dishes. Coverslips were coated by adding ca 100-200 μ l lysine onto each coverslip for 30 min. After removal of lysine, coverslips were rinsed with PBS and left to dry for a further 30 min. For each pancreas, 6 coverslips were prepared. After 4-7 h in the incubator at 37°C and 95 % air / 5 % CO_2 , 2 ml RPMI (10 mM glucose) were added to each dish.³ Dishes were placed in an airtight container and transported to the Department of Pharmacology where they were placed in an incubator for a further 15-17 h before use to allow them to recover from the digestion process. For experiments pertaining to the suitability of β -cells of increasing age in culture, some cells were used after ca 3 h and some were kept for up to 4 days. If cells were kept for longer, the culture medium was replaced every 24 h.

³Concentrations above and below this level of glucose have been shown to progressively lead to apoptosis (see Efanova et al., 1998).

2.2.5 Identification and Selection of β -Cells for Calcium Imaging

Cells were viewed at 60x or 100x magnification under brightfield to assess their viability and select suitable candidates for imaging. Due to the large number of cells investigated and the experimental set-up used, it was not feasible to stain for Insulin in each experiment.⁴ β -cells were identified on the basis of their large size (see Fig. 2.4a) and typical round shape (see Fig. 2.4b) compared to other cells present in the dispersed cell culture, as well as their characteristic responses to glucose (Quesada et al., 2006; α cells are constitutively active in low glucose and stop responding as glucose is raised; β cells show the opposite pattern; δ -cells are constitutively active in the presence of glucose; see Fig. 2.5). Selection on the basis of the above-mentioned criteria is used routinely (see for example Chen et al., 2003 or Johnson and Misler, 2002).

⁴Mouse islet cells are sometimes treated with tolbutamide to identify putative β -cells, but this approach was avoided because it alters calcium homeostasis and insulin secretion (Aspinwall et al., 1999) and because it has been shown that α -cells can also respond to tolbutamide (Gopel et al., 2000).

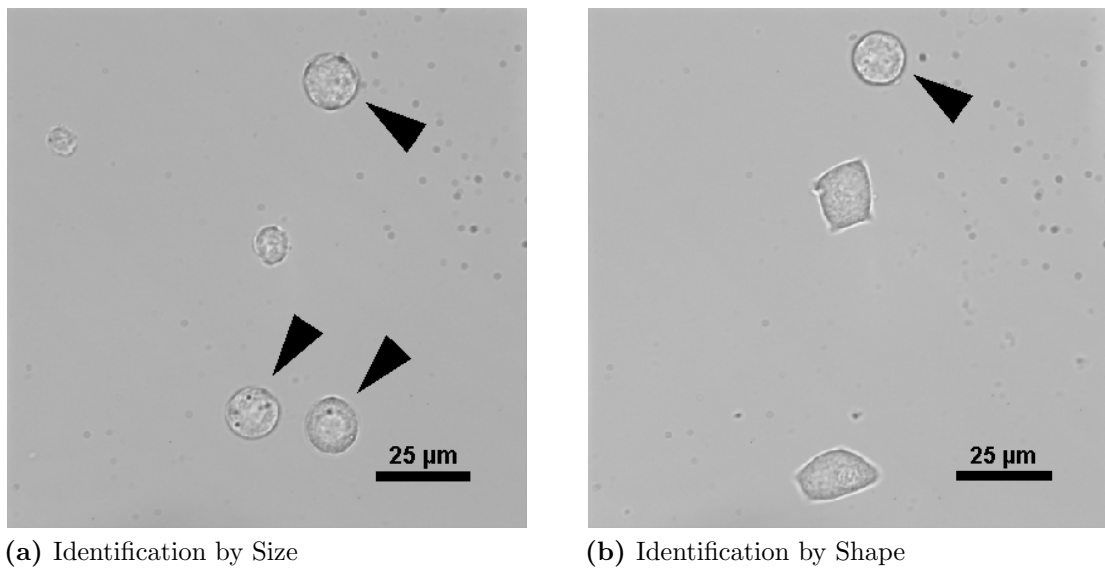


Figure 2.4: Examples of β -cells and non- β -cells under 60x Magnification. β -cells were identified by their characteristic round Shape and large Diameter in Comparison to other Cell Types that differ in (a) Size or (b) Shape (β -cells are marked with Arrows).

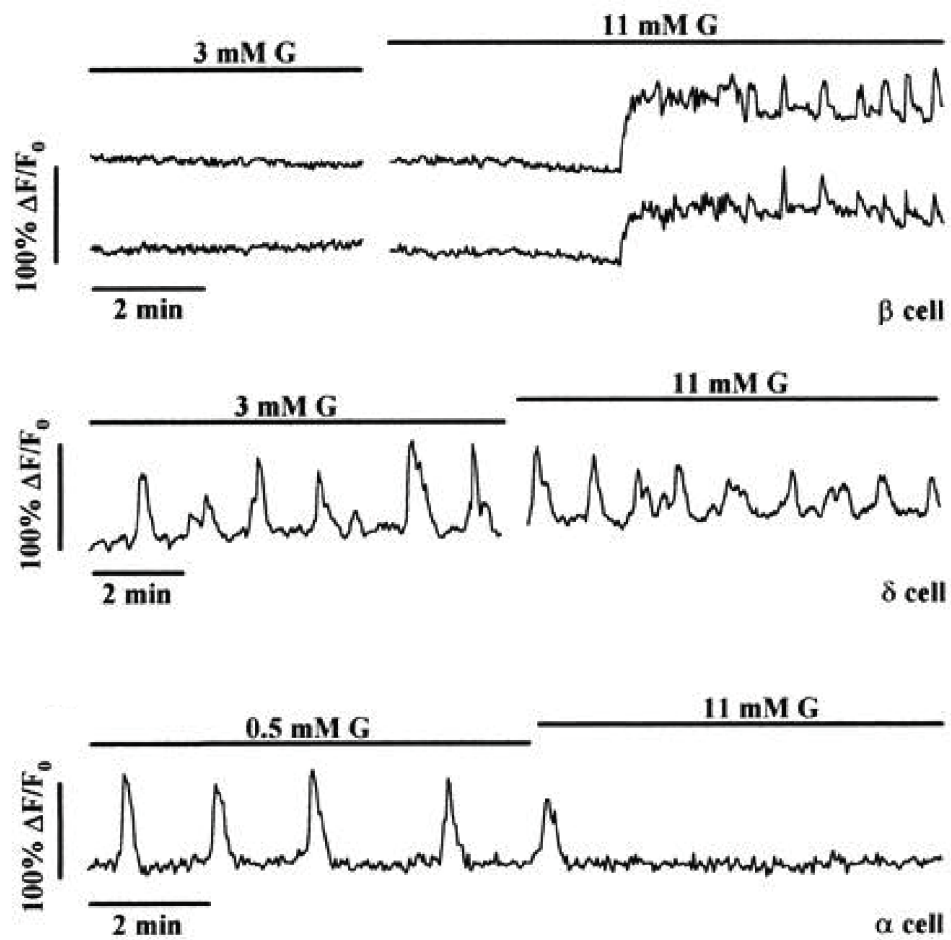


Figure 2.5: Characteristic β -, δ -, and α -cell Responses to Glucose (Modified from Quesada et al., 1999)

2.3 TIRF Microscopy

2.3.1 Fluorescent Calcium Indicator Dyes

Cells were loaded with the non-ratiometric fluorescent calcium indicator dyes fluo 4-AM or fluo 5F-AM (Invitrogen). Cells were incubated with these compounds at 500 nM and 1 μ M, respectively, in the dark for 1 h at 37°C in RPMI containing 10 mM glucose.

LysoTracker Red	200 nM
Fluo 4- AM	500 nM
Fluo 5 F- AM	1 μ M

Table 2.3: Concentrations of Fluorophores used for Imaging Experiments

Coverslips were removed from the incubator and used to form the glass base of a recording chamber (see Appendix A.1). Before imaging, they were washed once with imaging buffer at 3 mM glucose, and then left in 1 ml of the same for 10 min to allow any remaining AM dye to get de-esterified.

For the experiments to visualise acidic stores, cells were preincubated for 10 - 30 min with LysoTracker Red (Invitrogen) at 200 nM.

Cells were excited with an Argon-Ion laser (Andor DU-897, 40 mW; Melles Griot) at 488 nm (for Fluo) or 560 nm (for LysoTracker Red). Appendix A.2 shows the excitation emission spectrum for fluo-4 and Appendix A.3 that for LysoTracker Red.

2.3.2 Microscope Setup

Images were obtained using a Nikon Evanescent Wave Imaging System; an Inverted Total Internal Reflection Microscope (Nikon Eclipse Ti) equipped with 60x and 100x CFI Apochromat TIRF Series oil-immersion lenses. These lenses have a numerical aperture of 1.49, which allowed for a maximal incident angle of 76.87°, calculated by equation 2.1 below.

$$\alpha \geq \sin^{-1} \left(\frac{n_2}{n_1} \right), n_1 > n_2 \quad (2.1)$$

where n_1 is the refractive index of the coverglass (1.53), and n_2 the numerical aperture of the lens (1.49).

As established earlier (see equation 1.2), the critical angle α when imaging cells attached to a glass coverslip, is 64.41° . The exact angle for experimentation was determined using a Bertrand lens and the beam adjusted. All parameters were controlled using NIS Elements (Nikon). Images were acquired at a rate of 3.3 Hz for single channel recordings (i.e. only TIRF or Epifluorescence, only green or red TIRF) and at a preprogrammed rate for dual channel recordings. A CCD camera (Andor iXon+) was used to capture emitted fluorescence (at 515-555 nm or 600 nm, depending on the fluorophore).⁵

2.3.3 Temperature Control and Perfusion

All experiments were conducted at 37°C . Pharmacological agents were added to the chamber diluted in 1ml of the recording buffer using a Gilson pipette. Additions were mixed with the already present solution using the pipette. Solutions were removed from the chamber (for reversibility effects of antagonists) using disposable pasteur pipettes.

2.3.4 Perfect Focus System (PFS)

Manual additions to and removal of fluids from the chamber without disturbing the TIRF image were made possible by the presence of the perfect focus system. It recognises and counteracts drift between objective and cover-glass by reflecting a light-beam off the glass coverslip (see Appendix A.4). As with very low fluid levels in the chamber, the beam can reflect off the fluid level instead of the cover-glass and

⁵Binning: 1x1, Exposure: 300 ms, Multiplier: 89, Readout Speed: 10 Hz, Conversion Gain: 1x, Dimensions: 512 x 512, 160nm / pixel.

misalign the focus; this required the chamber to have large volumes of fluid in it at all times (a 3 mm fluid level is specified by the manufacturer). A minimum of 1 ml of fluid was present in the chamber at all times. The PFS was controlled using the hardware, and uncoupled from the NIS Elements software driving the experiments, as its control by the software slowed down the filter change during simultaneous recordings. The offset for the PFS was (like the TIRF angle) set up once and then fine-tuned at the start of every experiment when necessary.

2.3.5 Parallel Imaging of Epifluorescence and TIRF

In the case of parallel TIRF and epifluorescence recordings, 3 sequential images were taken in TIRF mode, 300 ms apart, followed by a filter change and one image being taken in epifluorescence. This filter change, though recorded as 300 ms by the software, was very lengthy in practice (several seconds); limiting the acquisition rate for parallel recordings substantially.

2.3.6 Parallel Imaging of Red and Green TIRF

For parallel imaging of red and green TIRF, the acquisition rate was 1 Hz overall, with a filter change after each acquisition; i.e. the acquisition rate for each individual channel was 0.5 Hz.

2.3.7 Visualising Elementary Events

Since most spark analysis software is based on line scans, most notably SparkMaster (Picht et al., 2007) for Image J (NIH), these could not be used to analyse 2-dimensional images for sparks. Practically all algorithms for spark detection are permutations of Cheng's original spark analysis algorithm (Cheng et al., 1999) and based on high temporal as well as spatial resolution. As with the current setup the frame rate of recording was 3.3 Hz, the temporal resolution was relatively poor and there was

never more than one time frame of any one spark. Thus no differentiation between sparks and random fluctuation can be made on the basis of the temporal shape of the events (sparks are symmetrical in time). Because many sparks recruited local calcium release that spread over a large number of pixels (up to a quarter of the cell), it would have been ineffectual to analyse regions of interest using a graticule. The larger events would have been analysed as multiple smaller events in different locations and have skewed the data. It was decided to analyse fluorescence across each whole cell by first normalising each image (time frame) pixel by pixel with respect to the average fluorescence across the last 100 frames before cell stimulation with glucose or NAADP-AM. To eliminate the additional problem caused by the presence of a moving baseline (ramp), a frame by frame subtraction function was used to eliminate this. Of these normalised, baseline-controlled images, the maximum intensity within each cell was measured. this way, every spark was selected, unless there was a bigger spark present simultaneously at another location within the cell. This algorithm for spark detection certainly will miss some smaller events and skew the distribution towards the larger events marginally, but given the limitations of the present raw data it was the most suitable analysis. A similar algorithm has been used for 2 D images by Banyasz et al. (2007) who elaborates on the above-mentioned issues. From the measured maximum intensities for each cell over time, frequency histograms were compiled to depict the quantal nature of the events. Events more than 2 standard deviations above the mean were considered genuine events. This is a standard estimate of minimum spark amplitude; see for example Fogarty et al. (2000).

2.4 TPC Knockout Mice

TPC Knockout Mice were generated by members of the Department of Pharmacology see Chuang (2011) in collaboration with MRC Harwell (Oxfordshire, UK). The

generation of the animals was complete before the start of the present study, and the methodology below is for explanatory purposes.

2.4.1 Generation of TPC1 and TPC2 Single Knockout Mice

To create TPC1 KO mice, an embryonic stem cell line (TPC1 D159) was used. In this ES line, portions of exons 4 and 5 of the TPCN1 gene were deleted, and the LacZ-neo marker gene introduced by homologous recombination.⁶ Mice heterozygous for TPC1 D159 (+/-) were obtained from Deltagen, and crossed to produce WT (+/+) and homozygous mutant (-/-) mice. To select mice homologous for the mutant allele, offspring were genotyped from ear clips using a 3-primer PCR (2 sense primers; one for each allele; and one antisense primer). Genotypes were identified by the presence of WT TPC1 only (376 bp), KO TPC1 only (459 bp), or both. To validate TPC1 D159 as a true TPC1 KO, RT-PCR was used to demonstrate that TPC1 mRNA (exons 6-16, 606 bp) expression was abolished (Chuang, 2011). Fig. 2.6 illustrates the principles of generating knockout mice.

For the generation of TPC2 KO mice, ES cells from the TPC2 YHD437 ES cell line were used. The gene trap vector used in this cell line led to the insertion of β -geo and a polyadenylation signal between exons 1 and 2 of the TPCN2 gene. ES cells were injected into blastocysts of C57BL/6 mice (a commonly used laboratory mouse strain with black fur) and then implanted into surrogate mice. The resultant mosaic embryos are heterozygous for the TPCN2 gene and when born have patchy black and white fur. These animals were crossed with WT C57BL/6 animals to confirm the mutant gene was included in the germline. The TPCN2 heterozygotic (+/-) offspring of this cross was further mated to result in mice that were homozygous for the mutant TPCN2 allele (-/-). Mice were genotyped using a 3-primer RT-PCR to determine

⁶A previous attempt at creating TPC1 KO mice using a gene trap vector (ES cell line TPC XG716) introducing β -geo between exons 2 and 3 had failed to produce a full TPC1 KO. This can occur by gene trap skipping, which results in some expression of the original protein despite the interruption of the gene by the vector. This is not possible in homologous recombination, as the exons themselves are partially deleted rather than relocated.

General strategy for gene targeting in mice

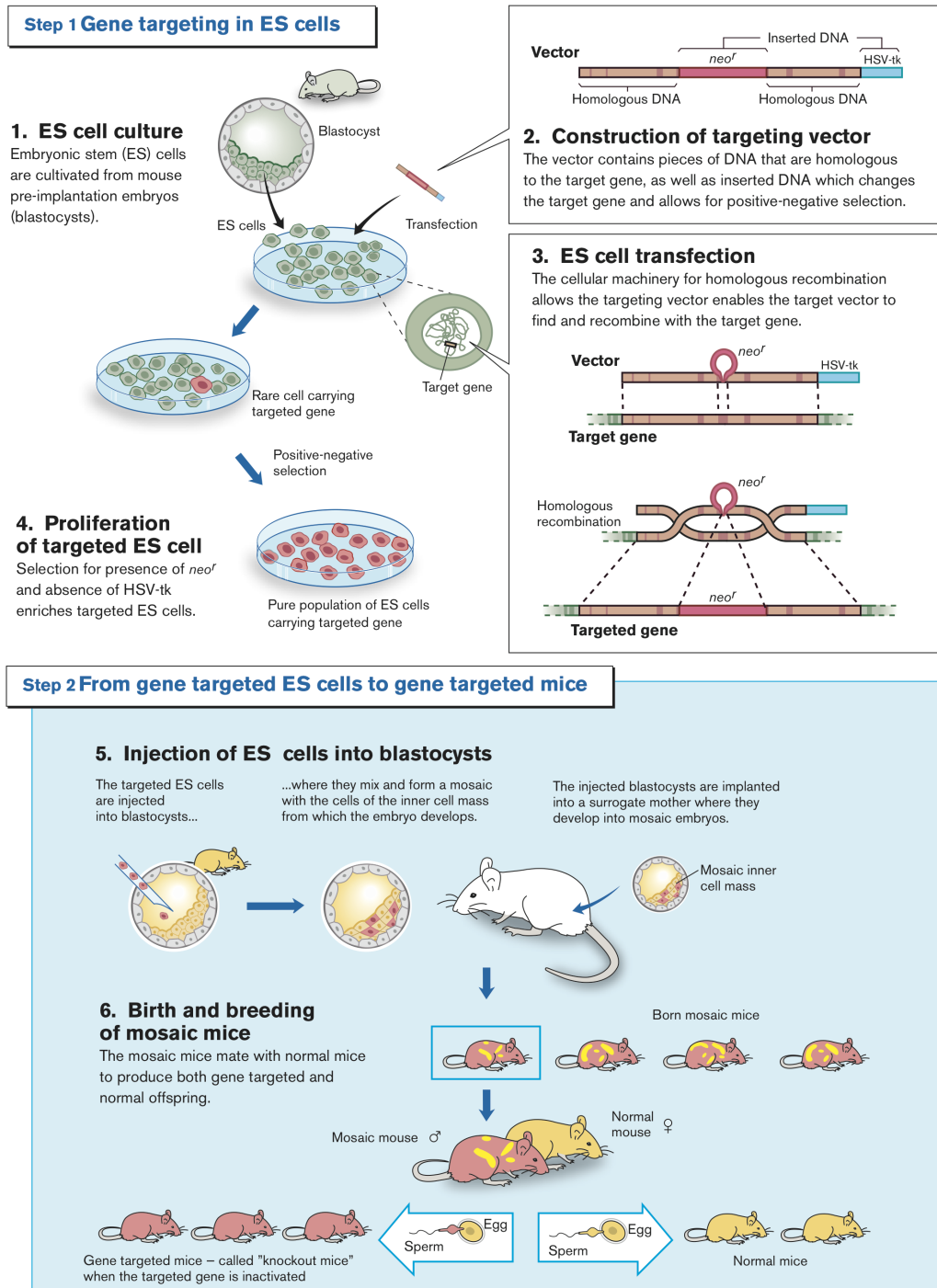


Figure 2.6: Generation of Knockout Mice (Reproduced from *The Nobel Committee for Physiology or Medicine, 2012*).

their genotype (TPCN2 WT only (493 bp); TPCN2 mutant only (336 bp); or both). Again, RT-PCR resolving the expression of TPCN2 (exons 4-8, 564 bp) was used to determine that TPC YHD437 was a true TPC2 knockout mouse (Chuang, 2011).

2.4.2 Generation of TPC Double Knockout Mice

In order to generate double knockout mice (DKOs), TPC1 single KOs (TPC1 D159) were bred with TPC2 single KOs (TPC2 YHD437). Offspring from breeding these should be heterozygote for both TPCN1 and TPCN2 (TPC1^{+/-} TPC2^{+/-}). These mice were then crossed with each other to obtain double homozygous mutants (TPC1^{-/-} TPC2^{-/-}) with a probability of 1 : 16 (according to Mendelian genetics). Offspring from crossing the heterozygous mutants were genotyped for their possessing the different TPC1 and TPC2 alleles using the same 3-primer PCR strategy mentioned above. For TPC1 the presence of WT TPC1 only (376 bp), TPC1 mutant only (459 bp), or both alleles was investigated; and for TPC2; WT TPC2 only (493 bp), TPC2 mutant only (336 bp), or both alleles. Using RT-PCR, it was determined that both TPC genes had been successfully knocked out and neither mRNA was expressed in the animal (TPC1 exons 6 - 16, 606 bp; TPC2 exons 4 - 8, 564 bp); see Fig. 2.7 (Chuang, 2011).

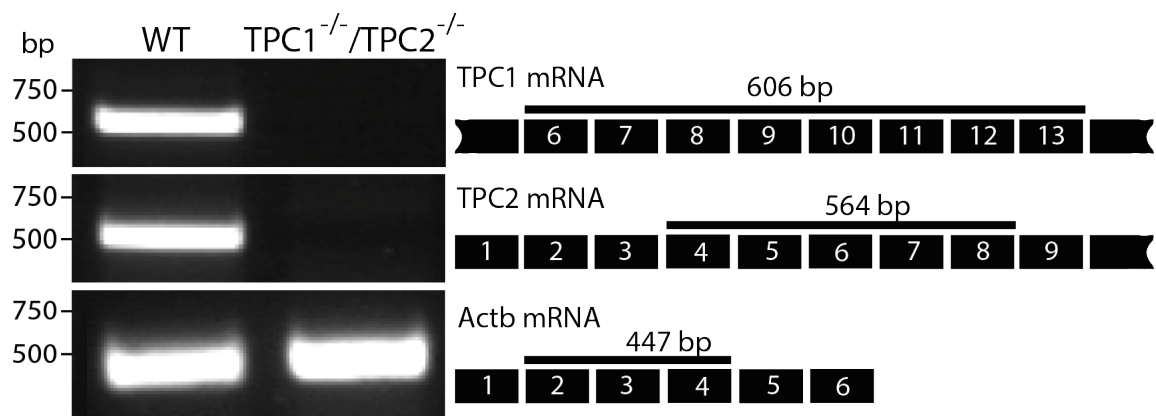


Figure 2.7: RT-PCR showing TPC Expression is abolished in TPC1^{-/-} TPC2^{-/-} but not WT Mice (left). Beta-Actin (Actb), a house-keeping gene, is expressed in both Genotypes. Amplified mRNA Regions (right) with numbered Exons (Reproduced from Chuang, 2011).

2.5 Electron Microscopy

Electron microscopy was conducted by Wendy Tynan of the Department of Pharmacology in collaboration with the MRC Neuropharmacology Unit, which houses the electron microscope.

2.5.1 Fixation of Islets and Preparation of Blocks

Pancreatic islets were isolated and picked as described above. From the clean pick, they were placed in Eppendorf tubes containing HANKS and kept on ice until further processing. This was started within 1 h of islet isolation.

An equal volume of 100 mM cacodylate buffered, double strength fixative was added to the tube containing HANKS, to give a final concentration of 2 % glutaraldehyde (TAAB Labs.) and 2 % paraformaldehyde. Samples were fixed for two hours at room temperature on an automatic rotator. Further processing was carried out on a tilting platform in a fume cupboard.

Samples were washed three times with 100 mM cacodylate buffer containing 20 mM calcium chloride and then post fixed for 1.5 to 2 hours in 1 % osmium tetroxide (TAAB Labs.) in 100 mM cacodylate buffer. This was followed by a further three washes with 100 mM cacodylate and subsequently three washes with maleate buffer (pH = 5.15; BDH). Samples were stained 'en bloc' in the dark for one hour in 1 % magnesium uranyl acetate (TAAB Labs.) at pH 6 (this pH drops to 5.15 during staining). This was followed by another three washes in maleate buffer.

Samples were split into two groups at this point. All dehydration and infiltration steps for both groups were conducted in a fume cupboard.

Group one was processed to the methacrylate LR White (TAAB Labs.). This was done by dehydration through an ethanol series (50 %, 70 %, 80 %) followed by infiltration with an ethanol-LR White mixture (80 % Ethanol : LR White; 30 : 70). This was succeeded by 4 changes of LR White over at least 4 hours. Samples were

then transferred to and embedded in labelled and pre-warmed gelatine capsules and polymerised overnight for a total of 20 hours at 50°C. Until the incubation at 50°C, all processes were conducted on a tilting platform and kept on ice.

Group two was processed to an araldite resin. Again samples were dehydrated through an ascending ethanol series on ice (50 %, 70 %, 80 %, 90 %, 95 %) followed by one change of dry alcohol. This last change was allowed to warm to room temperature (this took around 10 min), and the following steps were conducted at room temperature. Dehydration was completed by three 20 min changes of 100 % ethanol (dried over copper sulphate), followed by two changes of propylene oxide for 15 min. Samples were infiltrated with resin and propylene oxide (1 : 1) and whole plastic (with added accelerator; TAAB Labs.) for one hour. This was then replaced with whole plastic for two hours. This was done on a rotating platform. Samples were then transferred to labelled polyethylene capsules and allowed to sink to the bottom overnight. Finally these capsules were polymerised at 60°C for 48 - 72 hours.

2.5.2 Sectioning, Staining, and Imaging

The blocks produced as described above were cut on a Reichert-Jung Ultracut to produce 50 nm sections which were collected onto single slot copper grids (TAAB Labs.). Grids were stained with Reynolds lead (2 - 4 min), in a fresh solution of aqueous 1 % uranyl acetate (20 min), or a combination of both. Sections were viewed on a Philips EM10 transmission electron microscope and images acquired using a Gatan digital camera.

2.6 Analysis and Statistics

Data were analysed using NIS-Elements AR 4.0 (Nikon) and MS Excel 14 (Microsoft). Figures were prepared in Illustrator CS6 (Adobe). Calcium traces are of fluorescence change relative to baseline mean fluorescence ($\Delta F/F_0$). Baseline mean fluorescence

(F_0) was calculated from the fluorescence of the last 100 frames before stimulation. Traces are either representative, or expressed as means $\pm$ standard error of the mean (SEM) as indicated in figure legends. Calcium fluorescence images are pseudo-coloured so that changes in colour reflect changes in fluorescence. Warmer colours represent higher levels of fluorescence.

Statistical Analysis was conducted in MS Excel and SPSS 19 (IBM). Student's t-tests (paired or unpaired, one- or two-tailed; as applicable) and one-way Analysis of Variance (ANOVA) were used to determine the statistical significance of observed effects ($p < 0.05$; $p < 0.01$, as stated). Charts illustrating statistical differences between groups depict mean $\pm$ standard error of the mean (SEM) unless stated otherwise.

For the analysis of bafilomycin effects in Chapter 5, a repeated measures ANOVA was conducted with stimulus as the within-subjects independent variable and bafilomycin as the between-subjects independent variable. For the analysis of genotype effects and sex differences in Chapter 4, a univariate ANOVA was conducted with genotype, genetic background and gender as independent variables and reaction time and response peak height as dependent variables ($p < 0.05$; $p < 0.01$, as stated).

Movies for the supplementary material CD-Rom were prepared from Nikon .nd2 files and rendered using Premiere Pro CS6 (Adobe).

Chapter 3

Characterisation of β -Cell Calcium Responses using TIRF Microscopy

3.1 Introduction

In order to investigate how sub-membrane calcium levels as observed with TIRF differ from whole cell responses under epifluorescence microscopy, and to be able to compare these to responses seen in knockout animals, ‘regular’ calcium responses to 16.5 mM were first recorded in TIRF and characterised.

3.2 β -Cells show a Variety of Response Patterns

3.2.1 TIRF Imaging of β -Cells

As can be seen in Fig. 3.1 below, TIRF images of pancreatic β -cells show superior spatial resolution compared to those obtained with epifluorescence. The punctate substructure of the TIRF images suggests a heterogenous distribution of calcium immediately beneath the plasma membrane. This is conceivable given the abundance of insulin containing vesicles (putative calcium stores) and possibly co-localised membrane clusters of L-type calcium channels (Bokvist et al., 1995).

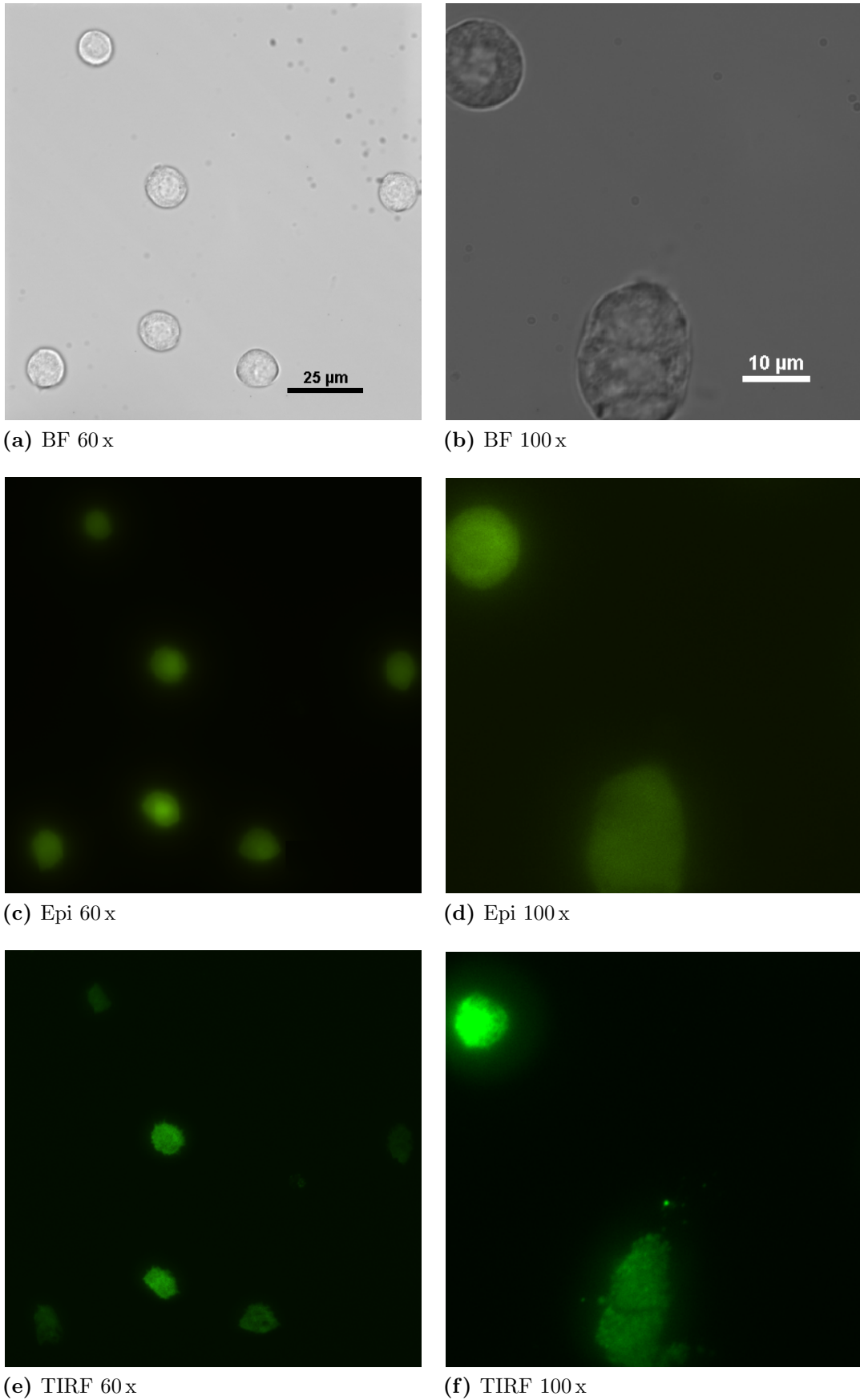


Figure 3.1: Representative Brightfield (a) (b), Epifluorescence (c) (d), and TIRF (e) (f) Images of single β -Cells and Cell Clusters loaded with Fluo 4 at 60x (a, c, e) and 100x (b, d, f) Magnification.

3.2.2 The Triphasic Calcium Response

Calcium responses to glucose in β -cells are usually described as having a typical triphasic shape as illustrated in Fig. 3.2, below. This is often seen in parallel with, and assumed to be the result of, simultaneously measured changes in membrane potential, which have a similar pattern and are in synchrony with the cytosolic calcium increase. The three phases of the calcium rise observed in response to an increase in extracellular glucose concentration can be summed up as follows; see Worley et al. (1994):

1. Phase 0: A ‘dip’, or initial decrease, in cell calcium concentration. This the result of increased SERCA activity removing calcium into the ER in response to stimulation by the rising ATP concentration in the cell due to increased glucose metabolism.
2. Phase 1: A transient rise in calcium (sometimes reaching concentrations 3 or 4 times higher than baseline calcium concentration), which is associated with calcium release from intracellular stores and CICR, followed by L-type channel activation, and has been suggested to underlie phase 1 insulin secretion.
3. Phase 2: Calcium oscillations on a steadily elevated plateau. These are thought to be the result of calcium influx through L-type channels, and responsible for phase 2 of insulin secretion.

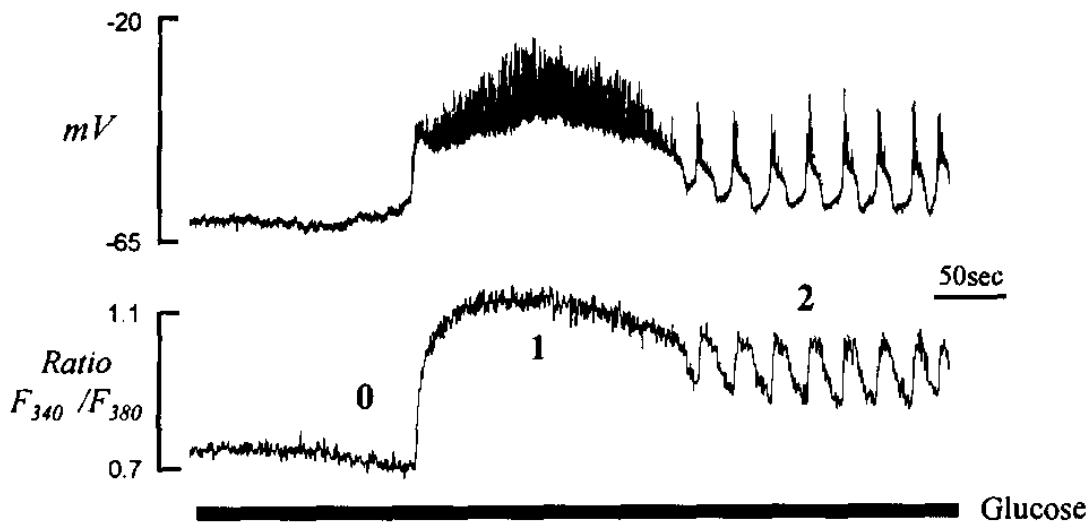


Figure 3.2: *Triphasic Calcium and V_m Response to Glucose (Reproduced from Worley et al., 1994)*

3.2.3 Distribution of Response Types

While this standardised model of ‘the’ triphasic calcium response to glucose is useful; it does not necessarily reflect what occurs in practice. It has been described that β -cells are extremely variable in their responses to glucose (and indeed other stimuli such as GLP1, Insulin, and non-glucose nutrients such as amino acids; see for example Herchuelz et al., 1991; Gustavsson et al., 2006). A recent classification of β -cell calcium responses is shown in Fig. 3.3. It illustrates Johnson and Misler’s response-type categories and their occurrence frequencies. Note that this is for primary human β -cells’ autocrine calcium response to insulin rather than glucose (Johnson and Misler, 2002).¹ As can be seen from Johnson and Misler’s study; β -cells display a variety of equally common but different calcium signals and no clear ‘standard’ response.

This heterogeneity of responses was replicated in the present study for stimulation with 16.5 mM glucose in mouse primary β -cells, as shown in Fig. 3.4. Responses from 1,017 cells were analysed. A further number of cells used for pilot studies was not considered, as experiments were conducted at room temperature (known to affect

¹A comprehensive listing of response types to glucose could not be found.

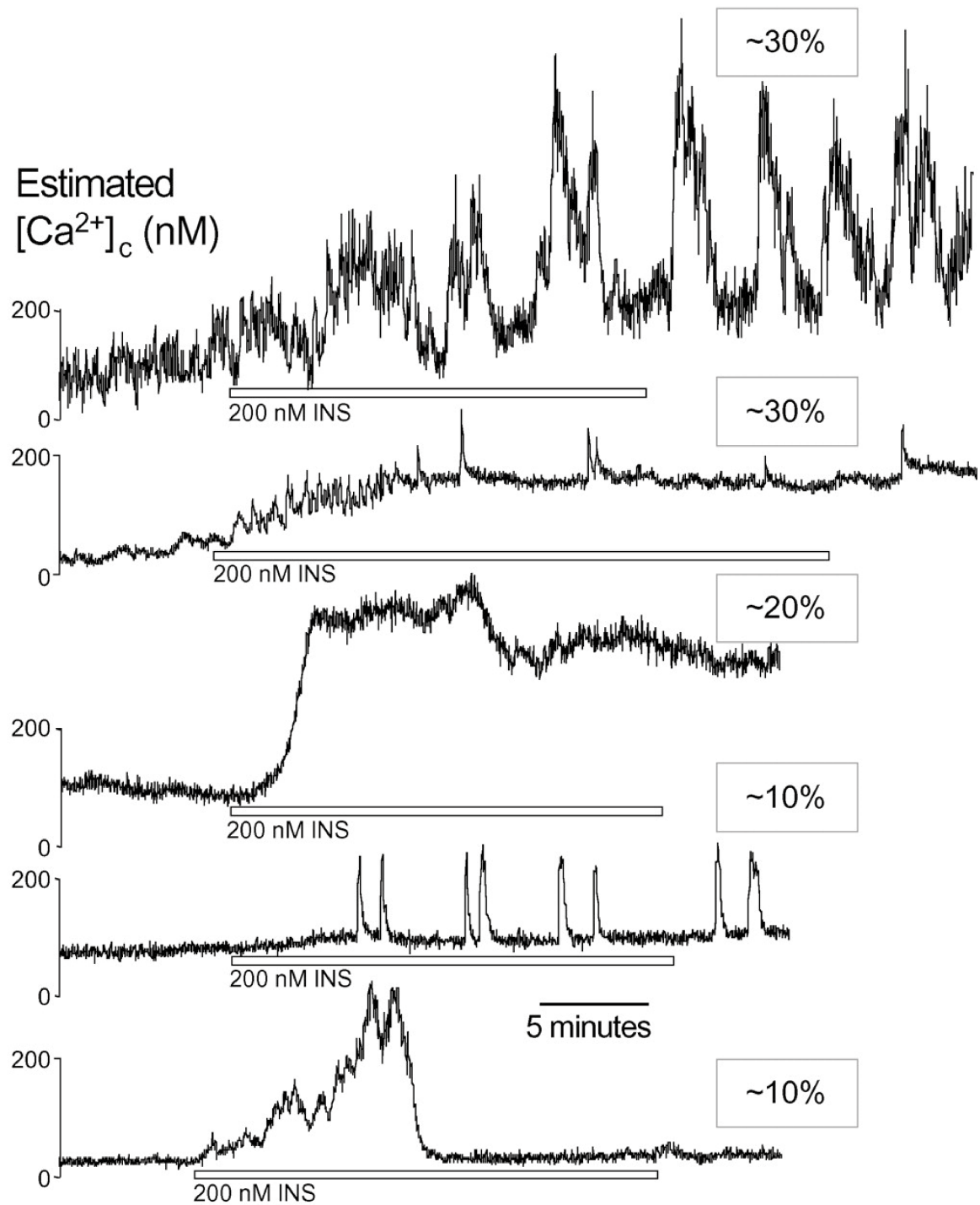


Figure 3.3: *Distribution of Calcium Response Types to Insulin. $n=101$. (Reproduced from Johnson and Misler, 2002)*

calcium responses to glucose; Rojas et al., 1994). Note that the percentages given for the frequency of the different types of responses in Fig. 3.4 refer only to the 239 male WT control cells of one genetic background that responded after glucose increase from 3 mM to 16.5 mM. The analysis of this pattern in female animals and the effects of different genotypes on this will be discussed in Chapter 4; and any changes in this pattern with pharmacological manipulations are covered where those agents are considered.

As can be seen in Fig. 3.4, the most common response observed ($\sim 60\%$ of responses), type 1, resembles the standardised triphasic response discussed above. This is in contrast to the cited responses to insulin (Fig. 3.3, where no response type seemed to be ‘dominant’. The next most common response type (type 4, $\sim 25\%$), resembled the triphasic response, but had a much less steep phase 1. The slow calcium oscillations classified as type 2 often occurred at periods of ~ 5 min, similar to that observed with whole islets (Beauvois et al., 2006). Response type 3 consists of numbers of large transients superimposed on a slow increase in calcium. These responses were rare under control conditions ($\sim 2\%$). A very small number of cells did not respond to glucose ($\sim 2\%$).² So in this sample of 239 cells, approximately 96% showed a prominent global calcium response triggered by 16.5 mM glucose. In 116 cells, 16.5 mM glucose evoked a mean peak height of $2.4 \pm 0.01 \Delta F/F_0$. While cells were rarely unresponsive to glucose, the present study found the spontaneous activity of β -cells to be very high. This phenomenon is discussed below.

For the experiments in this chapter and Chapter 4, the following parameters of the β -cells’ calcium response were analysed (see Fig. 3.5): Percentage or number of responding cells, peak height of response (PH, $\Delta F/F_0$), as measured from extension

²Two thirds of Johnson and Misler’s cells did not respond to insulin. This turned out to have been a non-linear dose-response effect for insulin which initially went unnoticed despite analysing 335 cells (Johnson, personal communication). This illustrates the difficulty of observing genuine effects in β -cells, as responses vary widely and it takes large numbers of cells to arrive at reasonable conclusions.

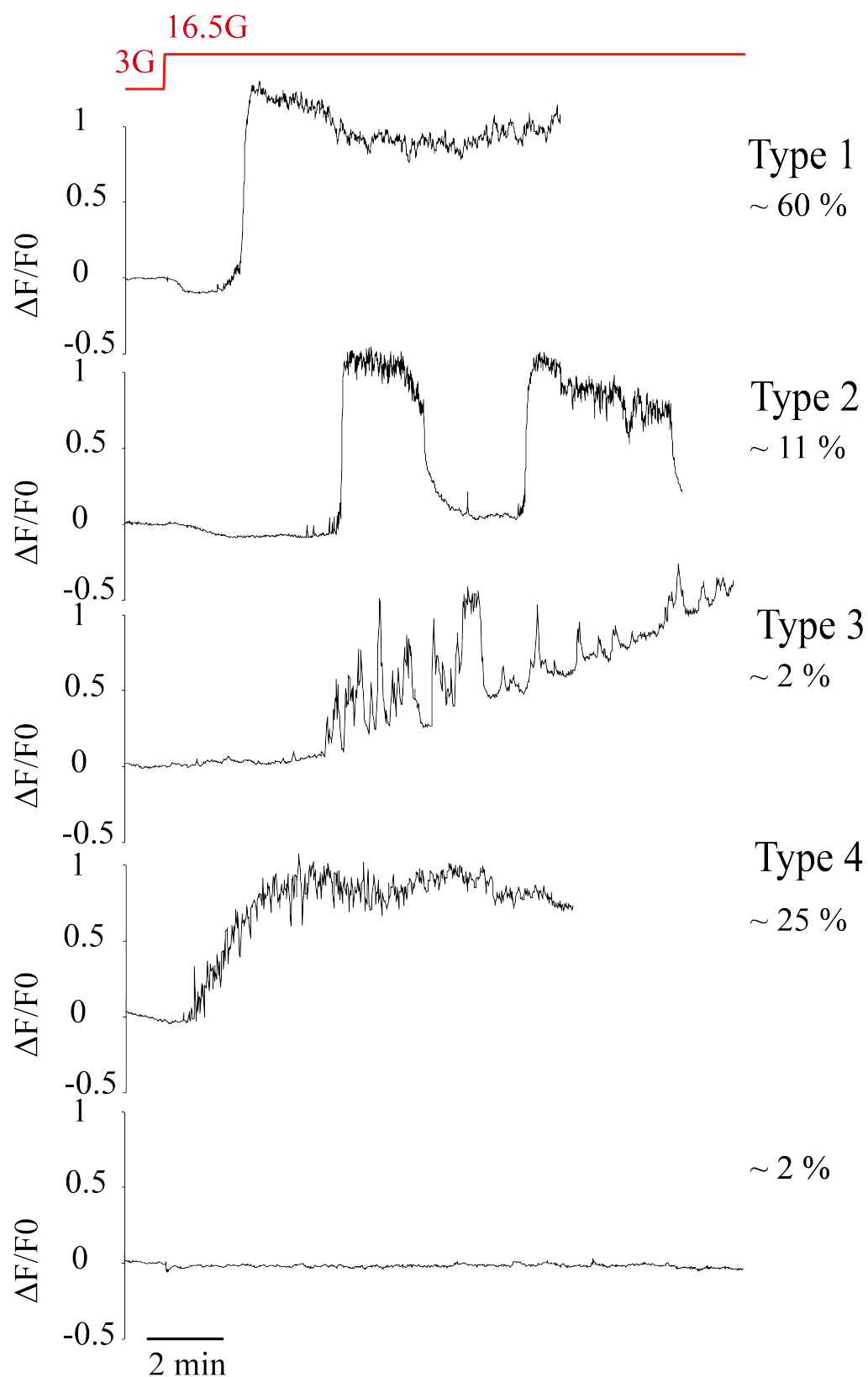


Figure 3.4: Distribution of Calcium Response Types to 16.5 mM Glucose at 37°C in Primary Pancreatic β -Cells from Male Mice. $n=239$.

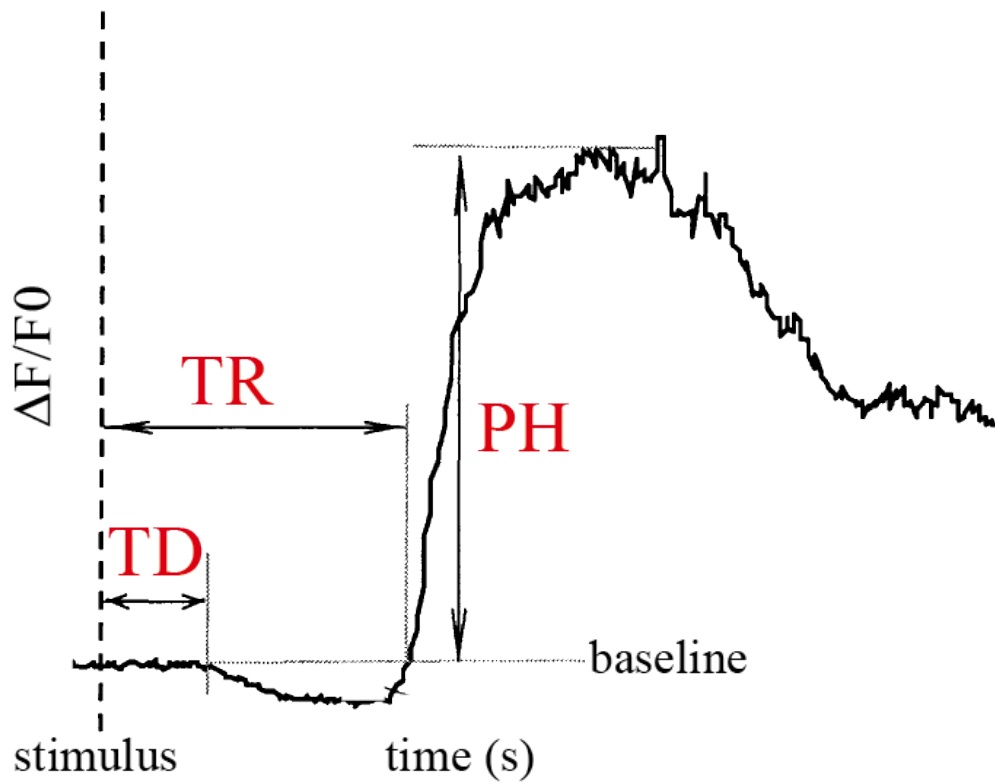


Figure 3.5: *Measured Parameters of the β -Cell Calcium Response. For Responses of Type 3, no Peak was measured. For Responses of Type 2, the initial Phase 1 was taken for Measurement. TR, Time to Response; TD, Time to Dip; PH, Peak Height from Extension of Baseline Line of Best Fit. (Modified from Pakhtusova et al., 2003)*

of baseline), time to response (TR), time to dip TD) and percentage of cells showing dip.³

³The slope of phase 1, the time to spikes pre-rise and the time to peak were also assessed, but did not add to the analysis of the parameters shown, and are thus omitted.

3.3 Spontaneous Activity in β -Cells

3.3.1 ‘Early’ Responses

As mentioned above; the present investigation found a large number of calcium responses to occur ‘early’; i.e. before addition of glucose had occurred.⁴ As is shown in Fig. 3.6; these amounted to 41 % of all responses recorded from male WT β -cells (n= 239). A response was classified as early if phase 1 was initiated before the point of glucose addition (this invariably still classifies some responses as non-early that must have been triggered before glucose addition; but it was the only systematic cut-off point that could be chosen). The highest proportion of ‘early’ responses observed was 57 % (n= 53). Comparing different experimental groups, the lowest value observed was 0 % (n= 33), with an overall average of 30 % (n= 631).⁵

⁴The fact that this large number of cells was falsely identified as β -cells is unlikely. The responses also did not subside with a rise in extracellular glucose; as would be expected with α -cells. Other islet cell types are so rare, it is very unlikely they made up this number of cells.

⁵N refers to the number of cells in the group for which that percentage of early responses was observed.

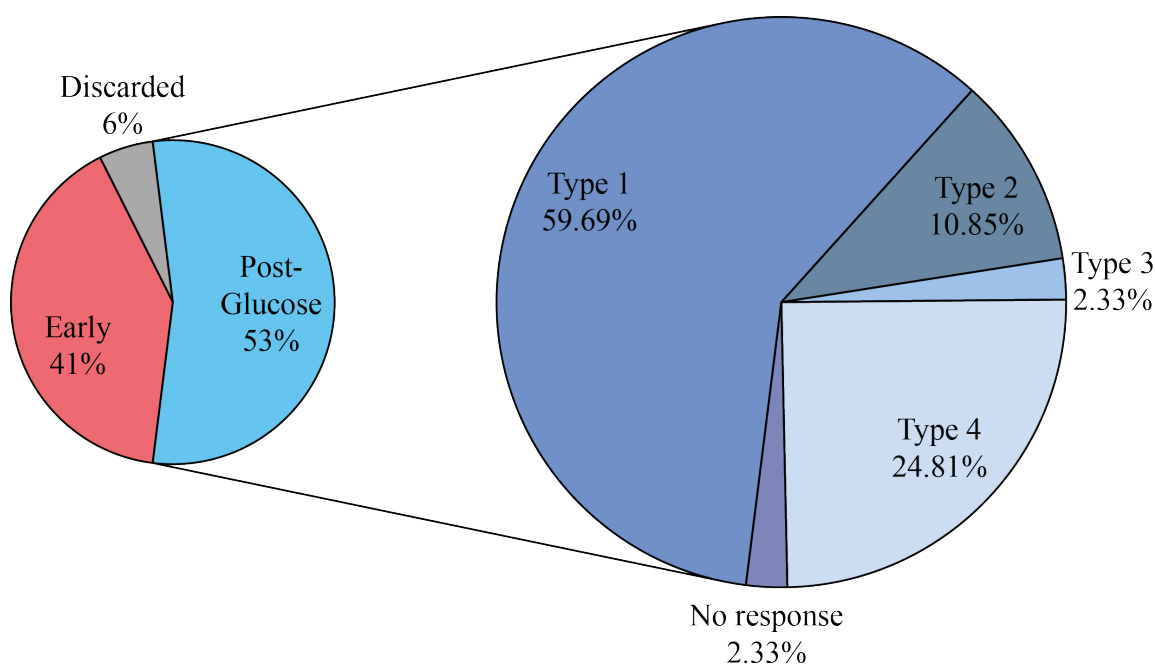


Figure 3.6: Responses of male WT Cells to 16.5 mM Glucose ($n=239$). Types are as explained in Fig. 3.4. ‘Discarded’ refers to Cells that exhibited α -cell behaviour as explained in Chapter 2: Methods.

3.3.2 Unstimulated Cells

To investigate the nature of the ‘early’ responses, a series of experiments without stimulation were conducted. Cells were treated as before, but instead of raising glucose, a ‘dummy’ addition of either baseline recording medium containing 3 mM glucose, or medium containing 2 % DMSO without solute were used. Spontaneous responses were recorded from 35 % and 38 % of cells, respectively. This resembles the level of spontaneous activity recorded for isolated mouse β -cells by Jacques (2009) and is lower than that recorded by Herchuelz et al. (1991) for isolated rat β -cells. In the present study, a disproportionate number of the spontaneous responses (two thirds and one third, respectively) occurred within the first minute of the at least 10 min recording period (equivalent to the ‘early’ responses seen in stimulated cells). This suggests that it is something inherent about the start of experimenting which triggers most of these responses. Likely causes of this activity might be a photosensitivity reaction

to laser illumination (as described for example by Grapengiesser, 1993; Knight et al., 2003), or a thermosensitivity reaction to addition of medium (see Hellman et al., 1990). Both of these factors are likely to have played a role in the present study as there appeared to be less ‘early’ responses in TIRF recording than combined TIRF and Epifluorescence recording (described below), as well as in pilot studies conducted at room temperature (results not quantified). As temperature and illumination should change minimally across experiments (unless a particular drug or genotype would render cells more photo- or thermo-sensitive, which is unlikely), it was assumed that these factors should be constant, and thus allow for comparison between experimental groups.

An additional concern are the effects of cell culture conditions; see (Gilon et al., 1994; Szollosi et al., 2007). Henquin and colleagues (Gilon et al., 1994) demonstrated that both TR and PH of whole islets changed significantly over 5 days in cell culture. Specifically, TR decreased and PH increased with time. However, as these conditions were the same for cells from the same batch of cell-culturing and investigated on the same experimental day; it was assumed that groups of cells that were tested in tandem could be compared with each other without unstimulated responses confounding data analysis; even if the quantitative description of the calcium responses for each group *per se* were not representative. Thus experimental population-statistics were avoided apart from the description of response types illustrated above and the genotype analysis presented in Chapter 4.

3.3.3 Comparison of Single Cells and Cell Clusters

A further factor determining the nature of the calcium responses recorded and the tendency toward spontaneous activity is the differences that can be observed between the responses of dispersed single cells, cell clusters of different sizes, and whole islets. Pakhtusova et al. (2003) conducted a thorough investigation into the differences in time to response (TR) and peak height (PH) of these, and found that with increasing

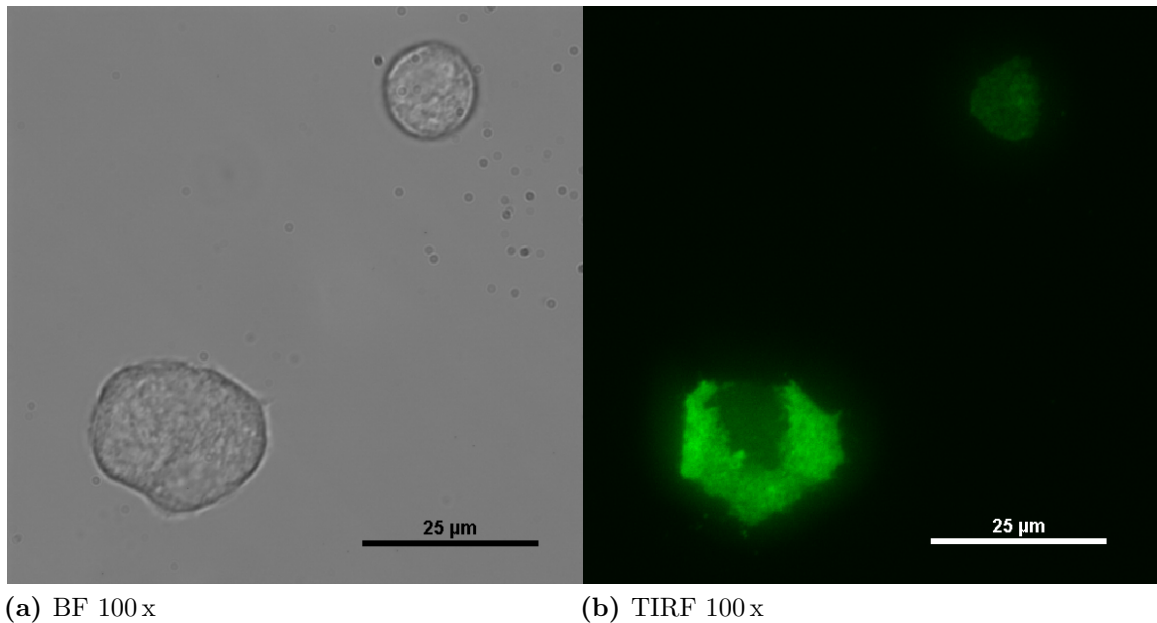


Figure 3.7: *Single Cells and Clusters under Bright-field (a) and TIRF (b) Microscopy at 100x Magnification.*

cluster size (from one cell to whole islet), TR and PH decrease. This is likely due to the ‘averaging’ of the responses of individual cells within a cluster or islet. Thus to make comparisons between groups it is important to work with a constant cluster size and/or ratio of cells to clusters. For the studies presented in this chapter and chapter 4, single cells were used almost exclusively. The experiments in Chapter 5 were conducted on both single cells and small clusters; at an estimated ratio of 50 % cells, 50 % clusters. As is illustrated by Fig. 3.7, clusters lend themselves to TIRF experiments, as they provide a large area positioned in the same focal-plane. Dispersed cells are usually at marginally different focal planes (due to the inherent curvature of the glass coverslip). Another reason one might prefer to work with cell clusters is that they appear to be less sensitive to the above-mentioned factors governing spontaneous activity (Jacques, 2009; Jaques et al., 2008); which seems to be dependent on cell-cell contact (see also Jo et al., 2005).

How β -cells within a cluster can interact in different ways is demonstrated by the fact that they can oscillate in synchrony or in asynchrony with each other when stimulated. As mentioned in Chapter 1, this phenomenon is potentially important for insulin secretion from the whole pancreas and is a field of study in itself. Movie 1, included on the CD-Rom (included with thesis), shows single cells and cell clusters as seen in TIRF oscillating in response to stimulation with 16.5 mM glucose. A synchronous and an asynchronous cluster are shown (please also see Appendix A.6 before watching Movie 1).

3.4 Differentiation of Calcium Responses into Local and Global

3.4.1 Parallel Recording of Epifluorescence and TIRF

Having established the general response pattern of β -cells to glucose in TIRF, it was investigated whether there might be a ‘time-lag’ between the TIRF calcium signal and the whole-cell signal observed with epifluorescence microscopy. This hypothesis was based on the notion that if intracellular calcium release from acidic stores triggered the global calcium response, an increase in calcium should occur first in the vicinity of the secretory vesicles, which constitute acidic organelles in the β -cell (Orci et al., 1986)), before recruiting a global calcium response that spreads throughout the whole cell (Arredouani et al., 2010).

To investigate this, parallel recordings were conducted in TIRF and epifluorescence (see Chapter 2: Methods). Specifically, it was investigated whether the small number of calcium oscillations that can be seen before the phase 1 rise in standard responses (see Fig. 3.4) also occurred in epifluorescence or could only be visualised by TIRF. Fig. 3.8 shows parallel recordings in response to stimulation with 16.5 mM glucose. As can be seen; there is no time-lag between the two traces (within the resolubility of the

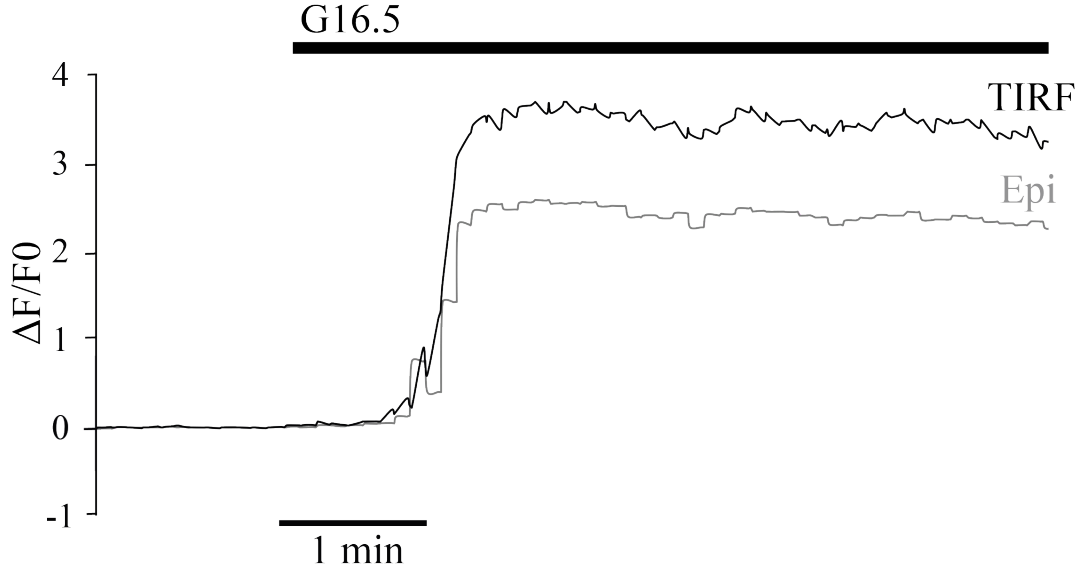


Figure 3.8: Representative Trace of parallel Recording of whole-cell (Epi-fluorescence; grey trace) and sub-membrane (TIRF; black trace) Calcium in Response to 16.5 mM Glucose. Frame Rate Epi: 1Hz. Frame Rate TIRF: 3.3Hz.

acquisition rate), and the pre-rise spiking occurs in both traces. This was observed in more than 100 cells. To ensure that there was no disproportionate representation of sub-membrane events in the global calcium trace, epifluorescence was measured at two different distances in the z-axis from the coverslip. At the same level as TIRF; and $5\mu\text{m}$ above it. The result remained as demonstrated in Fig. 3.8.⁶

3.5 Calcium Responses are dependent on Influx across the Cell Membrane

3.5.1 Experiments in Calcium-free Medium

As the visualisation of the time course of calcium signals showed no difference between TIRF and epifluorescence, it was investigated whether intracellular release of calcium

⁶A lower affinity calcium dye, fluo-5F ($K_d = 2.3\mu\text{M}$), was also trialed and its resolution compared to that of fluo-4 ($K_d = 0.35\mu\text{M}$). Fluo-5F did not provide a further increase to the resolution of calcium responses as compared to fluo-4. See Appendix A.5 for a representative trace recorded with fluo-5F.

	Control	Calcium-free	Significance (Student's t)
Total Cells	36	26	
Responding Cells	12	0	
'Early' Responses	19	0	
Cells Showing Dip (%)	50	84	
TD (s)	21 ± 0.9	17 ± 0.2	NS; $p = 0.057$

Table 3.1: *Effect of Removing Extracellular Calcium. (%) is % of non-early, non-discarded cells.*

could be seen in TIRF microscopy if the influx of calcium was eliminated by removing extracellular calcium. Cells were pre-incubated for 10 min in calcium-free medium and then kept in calcium-free medium for the duration of the experiment. As illustrated in Fig. 3.9, cells showed no increase in calcium in response to glucose under these conditions. This was observed in all 26 cells tested. Importantly, the SERCA-‘dip’ was still present, and TD unchanged (see Table 3.1), demonstrating the effect of glucose and functioning of SERCA, which is known to be unaffected by the removal of calcium (Ravier et al., 2011). When calcium was reintroduced at the end of the experimental run (after at least 10 min), a regular calcium response could be elicited, demonstrating the viability of the cell. Early responses were also eliminated in calcium-free medium; demonstrating that these, too, rely on the influx of calcium.

The fact that experiments in calcium-free solution eliminated all responses was surprising, since it is known that intracellular stores contribute to the calcium response. It is possible that calcium cycling between extracellular space, cytosol, and stores is so rapid that the lack of response observed in calcium-free solution is in fact due to store run-down having occurred during the 10 min pre-incubation period with calcium-free medium. An earlier pilot study used LysoTracker, an acidic organelle marker, to label insulin containing vesicles, while also imaging calcium dynamics using fluo 4. TIRF images were acquired in parallel at 515-555 nm and 600 nm wavelengths. The experiment was eventually abandoned because LysoTracker, while labelling acidic organelles within the TIRF field (see Appendix. A.7) more often than not dissipated

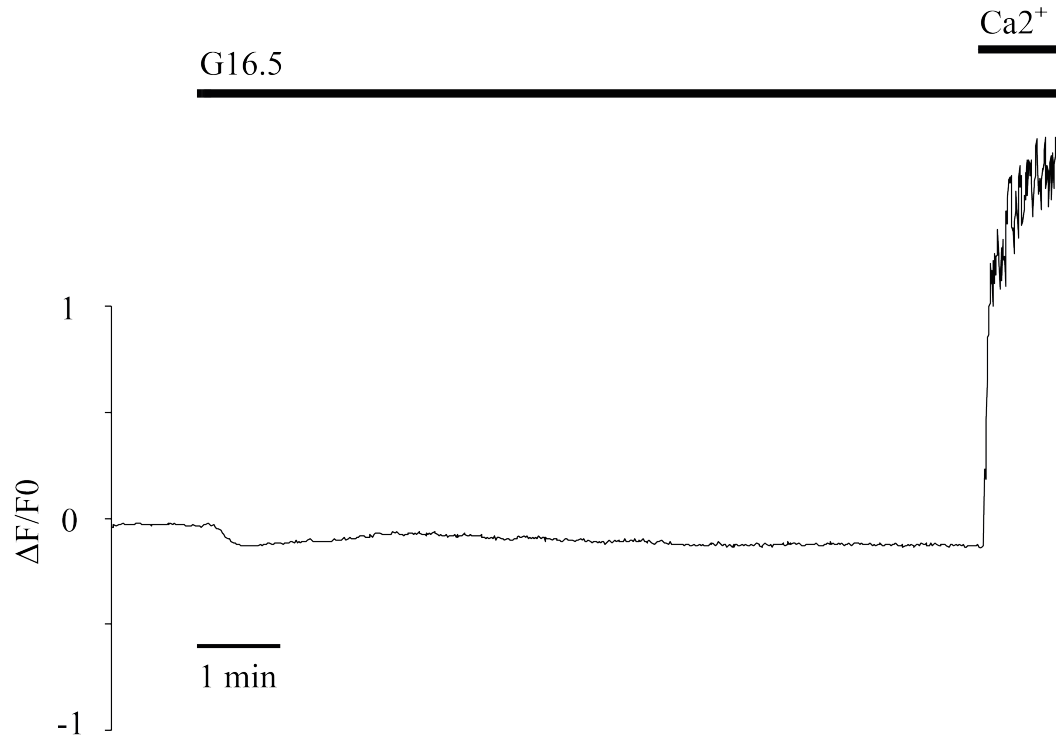


Figure 3.9: Representative Trace of a Calcium Response to 16.5 mM Glucose in calcium-free Solution as imaged using TIRF. Extracellular Calcium was replaced at the end of the Experiment; resulting in a global Calcium Signal.

within the first minute of experimentation. To examine whether this phenomenon is indeed store run-down, one could investigate the effects of different secretagogues on responses in calcium-free medium, for which the target organelle is known (for example stimulation with ACh to trigger calcium release from the ER).

3.5.2 Experiments with Nifedipine

In order to eliminate calcium influx without the possibility of affecting intracellular stores, experiments were conducted in the presence of the L-type calcium channel antagonist, nifedipine. The addition of nifedipine at 3 μ M diminished glucose induced responses, as shown in Fig. 3.10; though calcium did not return to baseline. This

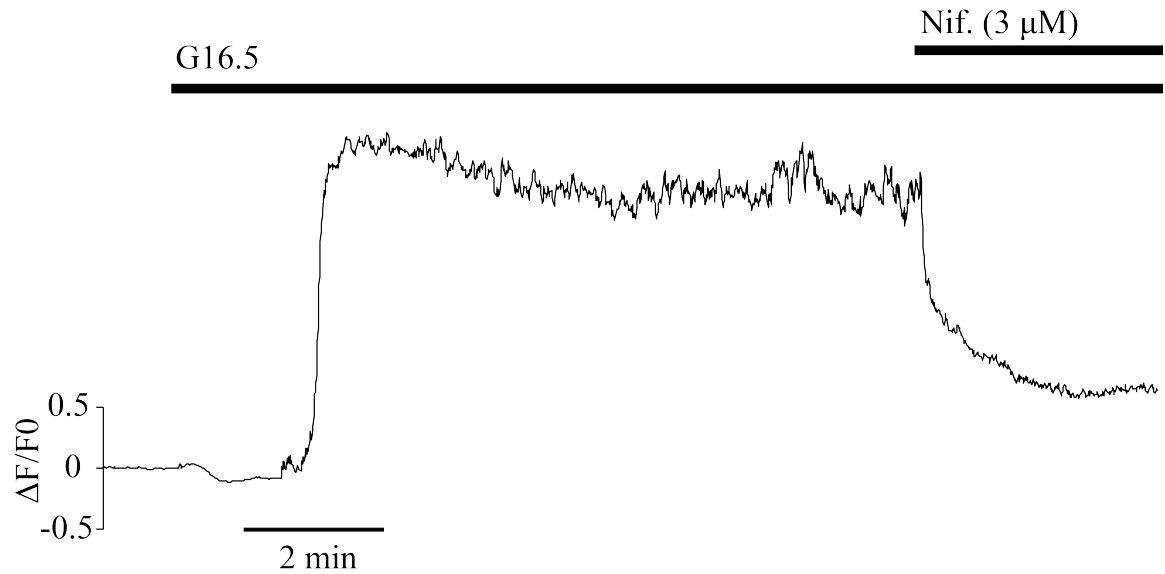


Figure 3.10: Representative Trace of a Calcium Response to 16.5 mM Glucose followed by the Addition of 3 μ M Nifedipine, as imaged using TIRF.

		Control	Nifedipine	Significance (Student's t)
Total Cells		26	17	
Responding Cells		13	8	
PH	($\Delta F/F_0$)	1.95 ± 0.04	0.21 ± 0.02	S; $p < 0.01$
'Early' Responses		8	0	
TR	(s)	160 ± 11	108 ± 10	NS; $p = 0.4$
Cells Showing Dip	(%)	46	50	
TD	(s)	19 ± 0.8	21 ± 1	NS; $p = 0.66$

Table 3.2: Effect of 10 min Pre-incubation with 3 μ M Nifedipine. (%) is % of non-early, non-discarded cells.

suggests there is some remaining calcium that does not depend on the influx through L-type calcium channels. When cells were pre-incubated for 10 min with 3 μ M nifedipine ($n=17$), responses were significantly reduced in amplitude (see Table 3.2). However, responses were not completely abolished. Even in experiments pre-incubating with 5 μ M nifedipine, miniature responses could still be observed (see Fig. 3.11). Notably, while the remaining responses in the presence of nifedipine are diminished in amplitude; there is no significant change in time to response; see Table 3.2.

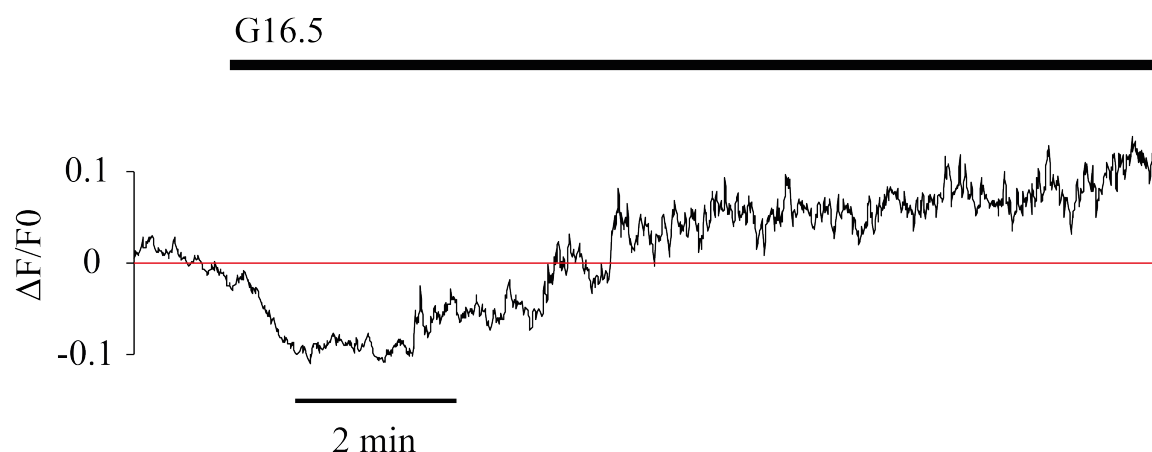


Figure 3.11: Representative Trace of a Calcium Response to 16.5 mM Glucose after Pre-incubation with 5 μ M Nifedipine for 10 min, as imaged using TIRF. Note the characteristic SERCA Dip followed by a Miniature Response. The Abscissa is extended in red to illustrate the Amplitude of the Response with Respect to Baseline.

3.6 Eliminating Acidic Store Calcium Content Alters Calcium Signals

If calcium responses to glucose are dependent on release specifically from acidic stores, they should be affected by Bafilomycin A1. Bafilomycin inhibits vacuolar-type H^+ -ATPases, leading to de-acidification of acidic organelles and consequent prevention of calcium uptake via the calcium-proton exchanger (Yoshimori et al., 1991). Insulin containing granules express the a3 isoform of this pump, which is bafilomycin sensitive (Sun-Wada et al., 2006). Glucose has been shown to decrease granule pH; bafilomycin increases the same, and pre-incubation with bafilomycin blocks the glucose-dependent decrease (Tompkins et al., 2002). Yamasaki and colleagues showed that β -cell responses to glucose, but not KCl or ACh can be eliminated with bafilomycin (Yamasaki et al., 2004). The elimination of global calcium responses to 16.5 mM glucose could be replicated in the present study after 10 min pre-incubation with 3 μ M bafilomycin (n=9); see Fig. 3.12. Shown in Fig. 3.13 are Images of β -cells that were pre-incubated with bafilomycin. They show a localised ‘smudged’ appearance where under normal

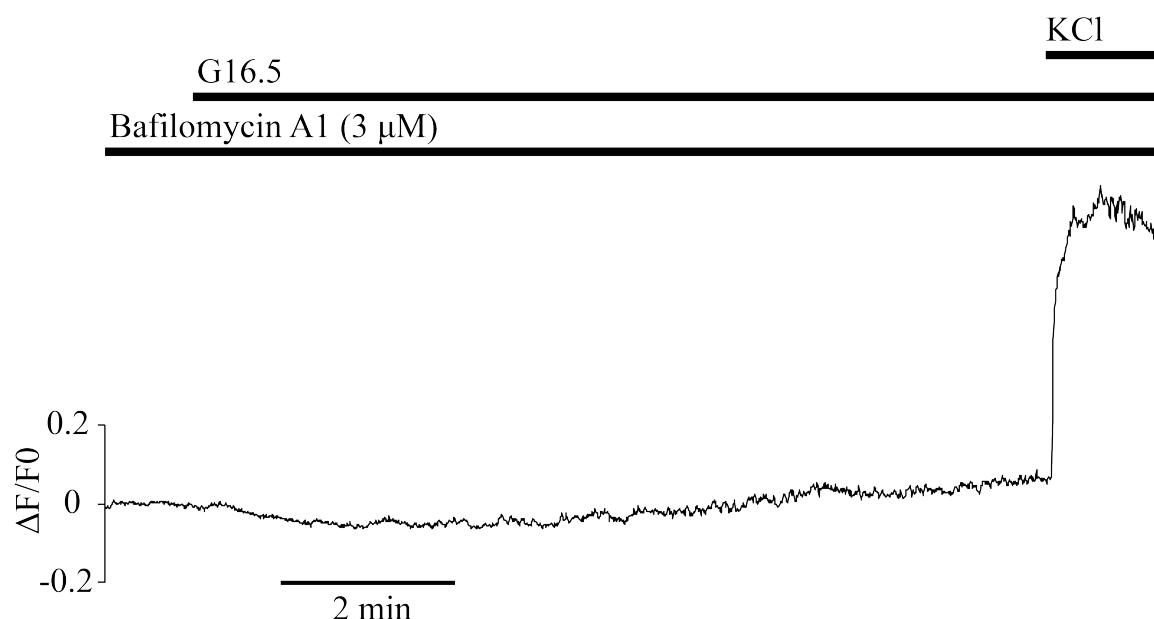
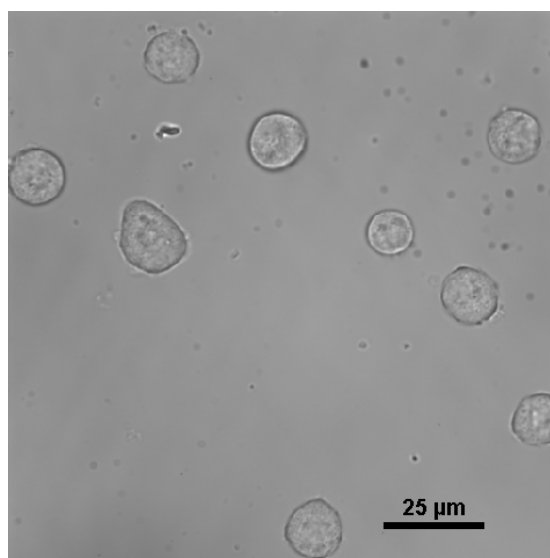
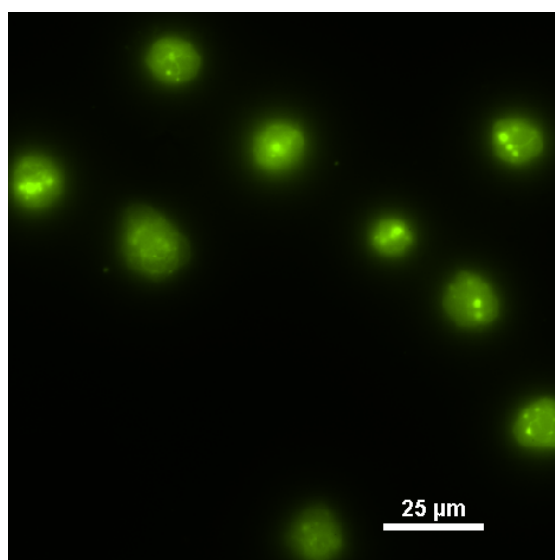


Figure 3.12: Representative Trace of a Calcium Response to 16.5 mM Glucose after Pre-incubation with 3 μ M Bafilomycin A1, as imaged using TIRF. The Cell was stimulated with KCl (45 mM) at the end of the Experiment to confirm its Viability.

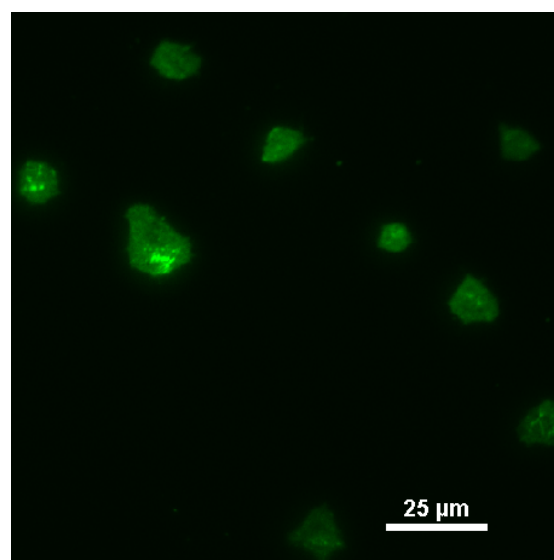
conditions, a regularly granular appearance was observed (Fig. 3.1), illustrating the effects of bafilomycin and their visibility in the immediate vicinity of the membrane. Interestingly, a 30 min incubation with 1 μ M bafilomycin did not eliminate responses, but caused a significant delay in their onset ($n=42$); see Table 3.3. This suggests that partial inhibition of acidic store calcium release might present with a delay in response. However, the time to the start of the SERCA ‘dip’ was significantly delayed also (Table 3.3), which could suggest an unspecific effect, or artefact. The latter is a possibility, as the TR for control cells in this sample seems much lower than in other experiments.



(a) BF



(b) Epi



(c) TIRF

Figure 3.13: Representative Brightfield (a), Epifluorescence (b), and TIRF (c) Images of single β -Cells after 30 min Pre-incubation with 1 μM Bafilomycin A1 at 60x Magnification.

		Control	Baf. (1 μ M)	Baf. (3 μ M)	Sig. (Student's t)
Total Cells		34	42	9	
Responding Cells		20	26	0	
'Early' Responses		12	13	0	
TR	(s)	51 $\pm$ 2	168 $\pm$ 6	N/A	S; p < 0.01
Cells Showing Dip	(%)	85	29	11	
TD	(s)	16 $\pm$ 0.2	21 $\pm$ 0.7	(31 $\pm$ 0)	S; p < 0.05

Table 3.3: Effect of Pre-incubation with Bafilomycin A1. Statistical significance is between Control and 1 μ M Bafilomycin. (%) is % of non-early, non-discarded cells.

3.7 β -Cells can be stimulated with NAADP-AM

Having established that intracellular calcium contributes to the presently observed responses and that bafilomycin can inhibit these suggesting the involvement of acidic stores; it was investigated whether responses could be elicited directly by NAADP. In order to do this, the membrane-permeable acetoxymethyl ester version of the compound (NAADP-AM) was used. Cells were kept in 3 G medium and stimulated with NAADP-AM at 10 μ M.⁷ As can be seen in Fig. 3.14; distinct responses can be elicited with this compound in 20 of 53 cells. When glucose is added at the end of the experiment; a SERCA-‘dip’ is superimposed on the NAADP-dependent calcium signal; but no further rise in calcium is observed. This suggests that maximal calcium responses can be elicited by NAADP-AM. Alternatively, it could mean that the intracellular NAADP concentration has reached desensitising levels at this point (this is $\sim$ 10 min after addition), preventing a potential further calcium release triggered by glucose. The drastically increased TR for stimulation with NAADP-AM as opposed to glucose illustrates that these responses are triggered by the compound, which takes time to cross the membrane at sufficient levels and be de-esterified; see Table 3.4.⁸

⁷NAADP is potent in the nanomolar range. However this refers to NAADP as added to the cell by microinjection or un-caging. As the present compound is the -AM form, and any potential response is dependent on the rate of uptake from extracellular space, this higher dose of NAADP-AM was recommended; Churchill and Bloor-Young, personal communication.)

⁸In previous studies, responses were seen at a delay of 15-20 min when using 60 nM NAADP-AM to stimulate β -cells (Arredouani et al., submitted for publication). The shorter delay in the present study with 10 μ M is consistent with the dose-related uptake of NAADP-AM by the cell.

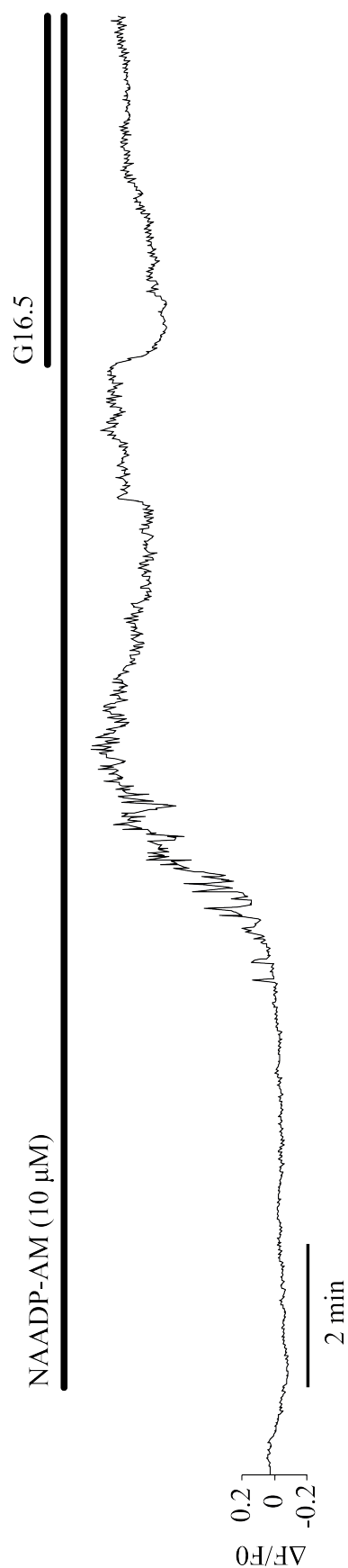


Figure 3.14: Representative Trace of a Calcium Response to 10 μ M NAADP-AM, as imaged using TIRF. Glucose was elevated to 16.5 mM at the Time indicated. Note the Lack of SERCA-‘dip’ in the NAADP-triggered Response; and its Presence upon Addition of Glucose.

		16.5 G	NAADP-AM (10 μ M)	Sig. (Student's t)
Total Cells		32	53	
Responding Cells		23	20	
PH	($\Delta F/F_0$)	1.8 ± 0.03	1.5 ± 0.04	NS; p=0.12
'Early' Responses		9	30	
TR	(s)	83 ± 2	307 ± 15	S; p < 0.01
Cells Showing Dip	(%)	35	0	
TD	(s)	19 ± 0.4	N/A	

Table 3.4: Effect of Stimulation with 16.5 mM Glucose or 10 μ M NAADP-AM. (%) is % of non-early, non-discarded cells.

3.8 β -Cell Calcium Responses can be diminished with rNed-19

Since it was possible to diminish calcium responses in β -cells with bafilomycin, and elicit them using NAADP-AM; the involvement of NAADP receptors was next investigated using the NAADPR antagonist Ned-19.

In the present study, commercially available trans-Ned-19 (tNed-19) was used initially (at a dose of 10 μ M). This neither eliminated previously elicited calcium responses; nor did it prevent calcium release in response to glucose after pre-incubation for 10-30 min (n=20, data not shown). This result was most surprising, since Ned-19 had been shown to inhibit β -cell calcium responses to glucose (Naylor et al., 2009;

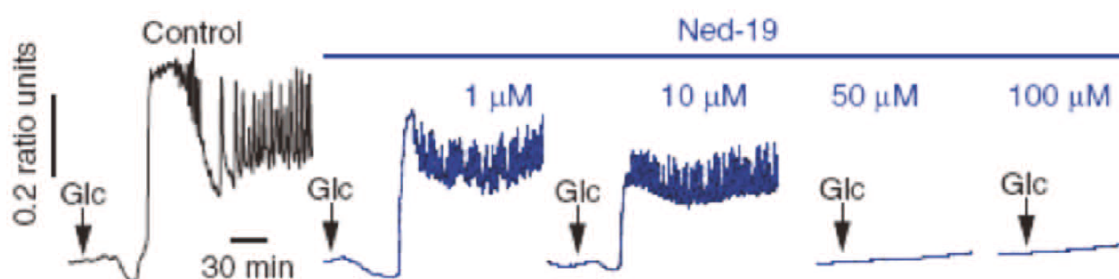


Figure 3.15: Glucose-induced Responses can be eliminated using rNed-19 (Reproduced from Naylor et al., 2009)

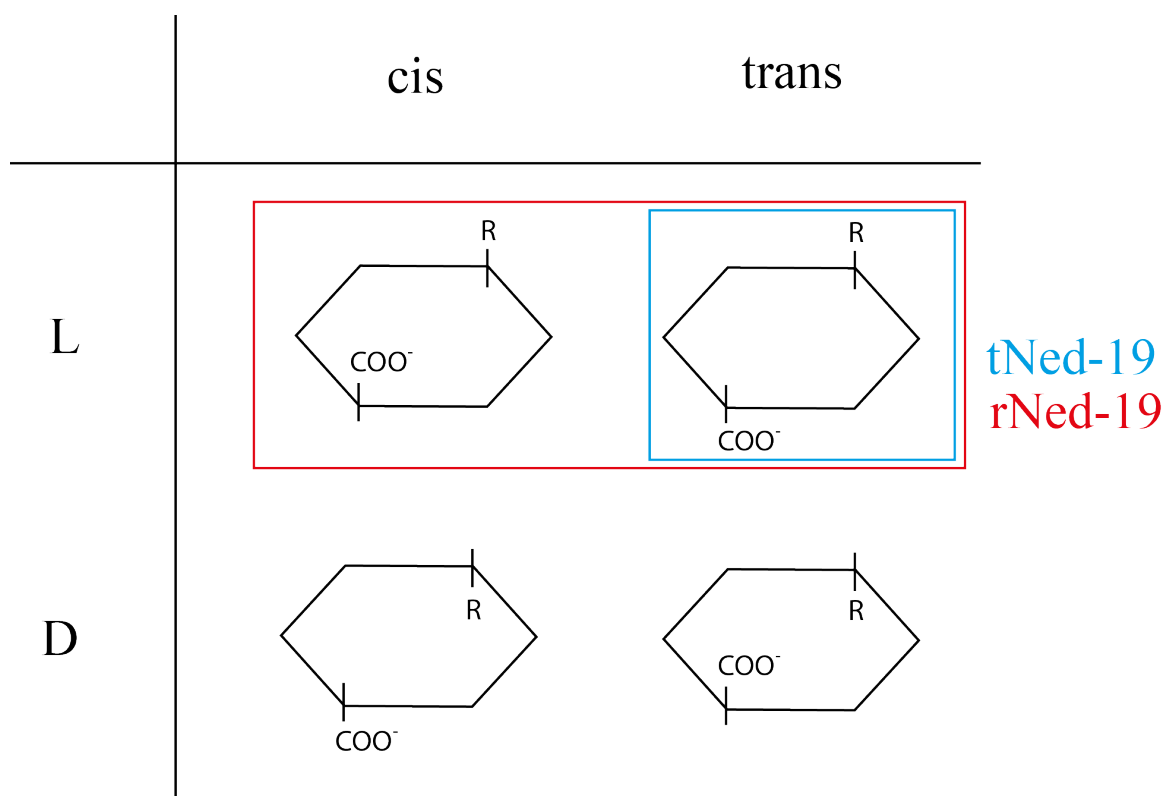


Figure 3.16: Illustration of the different Stereoisomers of Ned-19 using a schematic Molecule. L and D refer to the Tryptophan Isomers that the respective Ned-19 was synthesised from. The presently available Compounds were a racemic Mixture of cis- and trans- Ned-19 synthesised from L-Tryptophan (marked in red), as well as the commercially available pure trans-Ned-19 (marked in blue). The chemical Structure of these Molecules is shown in Fig. 3.18.

		Control	rNed-19 (100 μ M)	Sig. (Student's t)
Total Cells		43	29	
Responding Cells		27	9	
PH	($\Delta F/F_0$)	2.6 ± 0.02	0.6 ± 0.02	S; $p < 0.01$
'Early' Responses		14	3	
TR	(s)	38 ± 1	84 ± 2	S; $p < 0.01$
Cells Showing Dip	(%)	48	86	
TD	(s)	15 ± 0.2	25 ± 0.3	S; $p < 0.01$

Table 3.5: Effect of Inhibition with 100 μ M rNed-19. (%) is % of non-early, non-discarded cells. Note that those Cells that are listed as responding mainly produced Responses of Type 3 on a flat Baseline. In this Case the Amplitude of the largest Spike was taken for PH measurement. This was to ensure a conservative Estimate of Inhibition of Activity in the Presence of rNed-19.

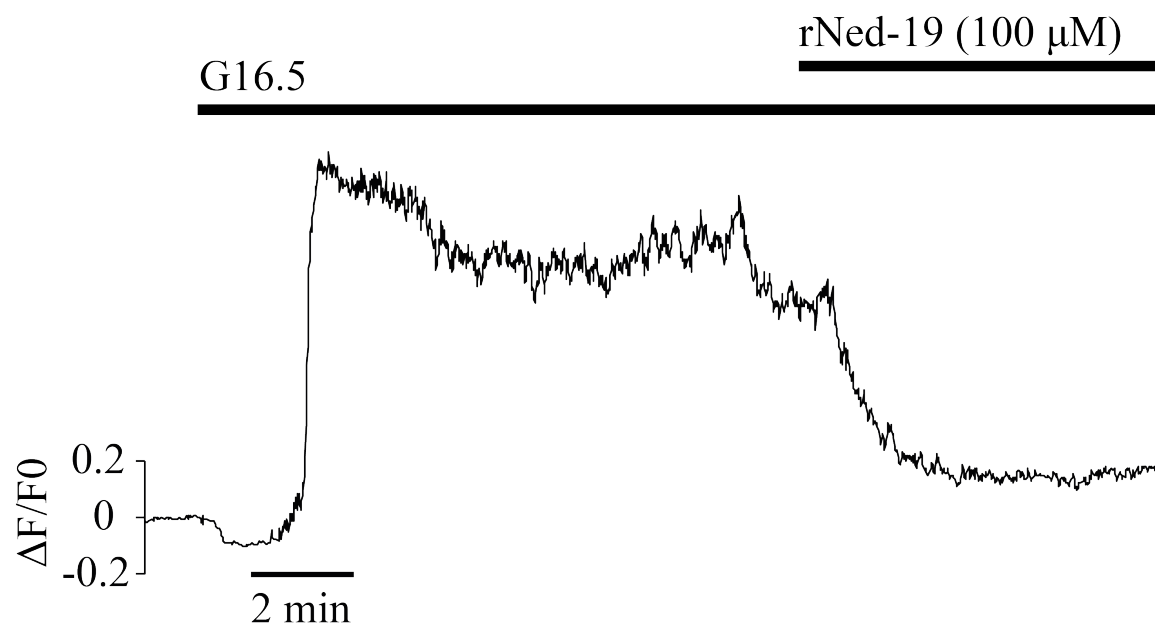


Figure 3.17: Representative Trace of a β -cell Calcium Response to Glucose, followed by Addition of rNed-19 at 100 μ M at the Time indicated.

Arredouani et al., submitted for publication), as shown in Fig. 3.15. However, Ned-19 exists as 4 stereoisomers (illustrated using a schematic molecular structure in Fig. 3.16). The efficacy of each isomer and different combinations of these appear idiosyncratic to particular tissues (Churchill, personal communication). It is the exception that publications reporting experiments involving Ned-19 (apart from those concerned with its initial synthesis) state which form of the compound was used. While tNed-19, the only form currently commercially available, is most common; all studies on β -cells to date have utilised the racemic mixture of Ned-19, consisting of its cis- and trans-stereoisomers. The separate concentration-effect curves of cis- and trans-Ned-19 are shown in Fig. 3.18. Thus it seems that while in some tissues, tNed-19 is much more potent than cNed-19 or rNed-19 (the concentration-inhibition curve of which lies between the former two); in β -cells, tNed-19 showed no effect at the recommended concentrations.

When experiments were repeated with 100 μ M rNed-19, as was used in previous studies; the above mentioned diminution of glucose-induced calcium responses could

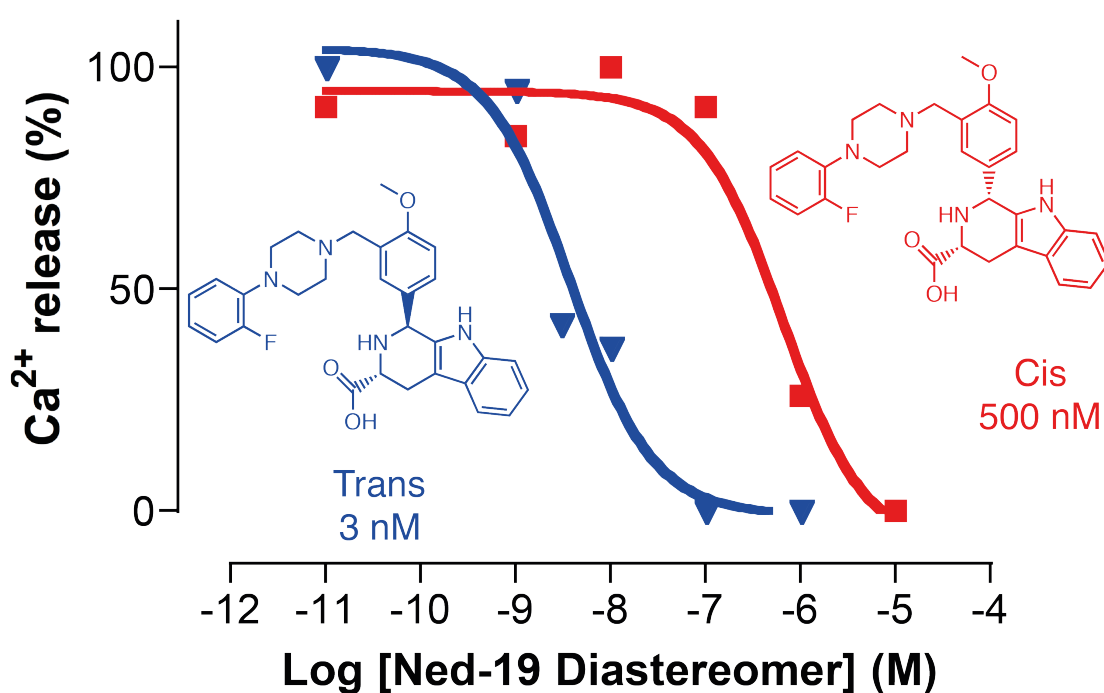


Figure 3.18: Stereoisomers of Ned-19 and their Concentration-Inhibition Curves for Calcium Release from Sea Urchin Egg Homogenate (*L. pictus*). Below their molecular Structures, the respective IC_{50} Values for the two Compounds are shown. (Reproduced with kind permission from G. Churchill, unpublished)

be replicated; see Fig. 3.17. When cells were pre-incubated for 10 min with rNed-19, glucose-induced responses were largely abolished ($n=29$), as shown in Table 3.5. Note that even the few remaining cells listed as ‘responding’, mainly showed responses of the type 3 classification- short transients on a flat or marginally sloped baseline. This is in marked contrast to control cells, where this sort of behaviour is very rare (see Fig. 3.6). Additionally, the remaining responses were significantly delayed as compared to control responses (Table 3.5).

Interestingly, at concentrations where rNed-19 was shown to eliminate calcium responses ($50\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$; see Fig. 3.15), tNed-19 diminished calcium responses, also (pilot studies; not quantified). The inefficacy of tNed-19 at concentrations chosen on the basis of what should be its IC_{50} , but efficacy at high concentrations has also been observed for electrophysiologically measured responses to glucose addition. Currents recorded by patch clamp on individual mouse β -cells were unaffected by concentrations of tNed-19 chosen on the basis of what should be its IC_{50} ; but could be diminished with $100\text{ }\mu\text{M}$ tNed-19 (Soanes, personal communication).

Thus it seems that either the concentration-inhibition curve for tNed-19 is shifted by orders of magnitude for β -cell calcium release to glucose; or cells react to a contaminant in tNed-19 (possibly cis Ned-19). It could, theoretically, also denote a general inefficacy of Ned-19 in β -cells (for lack of its target NAADP receptor or similar), and the effects observed purely nonspecific inhibition. However as rNed-19 has been tested across many preparations (at concentrations between 100 nM and $100\text{ }\mu\text{M}$) and produced a robust and specific effect (see Chuang, 2011, for review), as well as the fact that the cis- isoform of Ned-19 on its own is efficacious in other tissues (for example endothelial cells; Esposito et al., 2011); this is very unlikely.⁹ A possible reason for the stereoisomer ‘selectivity’ of tissues is that Ned-19 may need to be transported across the membrane. Thus its efficacy would depend on the expression of the relevant transport mechanism.

⁹Although it has been reported that cNed-19 can block L-type calcium channels in cardiac myocytes; Terrar, personal communication).

3.9 Conclusions

The above characterisation of calcium responses is by no means exhaustive, and many more parameters could be used to describe and classify them. With increasing knowledge of the underlying processes, the two could eventually be combined and responses characterised by origin. As things stand, the following conclusions can be drawn from the above experiments:

Firstly, β -cells are somewhat ‘volatile’ as experimental specimen, and do not lend themselves to being studied as a ‘model cell’ for the investigation of intracellular signalling. However, with large enough groups (see for example Johnson and Misler, 2002) and a thorough investigation of their ‘baseline’ behaviour, it is possible to draw conclusions about it from experiments with them.

It is worth remembering in this context that the present experiments were not conducted with 0 mM glucose at baseline, but with 3 mM glucose. This means metabolism and consequent insulin secretion were taking place. And while some of the mentioned ‘early’ responses will be the result of thermosensitivity, photosensitivity or culture conditions; this in itself might be a demonstration of the natural behaviour of β -cells. β -cells’ thermosensitivity, presently a confounding factor, could be an important part of their physiology. It is known that different members of the TRP channel family are expressed in β -cells (see for example Colsoul et al., 2010, for review); many of which are temperature-activated (Uchida and Tominaga, 2011). Some have been demonstrated to be involved in insulin secretion and are activated specifically at body temperature (Togashi et al., 2006). Since heat is generated by metabolism, and insulin secretion is known to be temperature dependent, it has even been suggested that heat might act as a second messenger in β -cells (Islam, 2010).

In addition to the challenges presented by the nature of the cells under investigation, the two types of channel mediating the main calcium components that were being assessed, TPCs and L-type calcium channels, are structurally related, and the unspecific binding of either of their main antagonists, Ned-19 and nifedipine, tends to be on the

other. This may need to be considered in the interpretation of any results obtained with those agents.

Because of the high level of spontaneous activity and the variability in responses (previously also described by Rojas et al., 1994 and references therein), pharmacological studies of β -cells proved difficult. However, it was demonstrated that:

1. Responses are eliminated by the removal of extracellular calcium.
2. Glucose-induced responses can be inhibited with Nifedipine, Bafilomycin, and rNed-19.
3. Responses to a similar magnitude as triggered by glucose can be elicited with NAADP-AM.

This replicates results from previous studies using confocal microscopy (Naylor et al., 2009; Arredouani et al., submitted for publication) with TIRF microscopy. The striking and unexpected difference between the racemic mixture and the homochiral form of Ned-19 highlights the importance of considering the different stereoisomers a drug can take and that their actions may be very different. It also raises the question of how Ned-19 crosses the cell membrane, and whether it is membrane transporters that ‘select’ the stereoisomer of Ned-19 for a cell, TPCs themselves, or potential binding proteins.

Further to the characterisation of responses in general; it was shown that using the current experimental setup, TIRF adds spatial resolution to the imaging of β -cells, but does not detect any calcium signals that precede or are not discerned by the epifluorescence recording of whole-cytosol calcium.

Chapter 4

Effects of TPC Knockout on β -Cell Structure and Function

4.1 Introduction

It was shown in previous studies that knockout of TPC2 affects NAADP- and glucose-induced calcium signals in β -cells, as well as NAADP-evoked whole-cell calcium currents (Naylor et al., 2009; Calcraft et al., 2009; Arredouani et al., submitted for publication); see Fig. 4.1 and Fig. 4.2. Specifically, it was shown that glucose-induced whole cell calcium responses were diminished in β -cells from TPC2 KO mice (see Fig. 4.2). The present investigation sought to further characterise the effects of TPC2 KO on calcium responses at the sub-membrane level using TIRF; as well as study the recently developed double knockout (TPC1^{-/-} TPC2^{-/-}, DKO) mice.

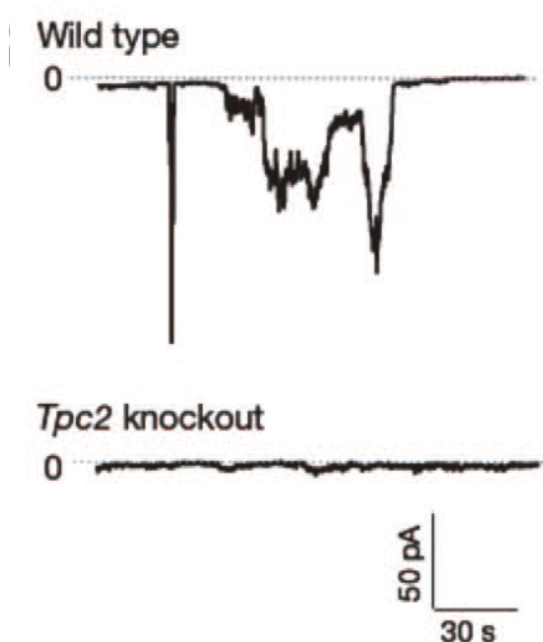


Figure 4.1: Calcium Currents in response to 100 nM NAADP in the Patch Pipette are abolished in TPC2 KO Mice. (Reproduced from Arredouani et al., 2010).

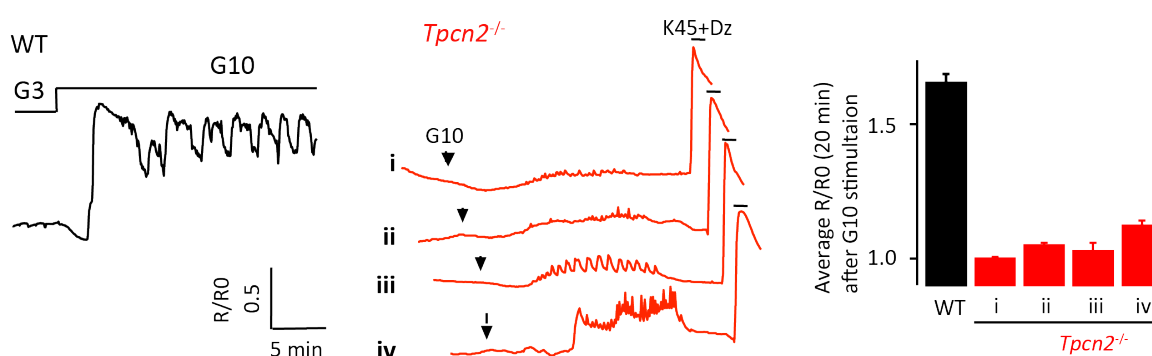
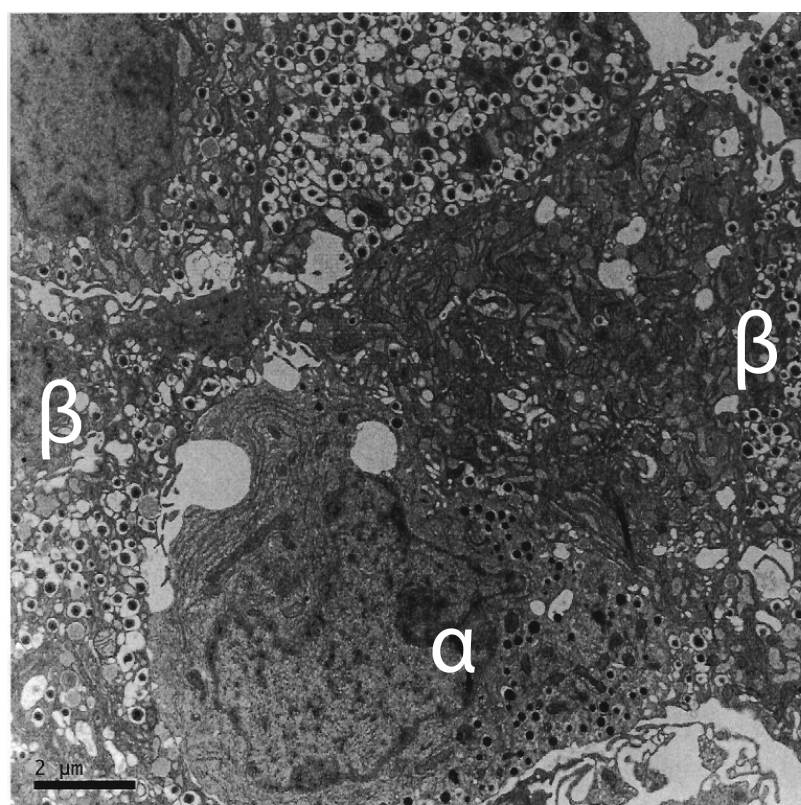


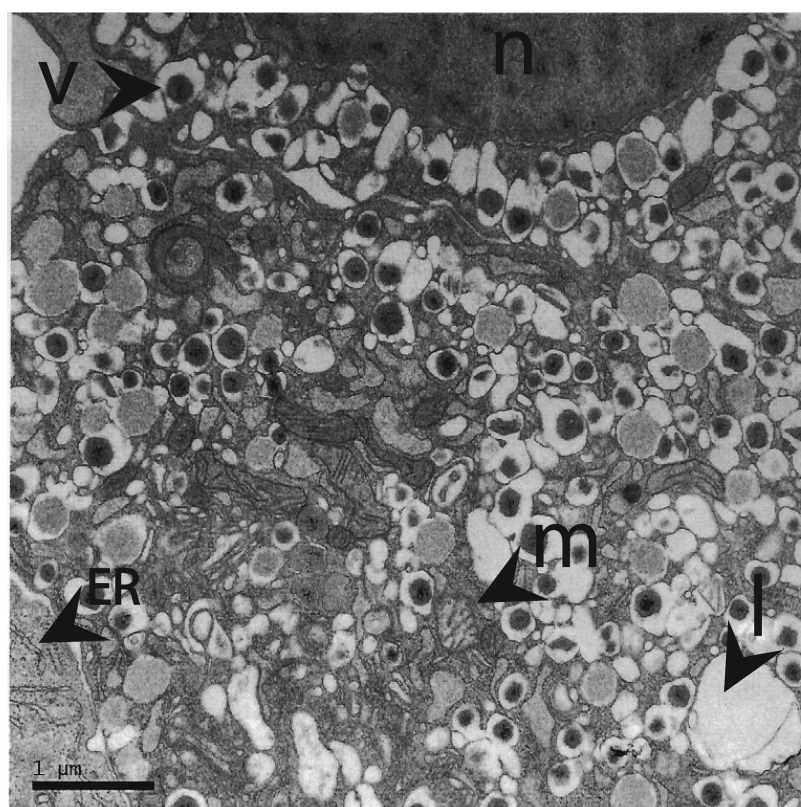
Figure 4.2: Calcium Responses to 10 mM Glucose are diminished in TPC2 KO Mice. The mean elevated Ratio (a normalised Measure of the Amplitude of the Plateau that Calcium reaches post-Phase 1) was measured for WT and TPC2 KO Mice in Response to Stimulation with 10 mM Glucose. Mean Elevated Ratio for 20 min after Onset of Response was measured and found to be significantly decreased in TPC2KO Mice (Reproduced from Arredouani et al., submitted for publication).

4.2 Assessing the Effect of TPC Knockout on Gross Morphology of β -Cells

Initially, electron micrographs were acquired to give confirmation of the micro-anatomical intactness of the islets and β -cells of the knockout mice. From the islet isolation procedure it was known that no obvious changes in the gross anatomy of the pancreas or the pancreatic duct were present in either of the knockout mice. The images below illustrate that after an initial assessment the same appears to hold true for the histological structure of the islets. A thorough assessment of islet (and specifically β -cell) morphology could be conducted on pancreata of TPC knockout mice if there was reason to suspect fundamental histological changes. One might wish to consider the proportion of α - and β -cells within the islets. Sub-cellularly, the number of docked insulin containing vesicles in β -cells, the proportion of the cell taken up by vesicles and/or lysosomes, as well as the presence and appearance of cell-cell contacts might be studied (see for example Lacy and Hartroft (1959); Lacy (1961); Dean (1973) for descriptions and illustrations of such measurements).



(a) TPC2 KO α and β Cells at 12300 x Magnification



(b) TPC2 KO Cell Organelles at 29500 x Magnification

Figure 4.3: Histology of TPC2 KO Islets. Images depicting whole α and β Cells (a) and β Cell Organelles (b). α , α -Cell; β , β -Cell; n, Nucleus; ER, Endoplasmic Reticulum; v, Insulin containing Vesicle; m, Mitochondrion; l, Lysosome.

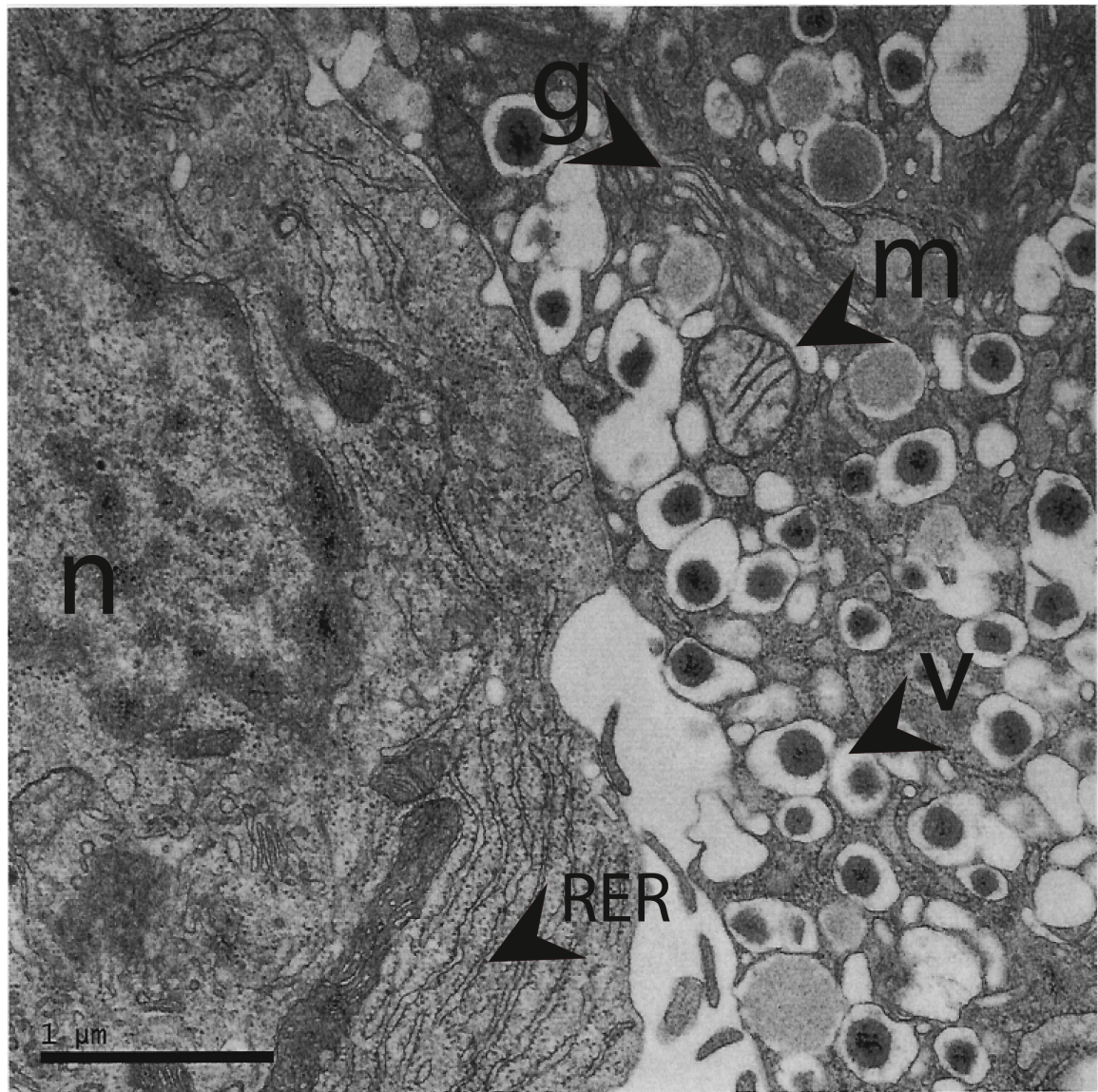
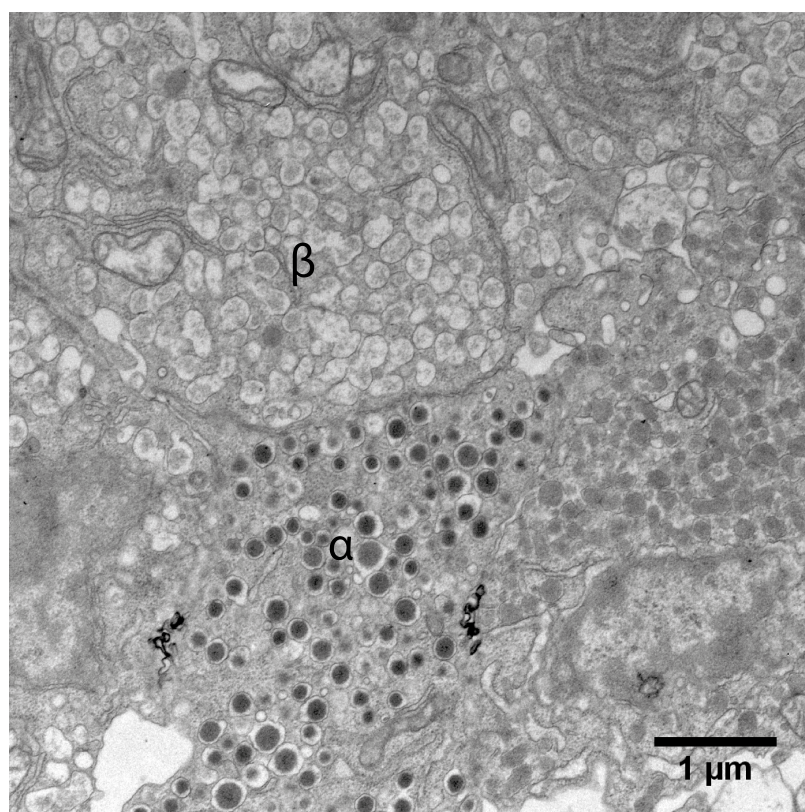
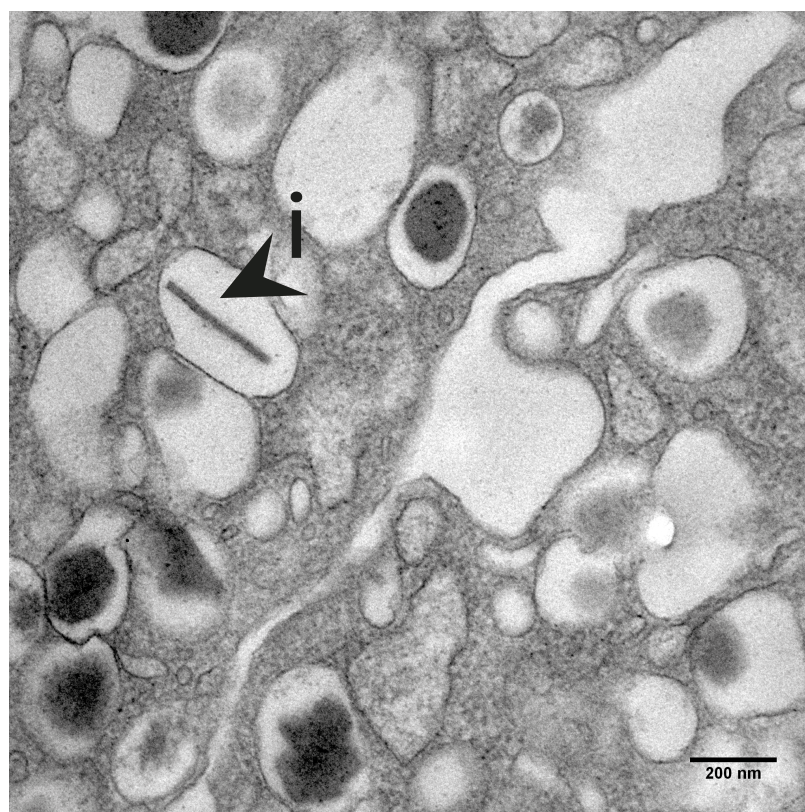


Figure 4.4: *TPC2 KO β -Cell (right) Sub-structure at 41500x Magnification. Cell-Cell Contact with Insulin-containing Vesicles docked at the Membrane. n, Nucleus; g, Golgi; RER, Rough Endoplasmic Reticulum; v, Insulin containing Vesicle; m, Mitochondrion.*



(a) DKO α and β Cells at 12300 x Magnification



(b) DKO Insulin-containing Vesicles at 54600 x Magnification

Figure 4.5: *Histology of DKO Islets. Images depicting α and β cells (a) and Insulin containing Vesicles (b) with characteristic Insulin Crystallisation. α , α -Cell; β , β -Cell; i, crystallised Insulin.*

4.3 Effect on Response Type Distribution

β -cell responses to 16.5 mM glucose were assessed for their response types as established in Chapter 3. This data is presented in Fig. 4.6. It should be noted that for the purposes of this chapter; data from ‘non-matching’ experimental days were pooled, because it was not feasible to study 8 different experimental groups in any one day. This necessarily means that there is more variability in the presented data, and there may be ‘seasonal’ differences presenting as group-effects because different groups of animals were tested at different points during the project.

From the qualitative data of the response type frequencies presented in Fig. 4.6, it appears that mice of the two different genetic backgrounds show different response patterns. Mice from background 2 (on which the TPC2 KO mouse was generated) show many more oscillatory responses (type 2), than do mice of background 1 (on which the DKO mouse was generated). There are also less ‘early’ responses among cells from the former. Although, as stated above, order-effects of experiments may contribute to the occurrence of these results.

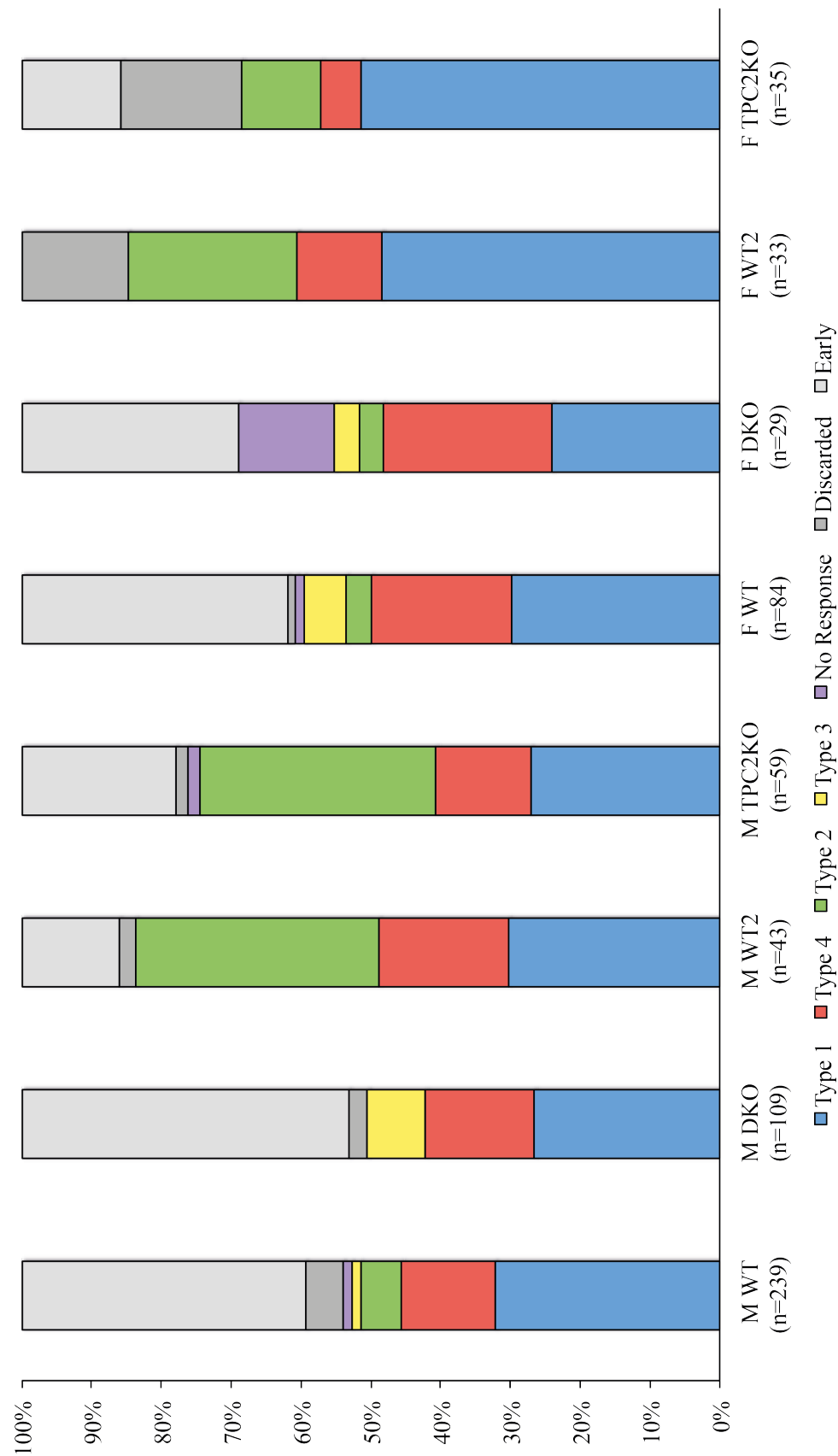


Figure 4.6: Response Type Frequency Distribution in TPC Knockout Mice. WT, Wild Type; WT2, Wild Type on Background 2; DKO, Double Knockout; TPC2KO, TPC2 Knockout; M, Male; F, Female. Response Types as Characterised in Chapter 3.

4.4 Effect on Response Amplitude and Time to Response

Akin to experiments in Chapter 3; measurements of peak height (PH) and time to response (TR) were made. Their respective distributions across the different genotypes can be seen in Fig. 4.7 and Fig. 4.8. In order to assess these data; a separate univariate ANOVA was conducted for each dependent variable (PH and TR). Besides determining the effects of knocking out TPC2 or both TPC1 and TPC2 (DKO) and the measured parameters, the analysis assessed the significance that gender and genetic background have on these (both in their own right, and in interaction with knockout of TPC). The results of these analyses are discussed below.

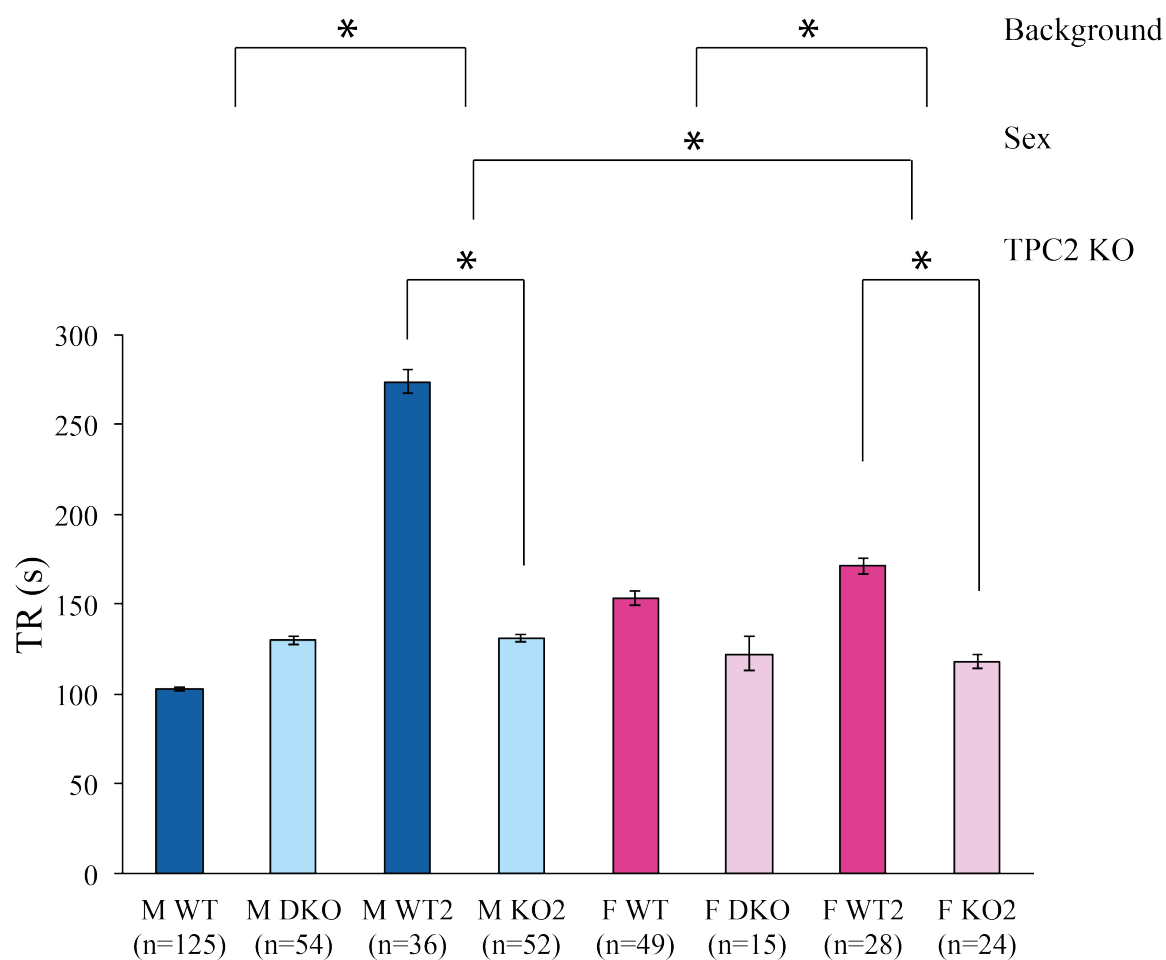


Figure 4.7: Mean Time (s) to Response (TR) of (16.5 mM) Glucose-induced Responses in TPC Knockout Mice. WT, Wild Type; WT2, Wild Type on Background 2; DKO, Double Knockout; TPC2KO, TPC2 Knockout; M (blue), Male; F (pink), Female; bright Colours, wild Types; pale Colours, Knockouts.

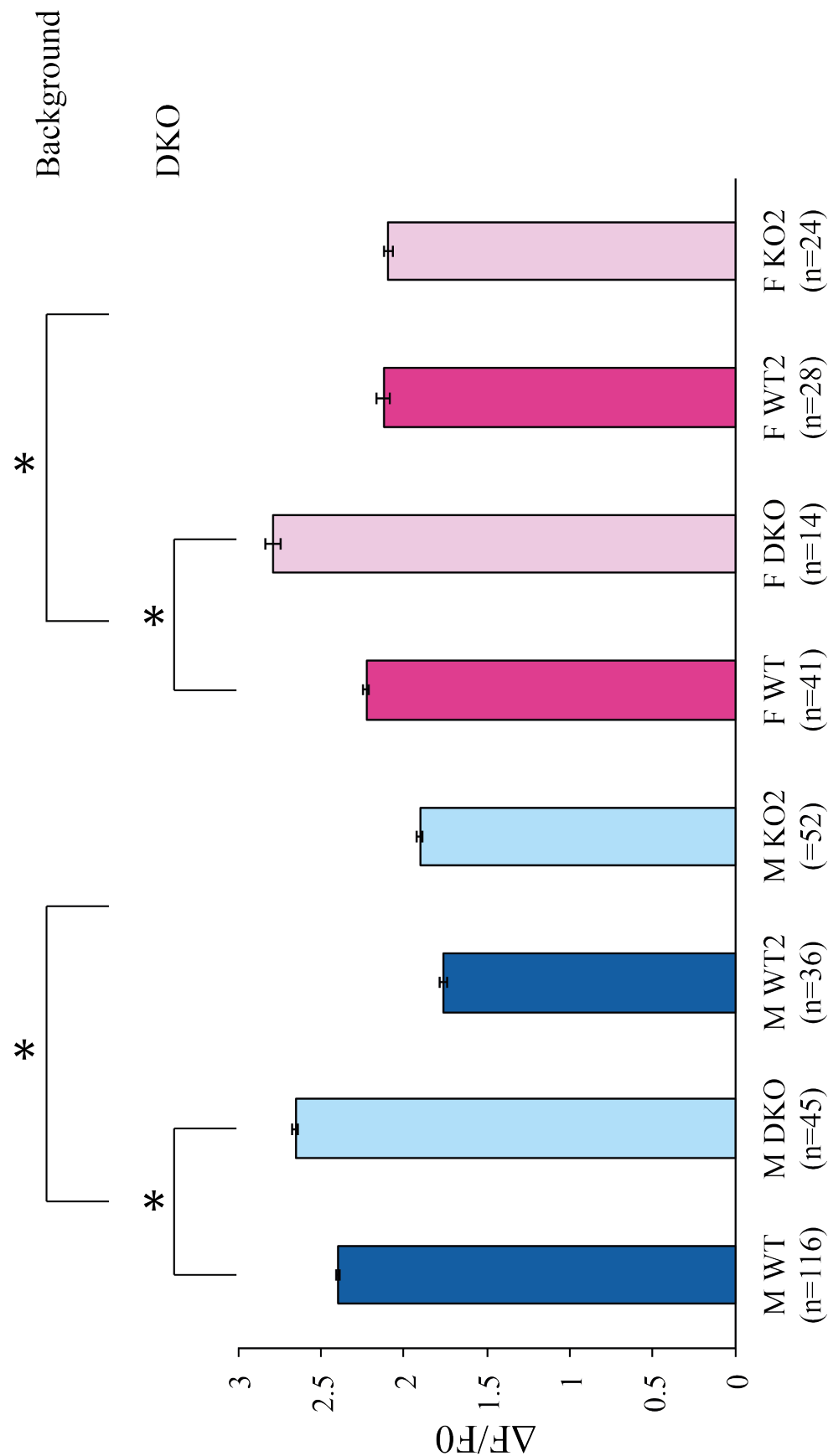


Figure 4.8: Mean Peak Height of (16.5 mM) Glucose-induced Responses ($\Delta F/F_0$) in TPC Knockout Mice. WT, Wild Type; WT2, Wild Type on Background 2; DKO, Double Knockout; TPC2KO, TPC2 Knockout; M (blue), Male; F (pink), Female; bright Colours, wild Types; pale Colours, Knockouts.

4.4.1 Effect of TPC2 Knockout

When analysing the time to response, it was found that TPC2 KO cells responded significantly faster than WT controls; $F(1, 136)=13.171$, $p < 0.01$). There was also a significant effect of gender, $F(1, 136)=4.608$, $p < 0.05$, within this group (i.e. background 2; TPC2 KOs and their respective WTs). This can be clearly seen in Fig. 4.7. This effect showed no interaction with the variable genotype. Thus with respect to the reaction time to glucose, there is a fundamental sex-difference within the genetic background that the TPC2 KO was generated on. This does not change with knocking out TPC2 ($F(1, 136)=2.765$, $p=0.099$). When analysing the peak height of glucose responses in TPC2 KOs, it was found that there was no significant effect of genotype ($F(1, 136)=0.115$, $p=0.753$); nor was there a significant effect of gender ($F(1, 136)=3.143$, $p=0.078$).

Thus in the case of TPC2 KOs, it appears that their reactions to glucose are not diminished compared to WT animals, as described previously (Arredouani et al., submitted for publication). Instead, there appears to be a paradoxical decrease in the response time to glucose for both males and females. Males of this genetic background react significantly slower than females; both in the WT as well as the TPC2 KO group. While there may be experimental caveats explaining the observed sex difference (males and females from background 2 were never tested on the same day); to observe the same effect of TPC2 KO in males and females in separate experiments suggests that this is a real effect of gene knockout.

The fact that TPC2 KO mice appear to react faster to glucose than WT mice but showed no difference in the peak height of their responses as compared to WT animals was surprising, given previous experimental data. A possible explanation for the lack of effect of TPC2 KO on peak height is given below. The decreased response time in TPC2 KO animals (a variable not assessed in previous experiments) is an interesting phenomenon, because according to Nunemaker and colleagues, idiosyncratic faster onset of calcium responses of whole islets is associated with faster release of insulin

(Nunemaker et al., 2005). It will be interesting to see, therefore, what the insulin secretion pattern of TPC2 KO mice is. Pilot studies have been conducted suggesting an increase in insulin release from isolated islets of TPC2 KO mice (Galione, personal communication).

One plausible explanation of such apparently paradox effects of knocking out one of the TPCs is the compensatory over-expression of the other TPC leading to an enhanced response. This has been shown to occur in hepatic tissue of TPC2 KO animals (Chuang, 2011).

4.4.2 Effect of Double Knockout

In order to circumvent potential compensatory over-expression of the second TPC, the recently generated TPC double knockout (DKO) mice were investigated for the same parameters. When analysing TR for DKO mice, it was found that there was no significant effect of genotype ($F(1, 212)=0.008$, $p=0.929$); nor a significant difference between males and females ($F(1, 212)=0.908$, $p=0.342$). Thus there was no significant difference in the response times to glucose of both male and female DKO mice compared to WT mice. When analysing the peak height for glucose-induced calcium signals in DKO mice; it was found that they had significantly larger responses than controls ($F(1, 212)=7.184$, $p<0.01$); and that there was no sex difference for this effect ($F(1, 212)=0.908$, $p=0.342$).

Thus in DKO mice; calcium responses to glucose did not change in their latency, but they increased significantly in amplitude compared to the responses of WT controls. This, again, is a most unexpected finding given previous experiments on TPC single knockout animals as well as the data presented in Chapter 3. It is known, however, that apart from over-expressing their TPC counterpart; TPC knockout mice up- or down-regulate the expression of many other genes. For the TPC2 KO mouse, Chuang (2011) found a change in expression of 140 different genes; suggesting that

compensation may occur by other genes as well as the over-expression of the remaining TPC.

4.5 Sex Differences in Responses

As it has been described previously that the expression of both TPC1 and TPC2 is sex dependent in liver (Chuang, 2011); observing sex differences in effects of TPC2 KO on β -cells was not unexpected. However, the interpretation of the presently observed sex difference in genetic background 2 should not be overemphasised because, as explained above, the population data analysed may have confounded the results in that regard.

4.6 Differences in Genetic Background

As mentioned previously, the use of the present knockout animals was somewhat limited by the fact that the two different knockouts were on different genetic backgrounds; requiring two separate WT control groups. This makes between group comparisons very difficult. In the present study, there was a significant main effect of genetic background in both the ANOVA for time to response ($F(1, 379)=5.659$, $p < 0.05$) and the ANOVA for peak height ($F(1, 352)=18.744$, $p < 0.01$). This illustrates the importance of comparing knockout results only with respect to their genetic background; and may well be the reason for the disparate results between the present study and previous experiments (Naylor et al., 2009; Arredouani et al., submitted for publication), which had used TPC2 KO mice which were rederived for housing in Oxford's new biomedical facility. The importance of the genetic background, or in this case the environment that the mice were in, has been demonstrated with respect to pancreatic cells. Indeed, one of the first reports of the effect of genetic background on gene expression was based on the observation that KO-induced diabetes was transient

on one background, but overt on another (Coleman and Hummel, 1973; Baetens et al., 1978).

4.7 Effect on Phenotype

At present, very little data has been collected on the behaviour and physiology of the TPC knockout mice. When they were originally generated, it had been suggested that the DKO of TPC might not produce a viable animal; as TPCs are ubiquitously expressed (Calcraft et al., 2009; Zong et al., 2009; Schieder, 2011) and involved in many key cellular processes (see Hooper and Patel, 2012 for review). This, however, was not the case, and in itself might suggest that the importance of TPCs lies in their interaction with other molecules. This again is a possible explanation for the different results observed with the different genetic backgrounds.

4.8 Using NAADP-AM on Knockout Mice

As the KO mice reacted in such an unexpected way, stimulation with NAADP-AM instead of glucose was attempted in male and female DKO mice. If NAADP bound to TPC directly, to no other target, and there were no compensation of any kind; responses should be abolished. If, on the other hand, NAADP bound via a binding protein, bound to multiple targets, or compensation via another protein was taking place; then responses might still be present. Note that because these experiments were conducted *post hoc*, the mice were not matched to DKOs of the same experimental run; but the population DKO response for males and females was used for comparison using Student's t-tests. The results of the experiment are presented in Table 4.1. As can be seen; there is no significant difference in TR for DKO mice stimulated with glucose and those stimulated with NAADP-AM. Neither for the males nor for the females did reaction time significantly change ($p=0.45$ for males, $p=0.87$ for females).

		M DKO 16.5G	M DKO NAADP-AM	F DKO 16.5G	F DKO NAADP-AM
Total Cells		109	12	29	13
Responding Cells		55	3	20	9
PH	($\Delta F/F_0$)	2.65 ± 0.02	1.98 ± 0.2	2.78 ± 0.05	1.37 ± 0.08
‘Early’ Responses		51	7	9	4
TR	(s)	129 ± 2	180 ± 16	122 ± 9	113 ± 7

Table 4.1: *Effect of Glucose or NAADP-AM Stimulation on DKO Mice.*

In PH, however, there was a significant decrease in the response amplitude for females, but not males ($p=0.66$ for males, $p<0.01$ for females). This, however, could be a sampling error, as discussed previously. The observation that the response times did not change in any of the groups is encouraging; as this indicates that the observed responses are most likely spontaneous rather than triggered by NAADP-AM. When calcium signals in response to NAADP-AM were observed in WT mice in Chapter 3, these were characterised by a highly significant increase in reaction time (due to de-esterification and compound accumulation within the cell being a slow process).

4.9 Conclusions

The study of the calcium responses to glucose of TPC knockout mice proved disappointing. Given previous results with NAADP-AM, rNed-19, and bafilomycin (see Chapter 3); as well as previous studies of these responses in TPC2 KO animals (Naylor et al., 2009; Arredouani et al., submitted for publication), it was expected that there would be some inhibition in both TPC2 KO and DKO mice.

It is not inconceivable that mice of different genetic backgrounds could produce very differently shaped calcium responses to glucose. Patterns of response have been shown to be idiosyncratic to particular islets, pancreata and individual animals (Pakhtusova et al., 2003; Nunemaker et al., 2005; Gustavsson et al., 2006). Thus observing a difference between WT mice from the different genetic backgrounds is

conceivable; even without considering the interaction between the knocked out gene and the respective genetic background.

For the purposes of further investigating TPCs it would thus be important to have the different types of KOs on the same, pure genetic background, in order to be able to compare them (and be able to use the same control group for both). It will be worth exploring whether the presently observed patterns persist when the knockout is moved onto a pure background. The results of glucose tolerance tests on the TPC knockout animals, currently in progress, will also be most interesting with respect to the observed increases in reaction time and peak height observed with TPC knockout.

Chapter 5

Elementary Calcium Events in β -Cells

5.1 Introduction

If stimulus-secretion coupling in β -cells relied initially on the release of calcium from intracellular stores rather than the closure of K_{ATP} channels; it should be possible to demonstrate the existence of elementary release events from these stores independent of calcium influx across the membrane. These kind of events have already been demonstrated in various cell types in response to the messengers IP_3 and cADPR in the form of puffs and sparks. The experimental results presented below suggest a possible equivalent event triggered by NAADP, which also occurs in response to stimulation with glucose in pancreatic β -cells.

5.2 Elementary Events are triggered by NAADP and Glucose

To visualise elementary calcium release in pancreatic β -cells, the global responses discussed in Chapters 3 and 4 must be eliminated, as they would mask any local events. To do this, cells were pre-incubated in recording medium (see Table 2.1) containing 1.7 mM calcium, 3 mM glucose, and 1 μ M thapsigargin. The pre-incubation with

thapsigargin was found to be imperative, as SERCA activity in β -cells is large and may obliterate any small amplitude calcium signals if activated (this point is further discussed towards the end of this chapter). Immediately before recording was started, the extracellular fluid was exchanged for calcium-free medium. This calcium reduction immediately before experimentation, rather than pre-incubation without calcium, was necessary as it was discovered that intracellular calcium stores run empty rapidly. Thus any experiment designed to investigate intracellular calcium release but not extracellular influx needs to be conducted in calcium free medium, but immediately after addition of the same. The change to calcium-free medium leads to an initial decrease in fluorescence across the cell, as free cytosolic calcium exits the cell and is no longer replenished. This process lasts around 30 seconds, when a new, lower, baseline fluorescence is reached. After a further 30 seconds of baseline recordings, cells were challenged with either NAADP-AM or an increase in extracellular glucose. At the end of every experiment, extracellular calcium was re-added to the bath to visualise a global response as a verification of the cell's viability.

5.2.1 Stimulation with NAADP-AM

Over the ~ 10 min recording period following addition of 100 nM NAADP-AM, cells showed clear localised calcium signals with amplitudes up to and above $2 \Delta F/F_0$. This is comparable in amplitude to calcium sparks observed with IP_3 , (Smith and Parker, 2009). The attached CD-Rom includes the very first recording of these events (Movie 2).¹ Examples of the individual calcium events observed are shown in Fig. 5.1. As can be seen, some events involve large areas of the cell (some include almost the entire cell), and some events are much smaller. The localised nature of the events in contrast to baseline levels of calcium, and, at the other extreme, global calcium influx, is illustrated in the 3D fluorescence intensity plots shown in Fig. 5.2.

¹The movie is of one β -cell cluster at 100x magnification, stimulated with 100 nM NAADP-AM in calcium-free medium. At the end of the recording, calcium is re-added to the bath, and a global calcium influx can be seen.

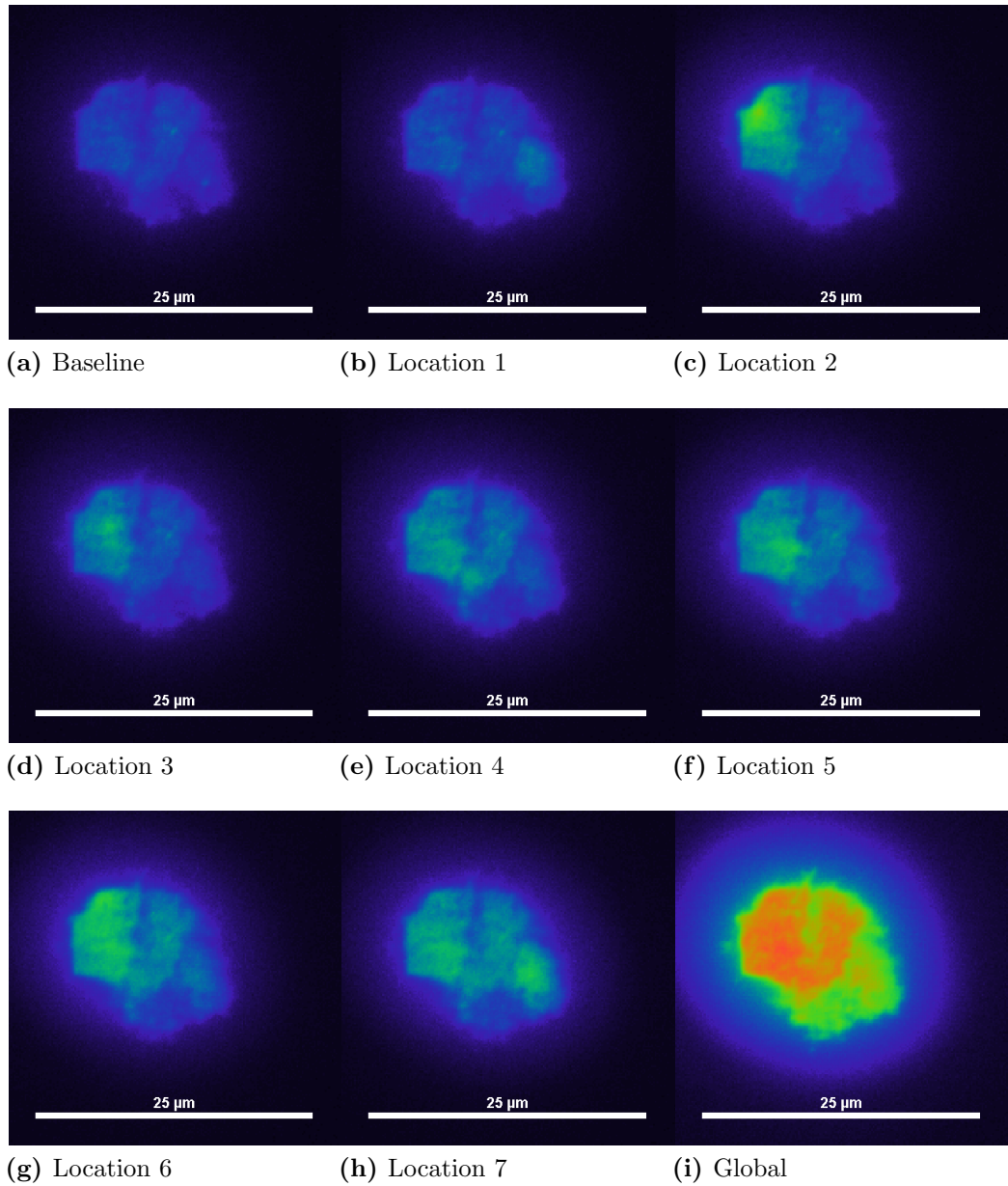


Figure 5.1: Localized Calcium Release after NAADP-AM Addition in a β -cell Cluster (3 Cells) as observed in TIRF. For Illustration Purposes, larger Events were selected. (a) shows Baseline Fluorescence, (i) shows Global Calcium Influx across the Membrane.

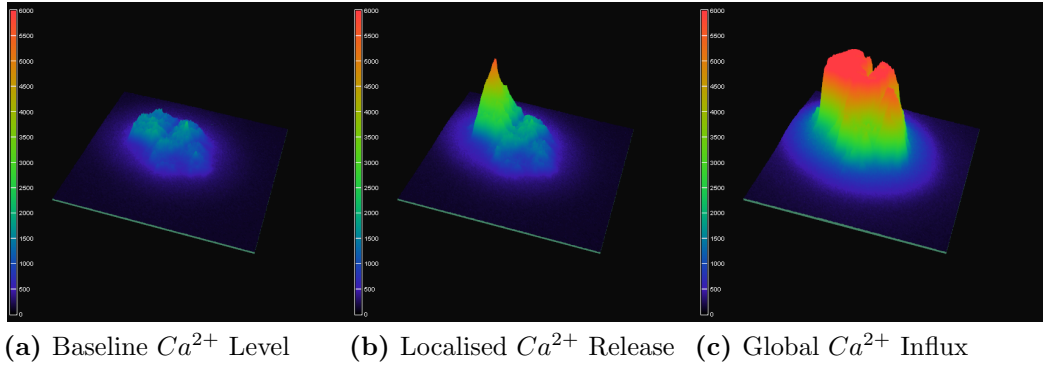


Figure 5.2: *Intensity Profiles of a β -cell Cluster at Baseline (a), in the Presence of an Elementary Calcium Event (b), and during Global Calcium Influx (c).*

The maximum $\Delta F/F_0$ can be plotted against time for any particular cell; as illustrated in Fig. 5.3, showing how the frequency of calcium events increases with time, settles to a plateau, and then decreases again. This is in accordance with the self limiting nature of these types of signals when stores cannot be replenished and stimulation remains (Cheng and Lederer, 2008). The Inlay of the Figure is a magnification of a range of calcium events of different sizes. Because the acquisition rate was 3.3 Hz, there is only ever one frame (one point on the graph) for each event.

Individual release sites within a cell can be visually identified and selected (see Fig. 5.4, for an example) and the fluorescence in those areas analysed independently. Fig. 5.5 shows the simultaneous fluorescence changes in seven different locations within one cell. As can be seen, the different areas react idiosyncratically, and they each show a variety of different spike amplitudes, the maxima of which are equally variable.

To assess whether there is a significant difference between the amount of spiking observed before and after the addition of NAADP-AM, the mean percentage of frames showing at least one spike (defined as an event 2 SDs above mean baseline $\Delta F/F_0$) were determined for the baseline and NAADP-AM conditions. The result can be seen in Fig. 5.6: NAADP-AM leads to a tenfold increase in spike frequency.

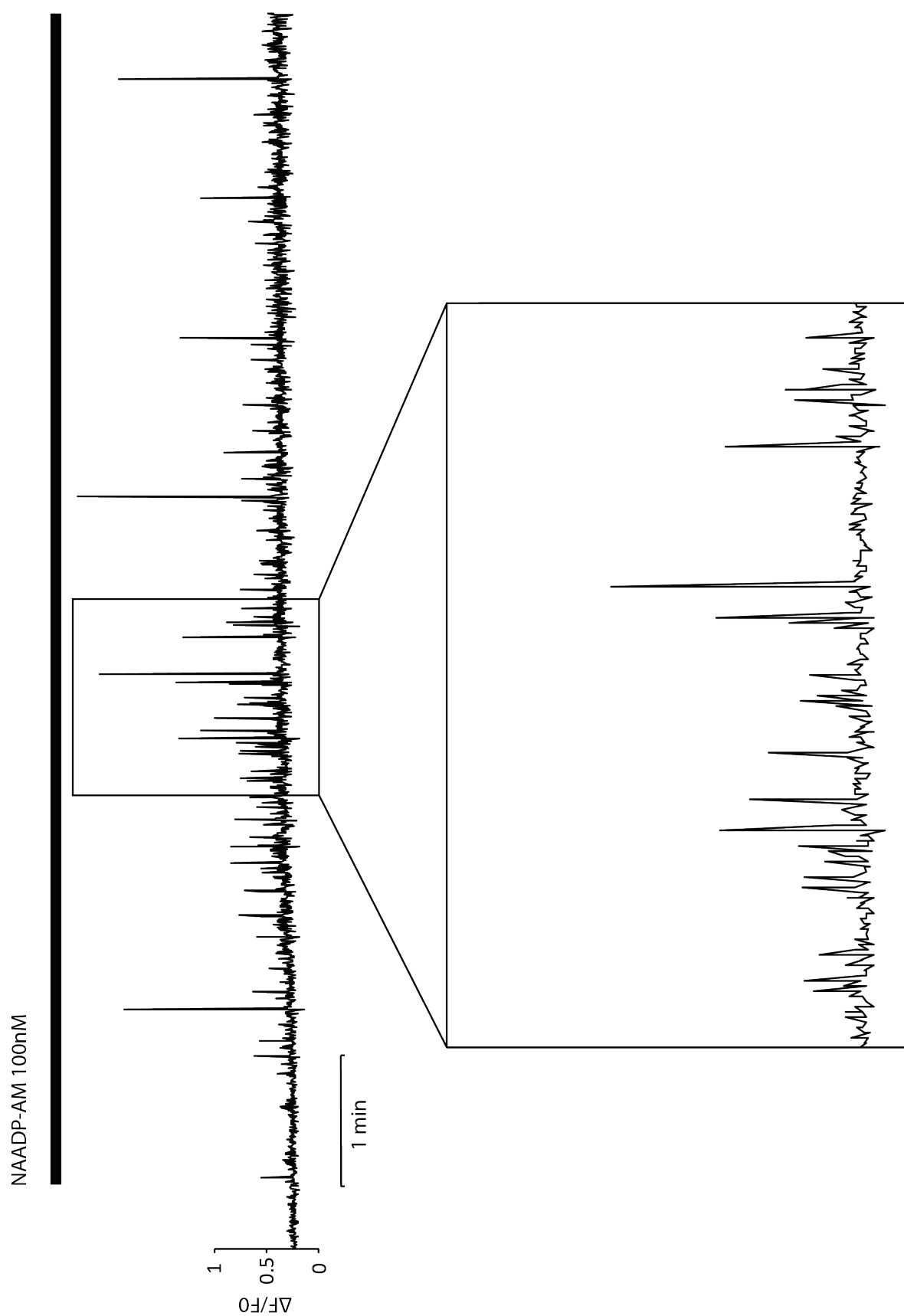


Figure 5.3: Representative Trace of a β -cell showing Elementary Calcium Events triggered by NAADP-AM (100 nM). Inlay shows a Magnification of Individual Events of different Amplitudes. Maximum Intensity Change of subsequent Frames after normalising to Baseline is plotted against Time.

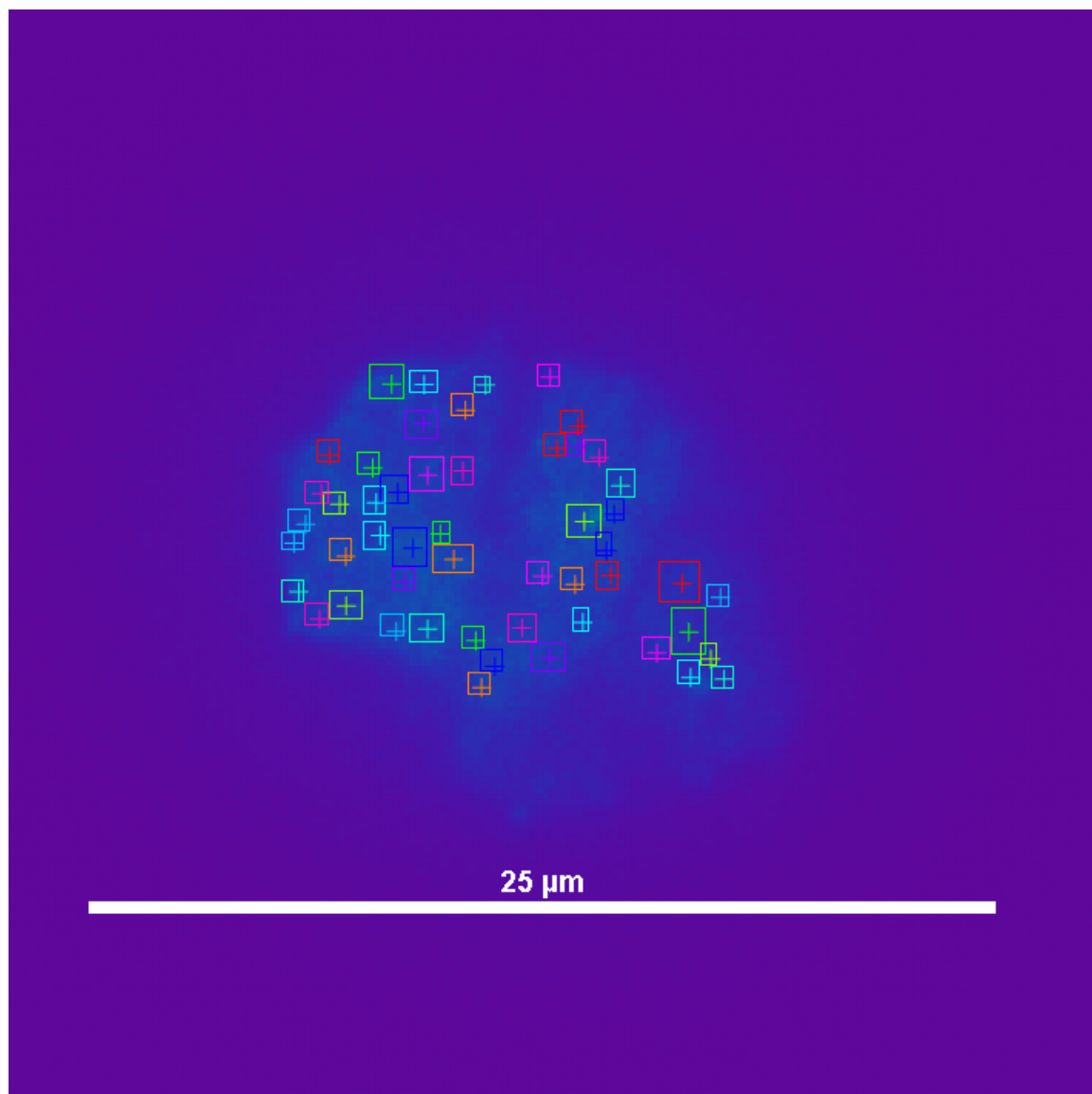


Figure 5.4: Example of multiple Areas of Interest in a β -cell Cluster selected for displaying Elementary Calcium Elevation in Response to NAADP-AM.

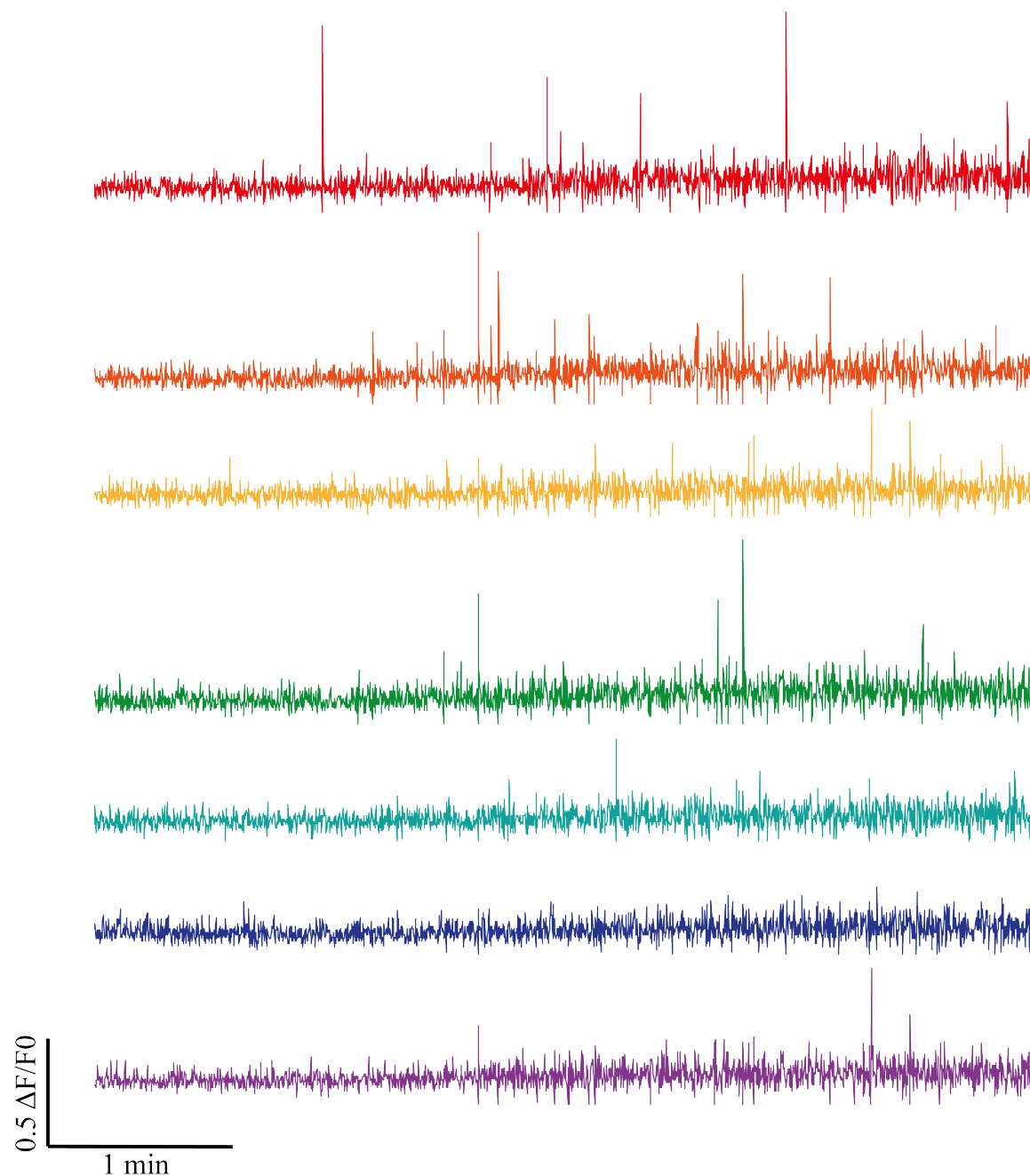


Figure 5.5: Maximal Fluorescence Change against Time for individual Areas of Interest within a Cell. Areas were selected manually Frame by Frame on the basis of their showing Elementary Calcium Elevation. The start of the Record is the Addition of NAADP-AM.

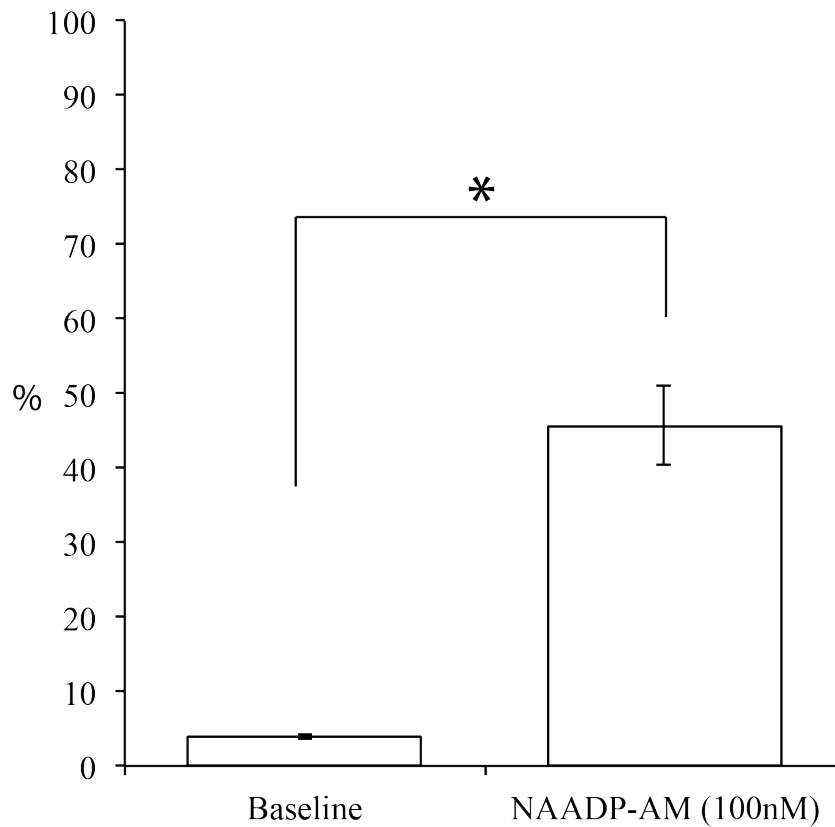


Figure 5.6: *Percentage of Frames Showing Elementary Events (defined as Events of an Amplitude more than 2 Standard Deviations above the Baseline Mean) before (Baseline) and after NAADP-AM Addition in the continued presence of 3 G. Results are from 20 Cells (8 experiments, 4 Animals). * denotes Significance (paired samples, one-tailed Student's t-test, $p < 0.01$).*

5.2.2 Stimulation with a Standard Experimental Stimulus

Having established that β -cells exhibit localised calcium spikes in response to NAADP-AM, their presence in response to glucose stimulation was examined. The experimental procedure for these experiments was exactly as described for NAADP-AM, except that glucose, which at baseline is 3 mM, was raised to 16.5 mM for stimulation. Concentrations of glucose between 10 and 20 mM (sometimes up to 30 mM) are standard practice in many experiments investigating stimulation of β -cells (see Islam, 2010 for review, and references therein).

As can be seen in Fig. 5.7, high glucose also produces the previously observed calcium spikes. And, as shown in Fig. 5.8, there are significantly more of these events present after glucose addition than during baseline recordings (56 %). The effect of 16.5 mM glucose is of the same order of magnitude as that observed with NAADP (increases of 52 % and 42 % from baseline, respectively).



Figure 5.7: Representative Trace of a β -cell showing Elementary Calcium Events triggered by 16.5 mM Glucose. Maximum Intensity Change of subsequent Frames after normalising to Baseline is plotted against Time.

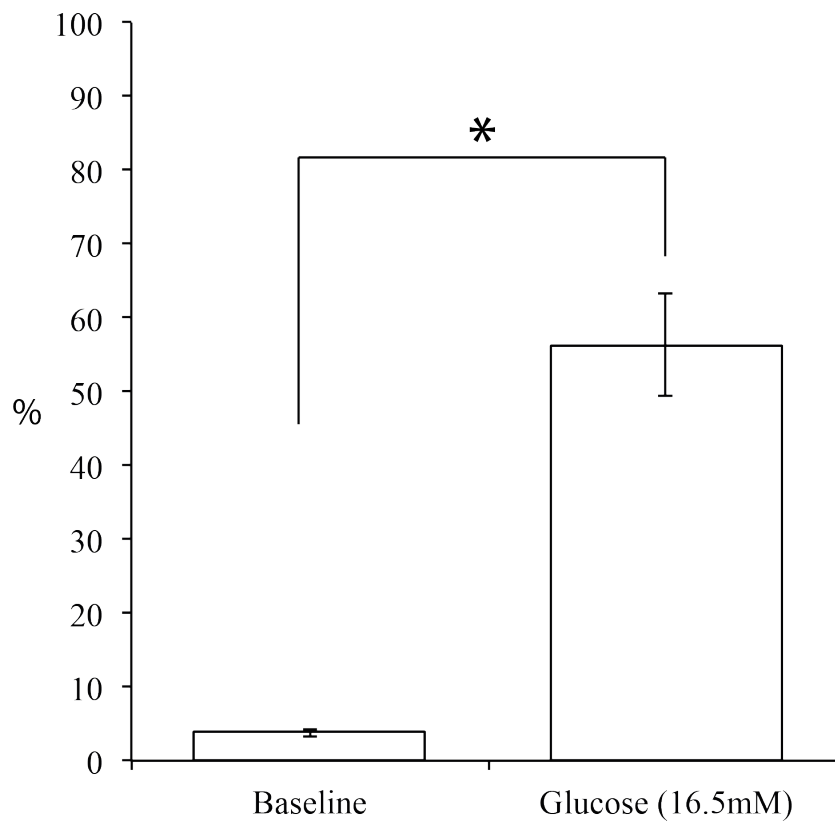


Figure 5.8: Percentage of Frames Showing Elementary Events (defined as Events of an Amplitude more than 2 Standard Deviations above the Baseline Mean) before (Baseline) and after Glucose Increase from 3 mM to 16.5 mM. Results are from 14 Cells (6 experiments, 4 Animals). * denotes Significance (paired samples, one-tailed Student's t -test, $p < 0.01$).

5.2.3 Stimulation with a Physiological Stimulus

It could be argued at this point, that if NAADP-AM and/or glucose induced calcium spikes were indeed the trigger for a larger calcium signal in the β -cell (which would eventually lead to opening of L-type calcium channels and exocytosis of insulin), how would any target channel or enzyme ‘see’ the spikes to allow it to act upon them? After all, we are only able to observe these signals in a fundamentally artificial system. In an intact pancreas in an organism, β -cells do not contain thapsigargin, and are bathed in a solution containing calcium as well as glucose.

The answer lies in the type of stimulation. In its ‘natural habitat’, a β -cell will not encounter 16.5 mM glucose (except, possibly, in a hyperglycaemic emergency state). These incredibly high glucose concentrations are routinely used in *in vitro* experiments; but are, in fact, not necessarily conducive to conclusions about the behaviour of β -cells *in vivo* (see Islam (2010), for review). To address this problem, it was examined whether increasing glucose from 3 mM (baseline) to 6 mM (a glucose concentration that is within the physiologically acceptable range) without having pre-incubated the cells with thapsigargin, would still lead to the occurrence of calcium spikes.²

As can be seen in Fig. 5.9, this is certainly the case. The data mimic those of high glucose stimulation in the presence of thapsigargin. Incidentally, the reason for there not being a ‘SERCA-dip’ present in this trace is the frame by frame subtraction algorithm used to increase spike resolution (see Chapter 2: Methods). If one looks at the ‘raw’ trace (Fig. 5.10), the effect of SERCA activity can be observed, and the spikes are actually sitting inside the ‘SERCA-dip’ (the calcium ‘ramp’ phenomenon that can be seen in this trace also will be discussed later in this chapter). Note the decreased signal-to-noise ratio in this trace; illustrating the purpose of the frame-by-frame subtraction used to visualise spikes.

²The assumption being that a lower stimulatory glucose concentration would cause a less marked increase in ATP production than do the experimental protocols involving exaggerated glucose increases; and thus a less marked stimulation of SERCA activity which obscures elementary events.

Again, if one looks at the percentage of frames containing spikes; this is significantly higher after raising extracellular glucose to 6 mM (15 %) than at baseline (4 %; Fig. 5.11). The increase in spike frequency from baseline seen with 6 mM glucose (11 %) is not as large as that observed with 16.5 mM glucose (52 %) or with NAADP-AM (42 %). It is not surprising to see something resembling a dose-response relationship with glucose stimulation. However, it is a possibility that this effect is in part due to the experimental protocol. The reason being that the coverslips used for the experiments with 6 mM glucose happened to have large numbers of cells within the same field of view, which were then imaged simultaneously. In TIRF imaging this can be an issue since coverslips are (unless they are special products) not perfectly smooth in themselves, and so when one side of the coverslip creates the perfect evanescent wave for TIRF, another area of the coverslip may not, as the laser will be reflected in a different way when the glass is at an angle. If one considers the cells from the 6 mM glucose experiment individually, it seems that some behave like those in the other experimental conditions, with large spike frequency increases; while others do not show much of an increase above baseline. It is usually the case that these cells are on the same coverslip but in different locations. However, the mean frequency increase of only those cells that showed a clear response (a mean frequency increase 2 SDs above BL); 24 %; is still significantly lower than that observed with 16.5 mM glucose; 52 % ($p < 0.01$). Thus it is most likely that technical caveats only exaggerated a dose-dependence effect for glucose.

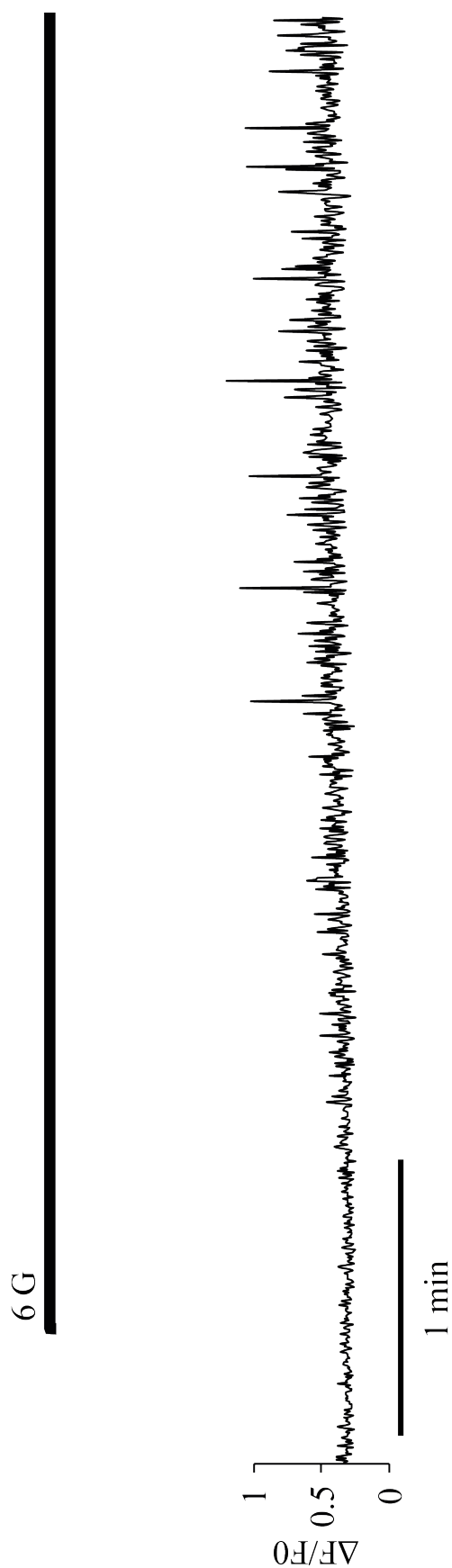


Figure 5.9: Representative Trace of a β -cell showing Elementary Calcium Events triggered by 6mM Glucose. Maximum Intensity Change after Normalisation to Baseline and Frame-by-Frame Subtraction is plotted against Time.

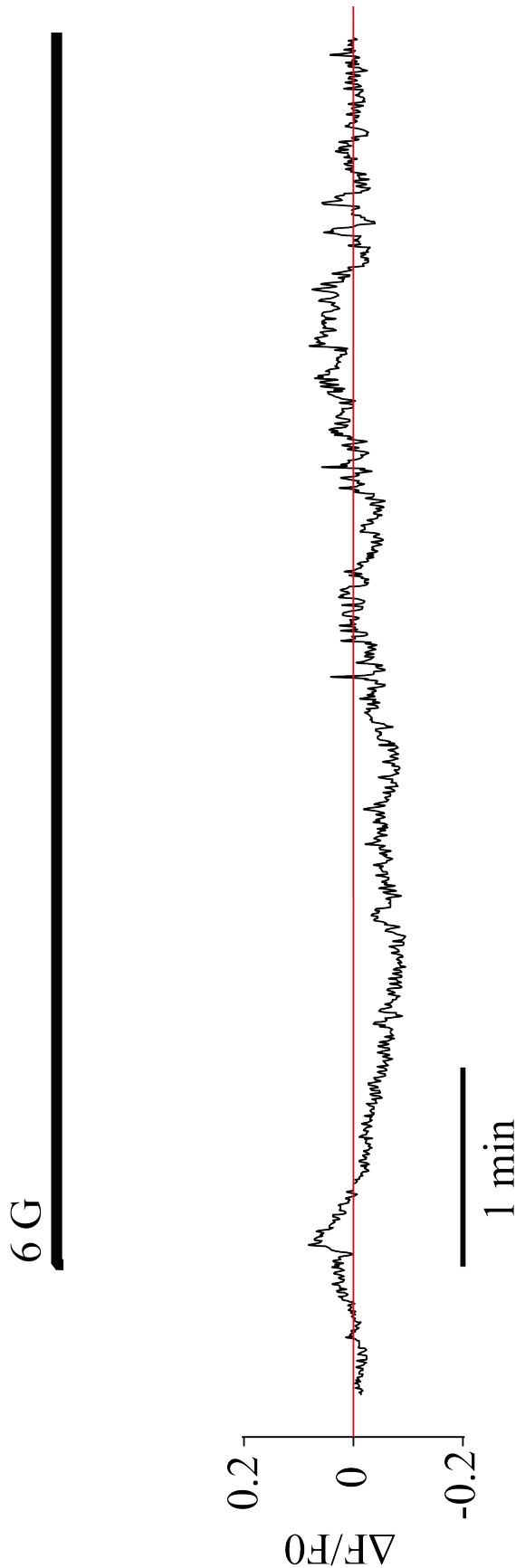


Figure 5.10: Representative 'Raw' Trace of a β -cell showing Elementary Calcium Events triggered by 6 mM Glucose. Maximum Intensity Change after normalising to Baseline is plotted against Time. The red Line denotes the Baseline Average. Note the characteristic Dip in the Response caused by Activation of SERCA followed by Calcium Spikes on a Ramp back to Baseline.

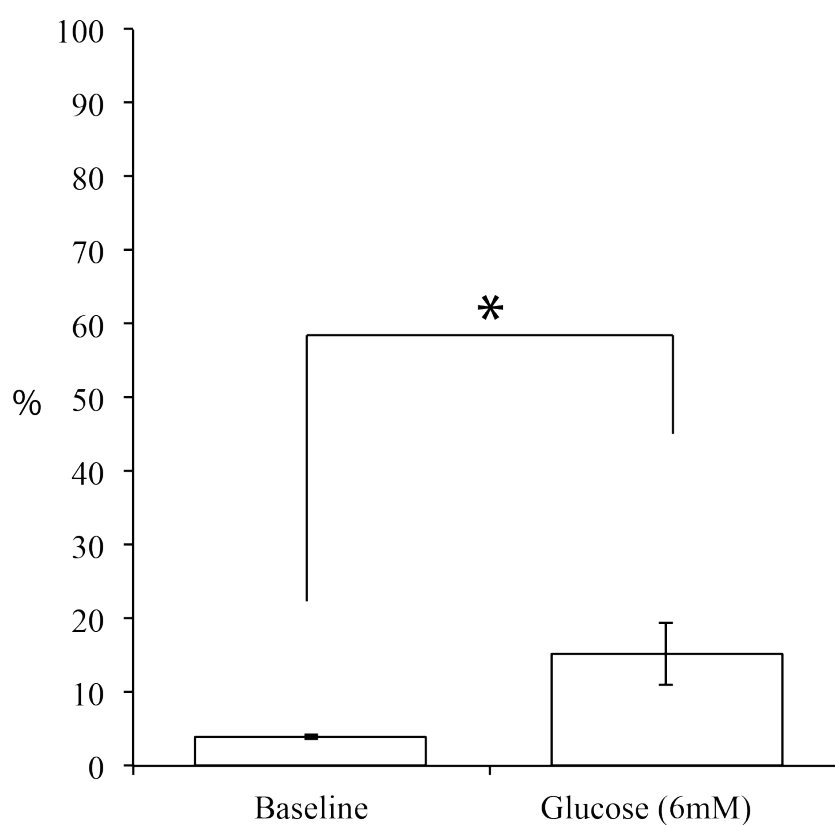


Figure 5.11: Percentage of Frames Showing Elementary Events (defined as Events of an Amplitude more than 2 Standard Deviations above the Baseline Mean) before (Baseline) and after Glucose Increase from 3 mM to 6 mM. Results are from 18 Cells (6 experiments, 6 Animals). * denotes Significance (paired samples, one-tailed Student's t -test, $p < 0.01$).

5.3 Elementary Events can only be visualised using TIRF at 100 x Magnification

The above mentioned calcium responses are so striking, and potentially so crucial in their physiological significance, it seemed most surprising that nobody had observed these before. It appears, however, that a very particular set of experimental circumstances is necessary to detect these events.

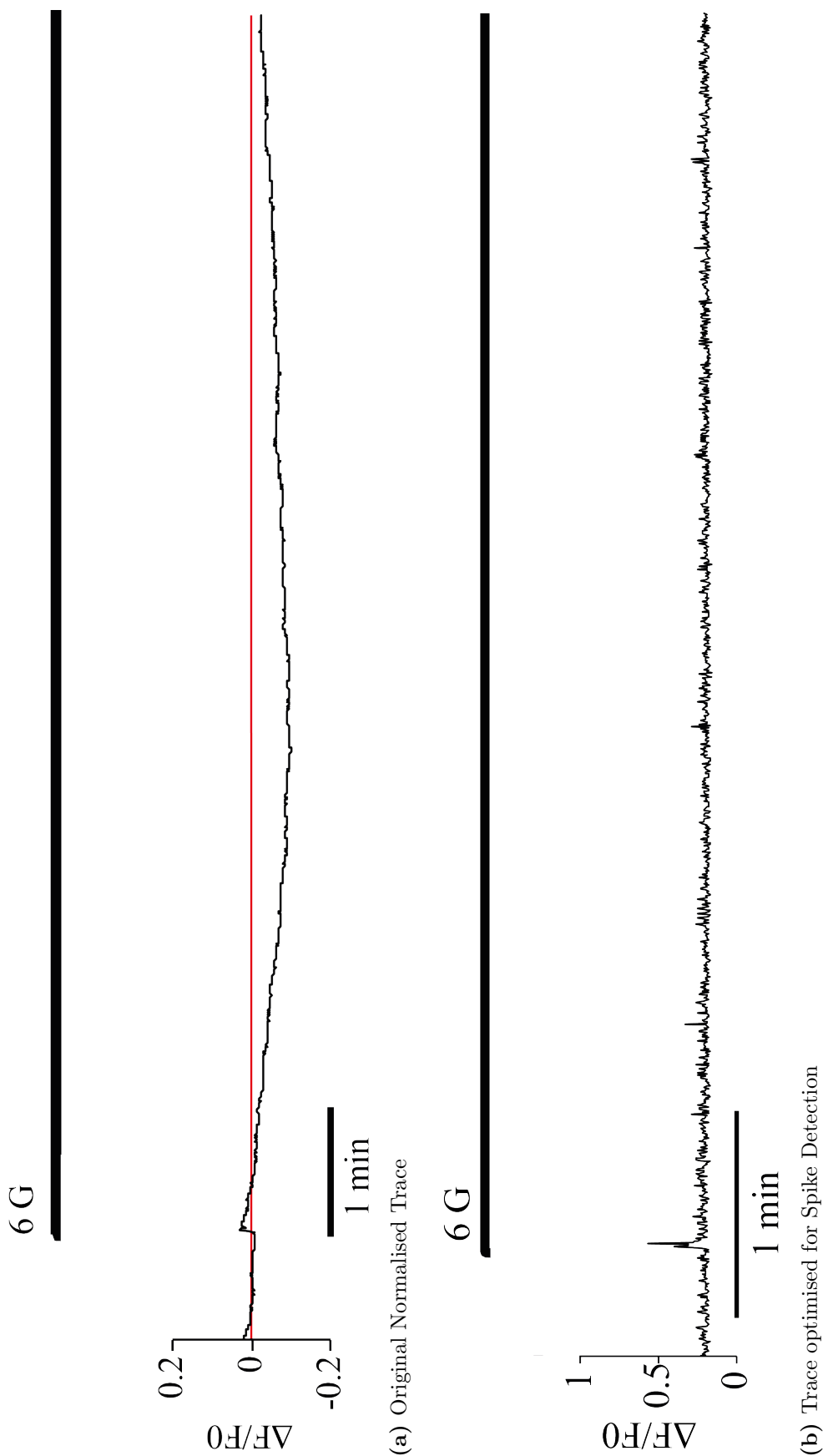
5.3.1 Experiments at 60 x Magnification

The first reason why these spikes are so easily missed is the sheer lack of magnification. For the major part of this project, a 60x lens was used under the misconception that the more cells were recorded within one field of view, the more likely it would be to observe potential effects. This magnification is simply not enough to resolve elementary calcium signals in β -cells. If one is certain of their existence, one might venture a guess as to their occurring in some cells imaged at 60x magnification- but at no point could individual events be clearly discerned.

5.3.2 Experiments under Epifluorescence

The second reason why these spikes may not have been discovered earlier, is that TIRF microscopy is necessary to visualise them; even at 100x magnification. If one repeats the above mentioned experimental protocol under epifluorescence, no calcium spikes are observed. Fig. 5.12 shows this very clearly. Note that the signal towards the beginning of the trace is a mechanical artefact of adding glucose to the bath. Parallel recordings as were conducted at 60x magnification for global responses were not attempted with this protocol, because the already slow acquisition rate would likely have become too delayed to observe any events at all if a filter change lasting several whole seconds was involved. This experiment was only repeated twice, once

with glucose at 6 mM, and once with glucose at 16.5 mM, as it was considered a mere proof of principle.



^aNote that the different timescales on what is the exact same record before and after analysis is not a typo. Trace (a) has time recorded in real time during the experiment; trace (b) has time assigned to it as 300ms per frame after analysis. The difference between the two timescales demonstrates a technical fault in the system- namely, that a nominal dictated acquisition rate of 1/300ms does not actually equate to taking an image every 300 ms, but taking an image every 400 or 500 ms. The work was clearly done at the edge of the hardware's capacities.

5.4 Elementary Events are not the Result of Calcium Influx

If the above events are the key to the K_{ATP} channel independent pathway of stimulus-secretion coupling, they must be shown to be entirely independent of calcium influx through the cell membrane. This can be demonstrated in three different ways:

1. Eliminate extracellular calcium
2. Pharmacologically prevent cell membrane calcium channels from conducting calcium
3. Electrochemically prevent the cell membrane calcium channels from opening

5.4.1 Experiments in Calcium-free Medium

While all the above experiments were conducted in calcium-free medium containing 100 μ M of the calcium chelator EGTA, it could be argued that because it was necessary to exchange the medium so shortly before experiment-start, and it was impossible to wash the bath several times with calcium free medium at that point (for fear of losing TIRF focus), that there might be residual calcium left in the bath which is entering the cell through the membrane upon stimulation. The following experiments were conducted to address this concern:

5.4.2 Experiments with High Extracellular EGTA

The first and most simple way to ensure there was minimal residual extracellular calcium, was to increase the EGTA concentration in the extracellular medium to a much higher concentration (i.e. a concentration of a buffering capacity a multiple of what could conceivably be necessary to bind the remaining calcium). The regular extracellular medium contains 1.7 mM calcium. If there were a small amount of this

medium left after exchange, 5 mM EGTA should certainly buffer the remaining calcium in this to nanomolar concentrations; and do so quickly. It is worth remembering at this point also, that relatively large quantities of fluid were used at all times (due to the PFS, see Chapter 2: Methods). Even if there were 100 μ l of regular medium left in the bath (which is unlikely), 1 ml of calcium-free medium containing 5 mM EGTA would be added to this. The EGTA to calcium ratio should be more than excessive under these circumstances.

Spikes are still present in the presence of 5 mM EGTA, and significantly more so than before addition of glucose at 6 mM or NAADP-AM at 100 nM ($p < 0.01$ and $p = 0.03$, respectively; Fig. 5.13). Note that in the presence of 5 mM EGTA 6 mM glucose produces frequency increases the same order of magnitude as NAADP-AM.

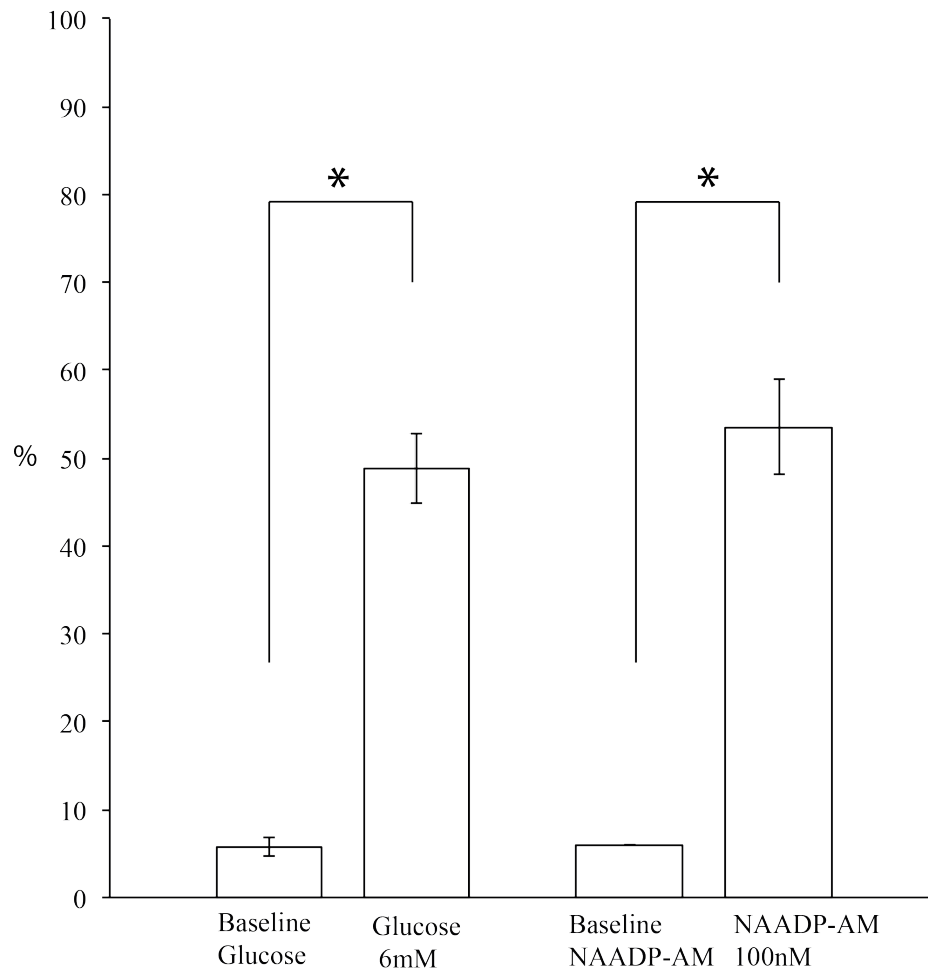


Figure 5.13: Percentage of Frames Showing Elementary Events (defined as Events of an Amplitude more than 2 Standard Deviations above the Baseline Mean) before (Baseline) and after Stimulation with Glucose (6 mM) or NAADP-AM (100 nM) in the Presence of 5 mM EGTA. Results are from 4 Cells (4 Experiments, 2 Animals) and 2 Cells (2 Experiments, 2 Animals) respectively). * denotes Significance (paired samples, one-tailed Student's t -test, $p < 0.01$, $p = 0.03$, respectively).

5.4.3 Experiments with Nifedipine and Diazoxide

Although the persistence of responses in the presence of 5 mM EGTA convincingly demonstrates that the observed events are most unlikely to arise through calcium influx across the membrane; pilot experiments were conducted in the presence of 1 μ M nifedipine and 100-400 μ M diazoxide, to address points 2) and 3), respectively. Nifedipine prevents influx of (any remaining) extracellular calcium by inhibiting L-type calcium channels, while diazoxide essentially acts as a chemical voltage clamp by keeping K_{ATP} channels in an open state. This prevents the cell from depolarising to the required V_m for L-type channel activation (~ -40 mV for the $Ca_v1.3$ type most common in β -cells (Braun et al., 2008)).

Spiking could still be observed in the presence of 1 μ M nifedipine (see Fig. 5.14; data not quantified). One might wish to conduct this experiment with a higher dose of nifedipine; for example 3 μ M, as was used in Chapter 3 to inhibit global responses to glucose. However, as discussed previously, this bears the risk of blocking TPCs as well as L-type channels (nifedipine has been shown to antagonise TPCs; Genazzani et al., 1997). As the present experiment was conducted in calcium-free medium, it was thought unnecessary to risk blocking TPCs by using a supra-maximal concentration of nifedipine.

Initially it was concluded that experiments with diazoxide confirmed intracellular release because cells kept displaying spiking behaviour in its presence. However, when it was attempted to demonstrate the efficacy of the compound by eliminating global calcium responses with it, it was discovered that diazoxide did not produce a reliable and significant reduction in the global response to glucose as is expected from previous reports (see for example Quesada et al. (1999)). It might be worthwhile to repeat this experiment; and possibly to combine diazoxide with nifedipine. Additionally it might be interesting to look at the negative control experiment and stimulate cells with agents known to rely on calcium influx for their actions. Adding K_{ATP} channel

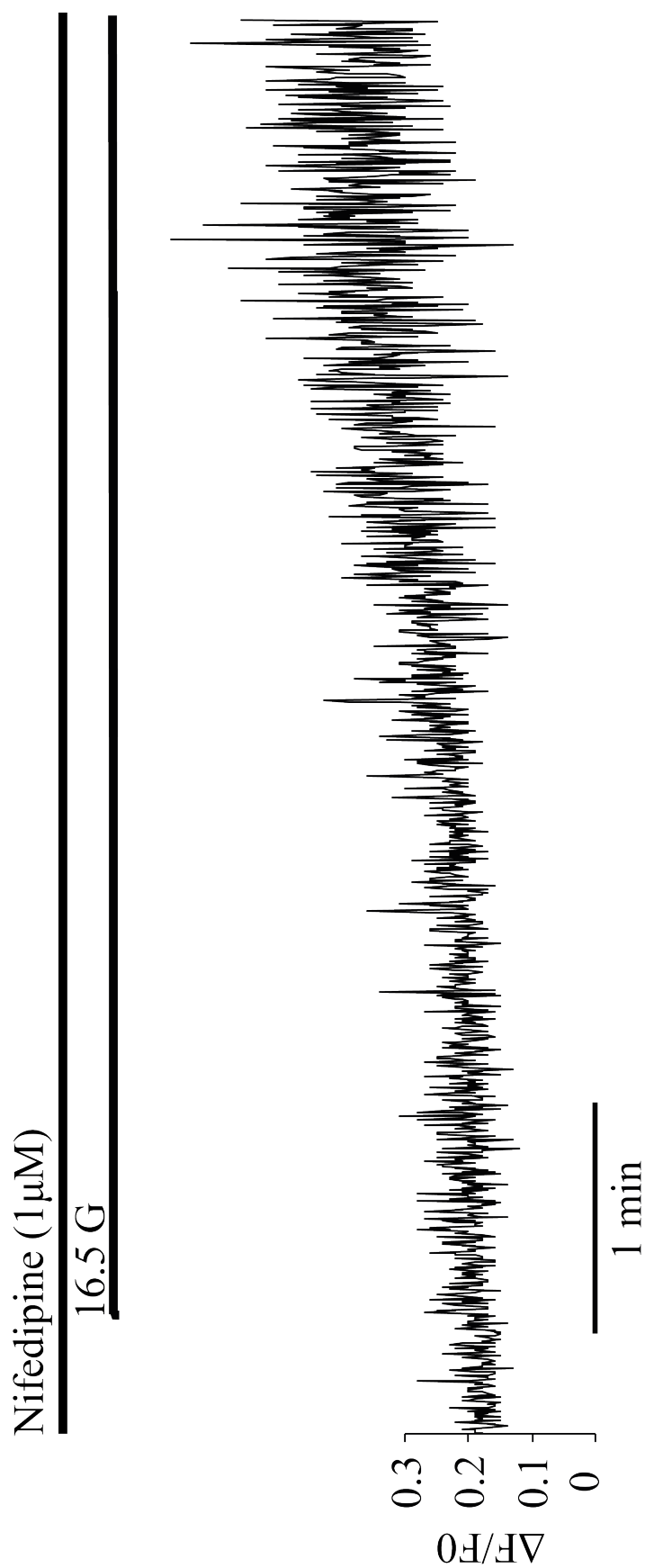


Figure 5.14: Representative Trace of a β -cell showing Elementary Calcium Events triggered by 16.5 mM Glucose in the Presence of 1 μ M Nifedipine. Maximum Intensity Change after Normalisation to Baseline and Frame-by-Frame Subtraction is plotted against Time.

antagonists such as tolbutamide, to see if spikes can be produced by action on the K_{ATP} channel or through the resulting depolarisation, would be one such example.

5.4.4 Experiments with Strontium

Another permutation of the experimental design aimed at distinguishing between intracellular and extracellular calcium influx, was to exchange extracellular medium for a medium containing strontium instead of calcium. This was in the hope (there were no published experiments on this matter) that strontium might not bind to fluo-4, and thus any signal observed would be from the intracellular calcium stores. It was observed, however, that strontium certainly did bind to fluo-4, and global calcium responses as described in chapters 3 and 4 could be replicated with it. Strontium also seemed to get taken up into the intracellular stores like calcium. This was discovered when cells were pre-incubated with strontium-containing medium, followed by recording in strontium- and calcium-free medium. Spikes as described here could be replicated with strontium. Thus strontium mimics calcium so perfectly that it could not be used for the dissection of this release pathway. It may be worth exploring other members of the beryllium family for this purpose; and possibly with other fluorescent calcium indicators.³ Gylfe and colleagues discovered that barium cannot be mobilised from IP₃-sensitive stores in β -cells (Liu et al., 1995). This may be a starting point to investigate interactions between different intracellular calcium pools in producing these elementary calcium signals.

5.5 Elementary Events can occur Spontaneously

Pilot studies on individual cells were conducted to explore the effects of different glucose and calcium concentrations on the calcium spikes (data not shown). These gave the impression that said responses can occur spontaneously. This was even the case in the complete absence of glucose (though it could be argued that a 10 min

³It has been reported that fluo-3 has a significantly lower affinity for strontium than calcium and can be used to distinguish store-mediated release and extracellular influx in β -cells (Lemmens et al., 2001). It may be worth investigating how the present responses are reported by fluo-3 and whether this allows for a dissection of the origins of the calcium stores with strontium replacement.

incubation without glucose is too short to have a significant effect on cell metabolism). The fact that spikes might occur spontaneously is not surprising, given the high level of spontaneous activity in β -cells (see chapter 3). It may be that these spontaneous spikes are in fact the trigger for the spontaneous global calcium responses observed previously. It is also the case that EGTA itself can lead to calcium release from intracellular stores (see for example Palade et al., 1983, or Wollheim and Sharp, 1981, and references therein). Further, it has been argued that removal of extracellular calcium can lead to calcium release from intracellular stores in cardiac myocytes (Cheng and Lederer, 2008); representing something like a ‘reverse-SOCE’. The tentative results presented here likely just illustrate yet again that there is no such thing as a ‘resting’ β -cell. It is probable that a certain level of spiking activity is always present, but may change frequency, amplitude or nature (combination of underlying channels) upon stimulation.

5.6 Elementary Events are superimposed on a Continuous Calcium Release (Ramp)

As was mentioned previously; the observed calcium spikes in β -cells are usually superimposed on a slow steady rise in intracellular calcium as is shown in Fig. 5.15. For quantal analysis of the spikes, a frame by frame subtraction algorithm was used to eliminate this baseline, as this was a hinderance when measuring the elementary events. However, this phenomenon is interesting in its own right. It could suggest that there are a number of separate calcium release processes present in response to glucose or NAADP stimulation. It could also be a general response to removing extracellular calcium⁴. It could simply be fluorescent indicator saturation. The latter is unlikely, as the ramp reaches a plateau. Dye saturation (as well as apoptosis, for that matter) would lead to a steady (and possibly exponential) increase in calcium

⁴It is not likely to be SOCE due to thapsigargin-mediated ER depletion, because it persists in experiments involving no thapsigargin.

signal. It is also the case that cells still responded with a regular global calcium signal at the end of the experiment; suggesting that they were physiologically intact and the dye had not saturated. Interestingly, the present pattern of calcium spikes superimposed on a calcium ramp fits perfectly with Misler and colleague's suggestion that the two phases of insulin secretion are dependent on an initial and then subsiding spike train of electrical activity and an underlying plateau depolarisation. They even suggest that the former could be associated with very localised very large increases in calcium (though they suggest VGCCs for this), while the latter might be associated with a slower 'background' calcium increase. The former, according to Misler et al., leads to the exocytosis of the immediately releasable pool of insulin granules, while the latter activates the reserve granule pool (Mislér et al., 2009). Ohara-Imaizumi and colleagues have also proposed that granules in the docked pool are sensitive to localised rapid calcium rise, whereas reserve pool granules are sensitive to lower and more sustained calcium release (Ohara-Imaizumi et al., 2009). It seems the present calcium recordings are consistent with these results. Of course the present study was not designed to investigate this effect; and no quantitative information was collected about the underlying calcium 'ramp'; but from the mere observation that this is present in practically every recording that was made, it seems a genuine phenomenon. If it were indeed the case that this was what underlies biphasic insulin secretion, then it again underlines that this is not dependent on the K_{ATP} channel, but the result of intracellular calcium release (possibly via NAADP). Indeed it was suggested as early as 1980 that the first phase of insulin secretion might be the result of intracellular calcium release, see Wollheim and Sharp (1981) for review. It will be interesting to try and discern the two types of calcium signal observed presently and to see whether they can be separated pharmacologically to determine their respective origins. Ideally one would combine TIRF microscopy with electrophysiological measurements to investigate whether the observed calcium signals happen in synchrony with electrical activity as described by Misler and colleagues. One could even combine these two

with measurements of insulin secretion or exocytosis. This is a technical challenge, but it can be done (see for example Hoppa et al., 2009).

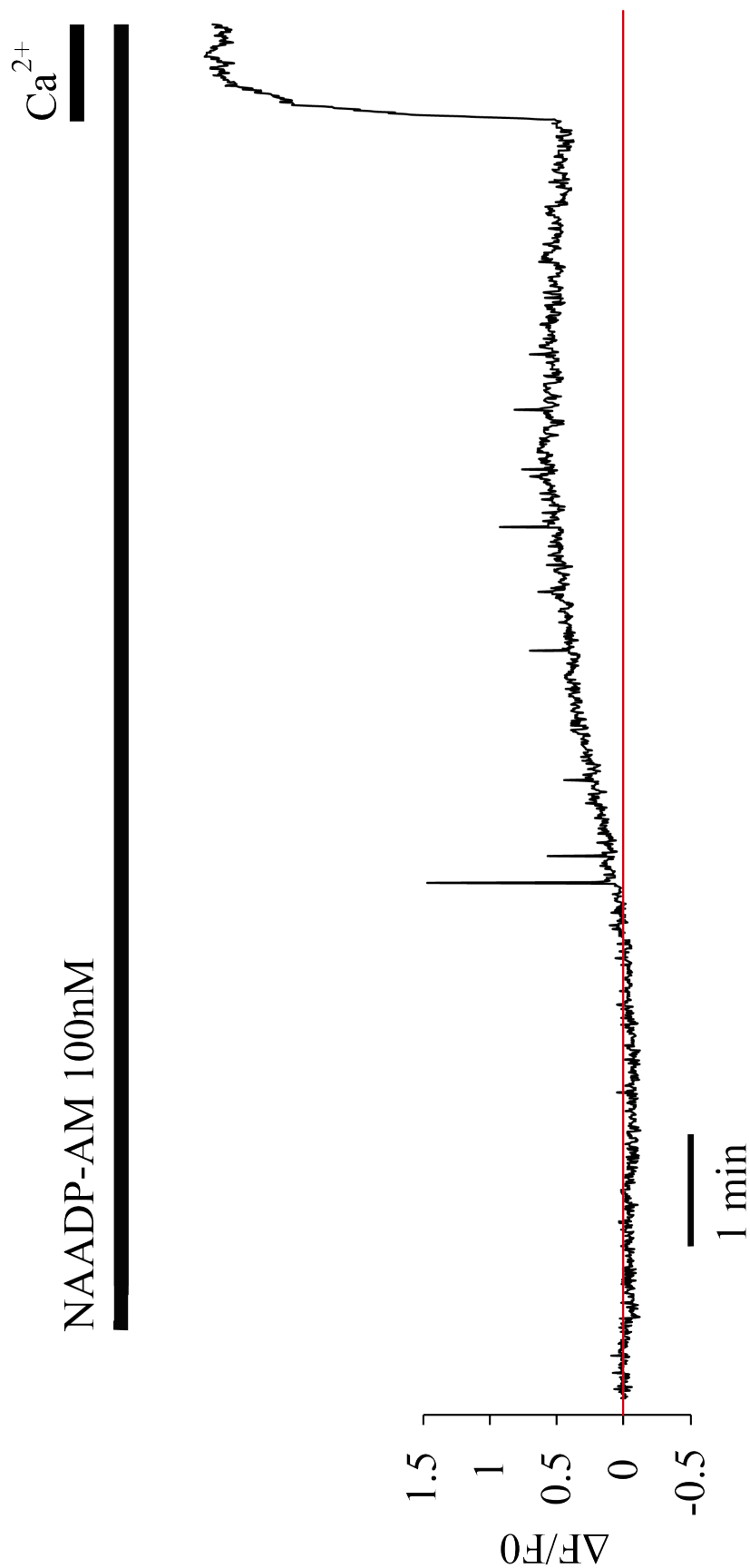


Figure 5.15: Representative Trace of a β -cell showing the characteristic Calcium Ramp underlying spiking Calcium Events triggered by NAADP-AM. Maximum Intensity Change after normalizing to Baseline is plotted against Time. The red Line denotes the Baseline Average. Extracellular Calcium was re-admitted at the End of the Experiment; leading to a global Calcium Response.

5.7 Elementary Events are not likely the Result of ER Calcium Release

5.7.1 Experiments with Thapsigargin

Thapsigargin, which acts by inhibiting the endoplasmic calcium pump SERCA, was used in all of the experiments described in this chapter, other than those with physiological level glucose stimulation. Thapsigargin is very specific and irreversible in its block of all SERCA isoforms (Lytton et al., 1991), and is used extensively to investigate the role of ER calcium in many cell types. Its use in the present experiments was paramount, as SERCA activity in β -cells is very high following glucose stimulation and the resultant ATP surge in the cell. This produces the characteristic ‘dip’ in intracellular calcium concentration, first described by Rorsman et al. (1984), and present in most recordings discussed in chapter 3. This increased SERCA activity is stimulated by glucose independently of intracellular calcium concentration. Tengholm and colleagues showed that the ER fills to the same extent in response to glucose irrespective of the calcium elevation it causes in the cell (Tengholm et al., 2001). As was shown in chapters 3 and 4, the SERCA dip remained unchanged across all experiments; most notably those involving pre-incubation and recording in calcium-free medium. After pre-incubation with thapsigargin, however, this dip is eliminated; and with it its capacity to mask any local calcium signals. Since it was necessary to utilise thapsigargin to visualise elementary calcium release, it is unlikely that this originates from the ER. However it was never tested (with IP_3 -BM, for example) whether the thapsigargin-sensitive stores were emptied completely; and so the possibility that some of the observed calcium originated from it cannot be excluded. Interestingly, if thapsigargin was left in the chamber for the duration of the experiment, rather than used for pre-incubation only, spikes could not be observed. This may be due to reversible

inhibition of various calcium channels at micromolar thapsigargin concentrations (see Toth et al., 2002, and references therein).

5.8 The Role of Acidic Stores in the Generation of Elementary Events

5.8.1 Experiments with Bafilomycin A1

To investigate whether acidic stores are the calcium source for the observed spikes, experiments were conducted in the presence of and after 30 min pre-incubation with 3 μ M bafilomycin A1. There is still a significant increase in the frequency of elementary events compared to baseline in the presence of bafilomycin when stimulating with 16.5G, or NAADP-AM ($p < 0.01$, $p = 0.013$, respectively). When stimulating with 6G in the presence of bafilomycin, there was no significant increase in spike frequency compared to baseline ($p = 0.058$). However, this is the result from the analysis of two cells (the effects from the other stimuli were so clear that this was only run as a pilot study). As can be seen in Fig. 5.16; there is no significant difference between increases observed in the absence and presence of bafilomycin. The background ramp persists in the presence of bafilomycin, also. There are several shortcomings of this particular experiment. Firstly the records were not all of the same length as during some experiments it looked as though bafilomycin had blocked spiking activity, and was taken out of the bath to show a recovery effect. Analysing the calcium signals after optimising the records for spike detection showed that small signals had been present after all; rendering the reversibility part of the trace uninterpretable (it could be a late response to the original stimulus rather than a recovery after bafilomycin removal). Thus only the first section of these records was used for analysis. The inherent problem with the self limiting nature of these sparks is that if one looks at frequency of frames as a quantitative measure of effect; having different length

records can skew the results one way or another depending on which phase of spiking a particular cell happens to be in. Even if bafilomycin showed no reduction in the frequency of these events, it would not necessarily mean that acidic stores are not involved. The system is likely so intertwined that taking out any one individual store or channel is unlikely to yield a striking result. This has already been suggested when discussing the global responses shown in Chapters 3 and 4.

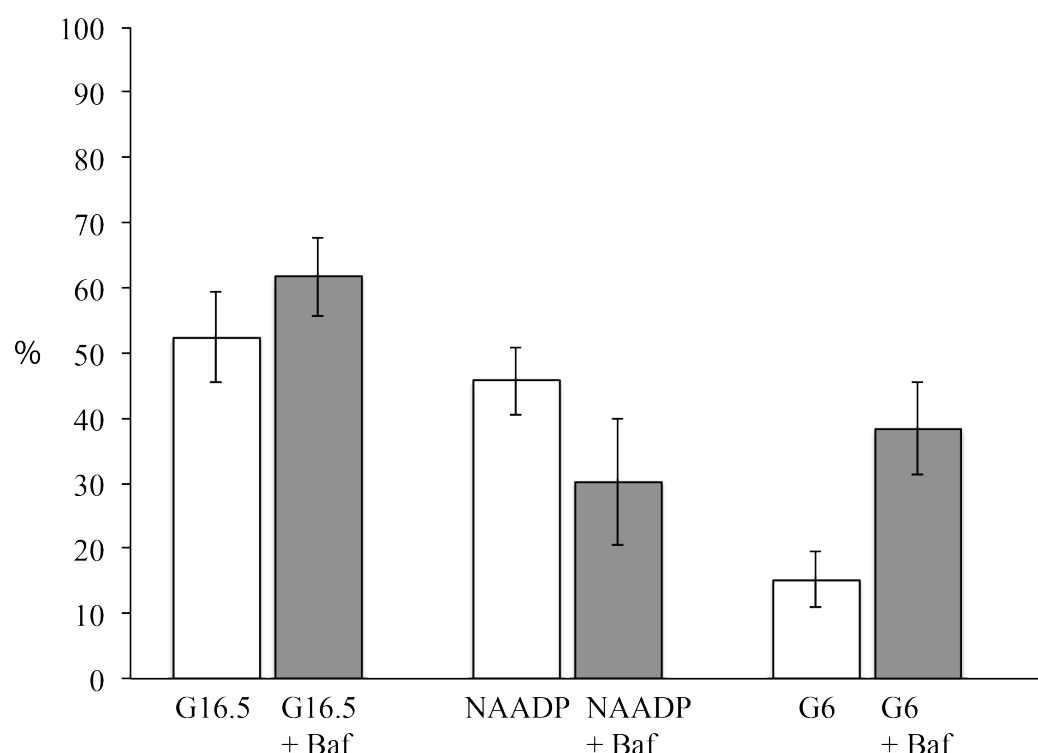


Figure 5.16: Percentage of Frames Showing Elementary Events (defined as Events of an Amplitude more than 2 Standard Deviations above the Baseline Mean) in the Absence (empty Columns) and Presence (filled Columns) of $3\mu\text{M}$ Bafilomycin after Stimulation with 16.5 mM Glucose, 100 nM NAADP-AM, or 6 mM Glucose. Results are from 13 Cells (4 Experiments, 4 Animals), 6 Cells (2 Experiments, 2 Animals) and 2 Cells (1 Experiment, 1 Animal), respectively. A Repeated Measures ANOVA was conducted with Stimulus (16.5 G, NAADP, 6 G) as the within-subjects independent Variable and Bafilomycin as the between-subjects independent Variable. For 16.5 G, there was a significant Effect of Stimulus $F(1, 25) = 149.18$, $p < 0.01$; no significant Effect of Bafilomycin $F(1, 25) = 0.99$, $p = 0.329$; and no significant Interaction between G 16.5 and Bafilomycin $F(1, 25) = 0.98$, $p = 0.331$. For NAADP, there was a significant effect of Stimulus $F(1, 24) = 42.8$, $p < 0.01$; no significant effect of Bafilomycin $F(1, 24) = 1.408$, $p = 0.247$; and no significant Interaction between NAADP and Bafilomycin $F(1, 24) = 1.078$, $p = 0.309$. For 6 G, there was a significant effect of Stimulus $F(1, 18) = 13.807$, $p < 0.01$. The Interaction with Bafilomycin was not analysed.

5.8.2 Experiments with GPN

Pilot experiments were also conducted after pre-incubation with GPN (data not shown).⁵ This paradoxically seemed to increase spiking (this observation being based entirely on those spikes detectable by eye). Though a 10 min pre-incubation with GPN may be too short to have lysed all acidic stores, and one may actually be imaging the action of GPN itself- destroying stores and releasing their contents into the cytoplasm, as suggested by Hille and colleagues (Duman et al., 2006) (see also Churchill et al., 2002). This is an experiment that might be interesting to conduct in the future.

5.9 TPCs may be involved in triggering Elementary Events

5.9.1 Experiments with tNed-19 and rNed-19

As was the case with global responses; attempting to use tNed-19 to determine the involvement of TPCs in the above-mentioned signals proved impossible. Neither pre-incubation with tNed-19 at concentrations between 10 and 50 μ M, nor post-hoc addition of the same had any effect on the responses (see Chapter 3 for lack of tNed-19 effect on global responses). Knowing that depending on tissue and cell type, certain isoforms and mixtures of Ned-19 may or may not be effective; the fact that it had no effect in this case does not suggest a lack of involvement of TPCs in the observed calcium spikes. Rather it points yet again to the importance of determining the correct subtype of Ned-19 for the tissue in question. It will be important to repeat these experiments with rNed-19, which was potent in the case of global responses (see Chapter 3); but, as it was produced in-house, was no longer available at the time of the experiment.

⁵GPN lyses acidic organelles osmotically by two methods; as a substrate for cathepsin C, and in a protease-independent mode, likely to involve the organelles acidic pH (Duman et al., 2006).

5.9.2 Experiments with Knockout Animals

A pilot study was conducted using a TPC double knockout mouse to see if spikes were still present when neither TPC1 nor TPC2 were available to bind NAADP. It looked as though NAADP-AM could still elicit spikes in the DKO animal. However, looking at small numbers of cells and given the high level of spontaneous activity seen in β -cells, this does not necessarily reflect a response to the stimulus in question. It will be interesting in future to examine the nature of these responses in TPC knockout animals in detail.

5.10 Elementary Events are Quantal in Nature

As can be seen in Fig. 5.17, the employed spark detection algorithms (see Chapter 2: Methods) certainly identify and visually isolate elementary calcium events in response to NAADP-AM. From frames such as the above, the maximum intensity (inevitably the spark) was recorded for quantitative analysis. This was measured for each cell in each frame for each experiment. Plotting the frame-maxima across all cells stimulated with NAADP-AM gives the frequency distribution depicted in Fig. 5.18. (Note that this represents the maximum intensity values for all frames, before excluding baseline noise; i.e. ‘empty’ frames that do not contain sparks).

As can be seen, this is not the Poly-Gaussian distribution one might expect to find with quantal events (see Appendix A.8); even if the lefttestmost mode within such a polymodal distribution (noise) would be expected to be very large because of the ‘empty’ frames included in this dataset. To remove these baseline fluctuations in fluorescence, only intensities larger than two standard deviations above the baseline mean intensity were included in spark analysis (see red line in Fig. 5.18). To arrive at this baseline mean and standard deviation, those frames that were taken before glucose or NAADP addition were permuted using the same algorithms mentioned above, and their maximum fluorescence intensities (in this case noise or spontaneous

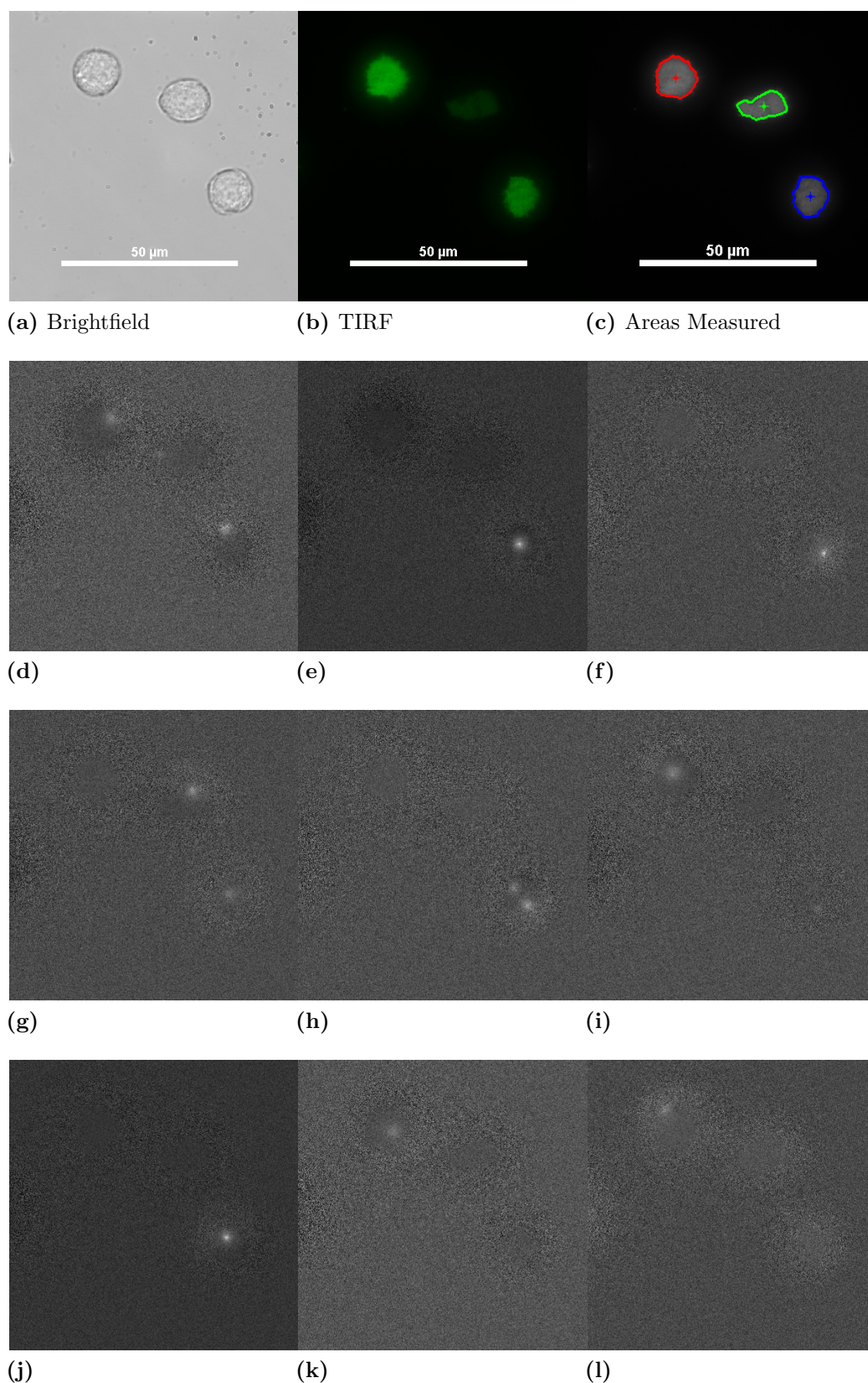


Figure 5.17: Examples of Elementary Release Events in Response to 16.5 mM Glucose (d) - (i) detected by Normalisation, Frame by Frame and Background Subtraction in 3 Individual β -cells. Cells are shown in original Brightfield (a) and TIRF (b) Recordings, as well as surrounded by the Boundaries Used in the Detection Analysis (c). Note that these Images are for illustrative Purposes; Quantal Analysis was carried out exclusively on NAADP-AM stimulated Cells.

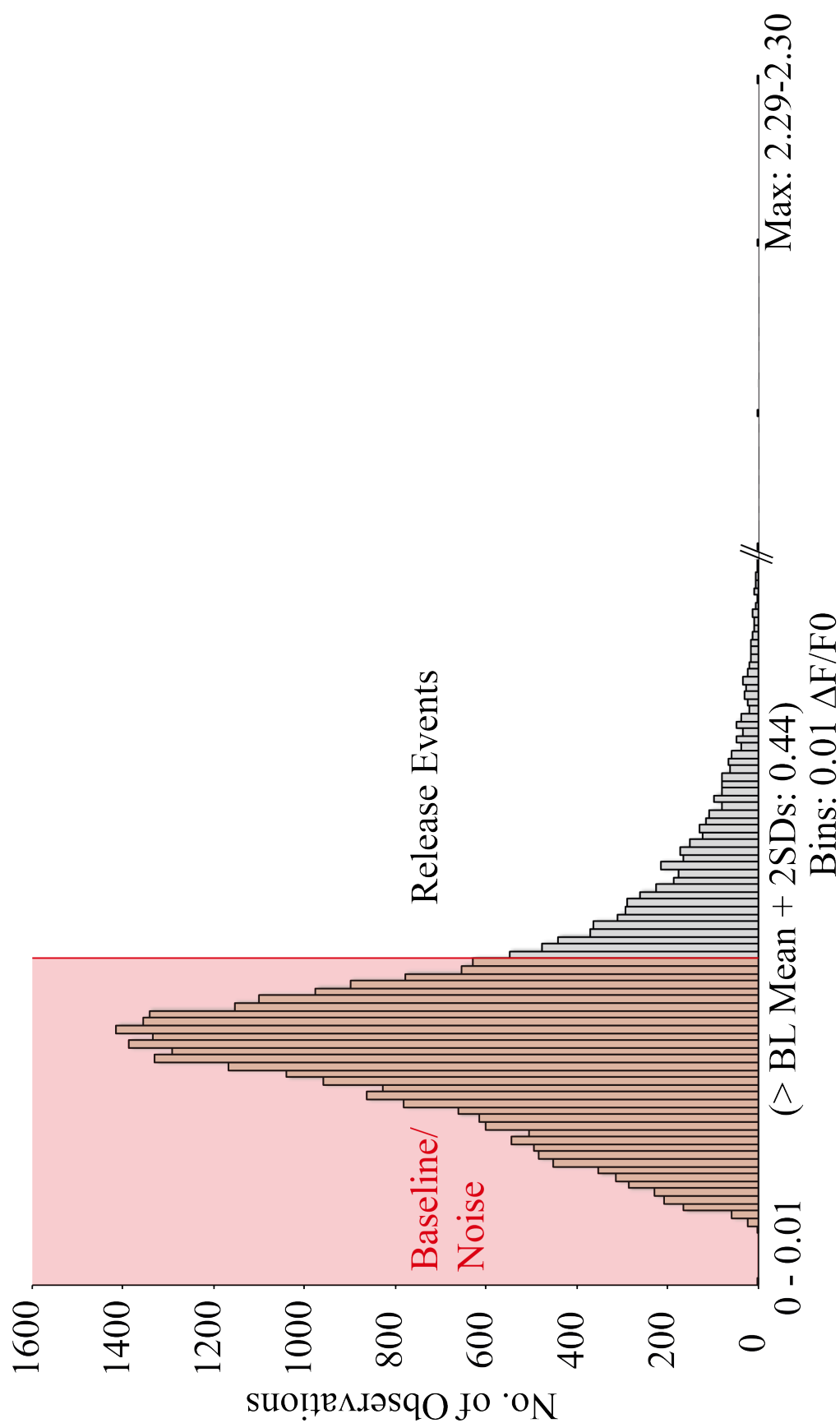


Figure 5.18: Frequency Histogram of Maximum Intensity Change Observed after NAADP Stimulation ($n=20$). The red Line marks the defined Cut-off Point for Inclusion in Spark Analysis (Two SDs above BL Average).

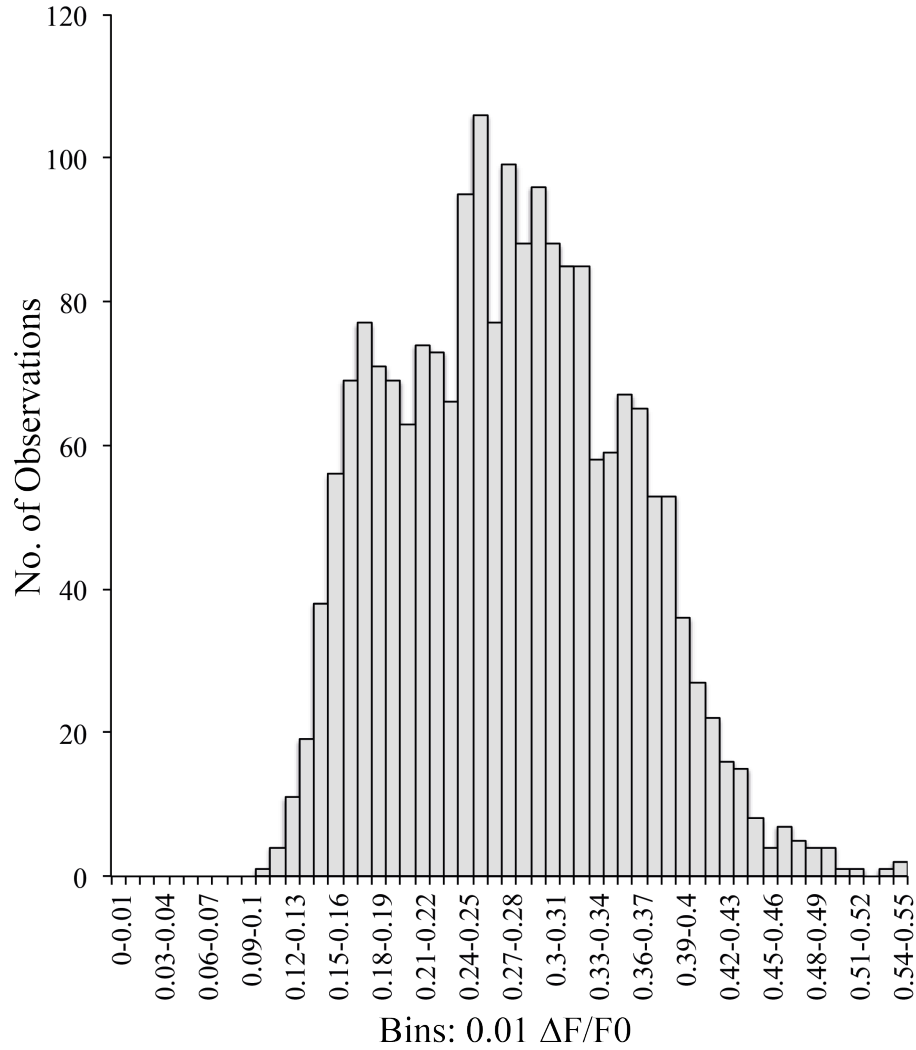


Figure 5.19: Gaussian Distribution of Baseline Fluorescence Variation (mean= 0.28, SD= 0.08 $\Delta F/F_0$)

activity) noted. This data showed a normal distribution for each cell, and also across the baseline condition for all cells (see Fig 5.19 for the distribution across all cells). The mean and standard deviation of this distribution were used to exclude values in the experimental data shown in Fig. 5.18 to distinguish between baseline fluctuations and actual signal.

As can be seen, the resultant frequency histogram (Fig. 5.20) is no more a clear poly-Gaussian distribution than that shown in Fig. 5.18. The observed event frequencies fit exponential decay more than any other function. There appear to be

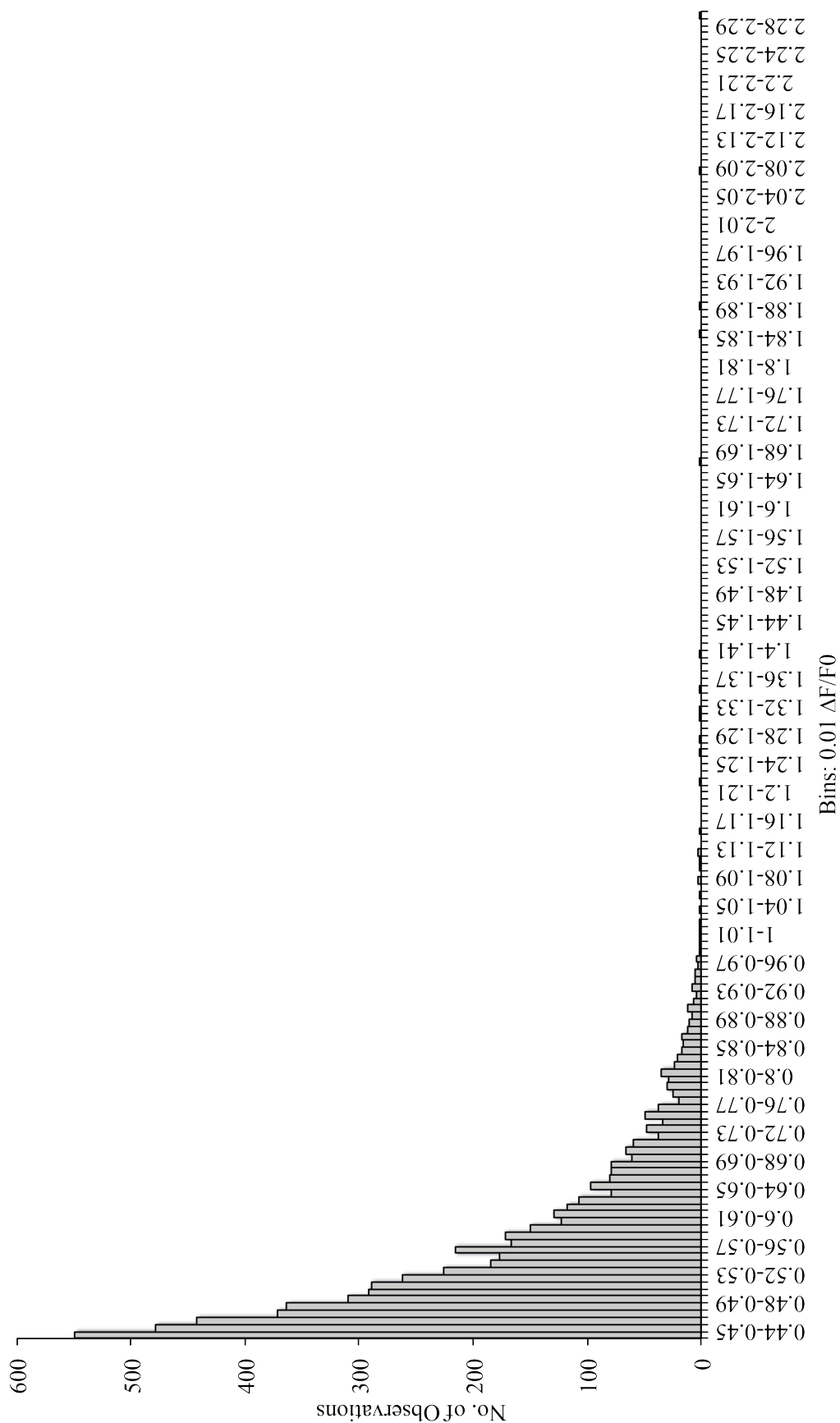


Figure 5.20: Frequency Histogram of Maximum Intensity Change $> 2SDs$ above BL Mean ($n=20$).

small modes visible in the ‘tail’ of this decaying function which occur at a period of $0.05 \Delta F/F_0$. Because these are so close together ($\sim 0.05 \Delta F/F_0$) it was assumed unlikely they represent multiples of real unitary calcium events. Though it should be noted that the order of magnitude is comparable to Parker’s optically-assessed calcium blips through IP_3 receptors ($0.1 \Delta F/F_0$; Smith and Parker, 2009), Thorn’s smallest imaged IP_3 signals in pancreatic acinar cells ($<0.1 \Delta F/F_0$; Fogarty et al., 2000), and Nelson’s elementary TRPV4 signals ($0.15 \Delta F/F_0$; Sonkusare et al., 2012). However, because the relationship looks tentative, and the potential unitary event size of $0.05 \Delta F/F_0$ lies below the baseline standard deviation of $0.08 \Delta F/F_0$, it was prudent to assume that the fluctuations are potentially the result of the summation of various cells’ variability in event amplitude rather than a real assessment of quantal release.

To eliminate this potential inter-cell effect, response frequencies of individual cells were plotted separately. Values were included if they lay above the ‘cut-off’ amplitude (baseline mean + 2SDs) of that particular cell. For each cell, fluorescence values were then normalised to the cell’s putative unitary event (i.e. the first apparent mode in that cell’s frequency distribution). The normalised frequencies for all 20 cells were then recombined to give a population frequency histogram. The result of this analysis is shown below (Fig. 5.21).

Again, a left-skewed Gaussian distribution is the result. This reflects the distribution from the whole population of cells we saw originally (see Fig. 5.18), with a tail that is not clearly polymodal (again, as seen in the whole population data; see Fig. 5.20).

It may still be the case that by choosing only the maximum fluorescence in each cell (which essentially means one pixel), one might eliminate a polymodal distribution for the smaller events, as random fluctuations in individual pixel brightness might be on the same order of magnitude as unitary events. To minimise this intra-cell variability, 51 specific areas of interest within a single cell (as illustrated in Fig. 5.4)

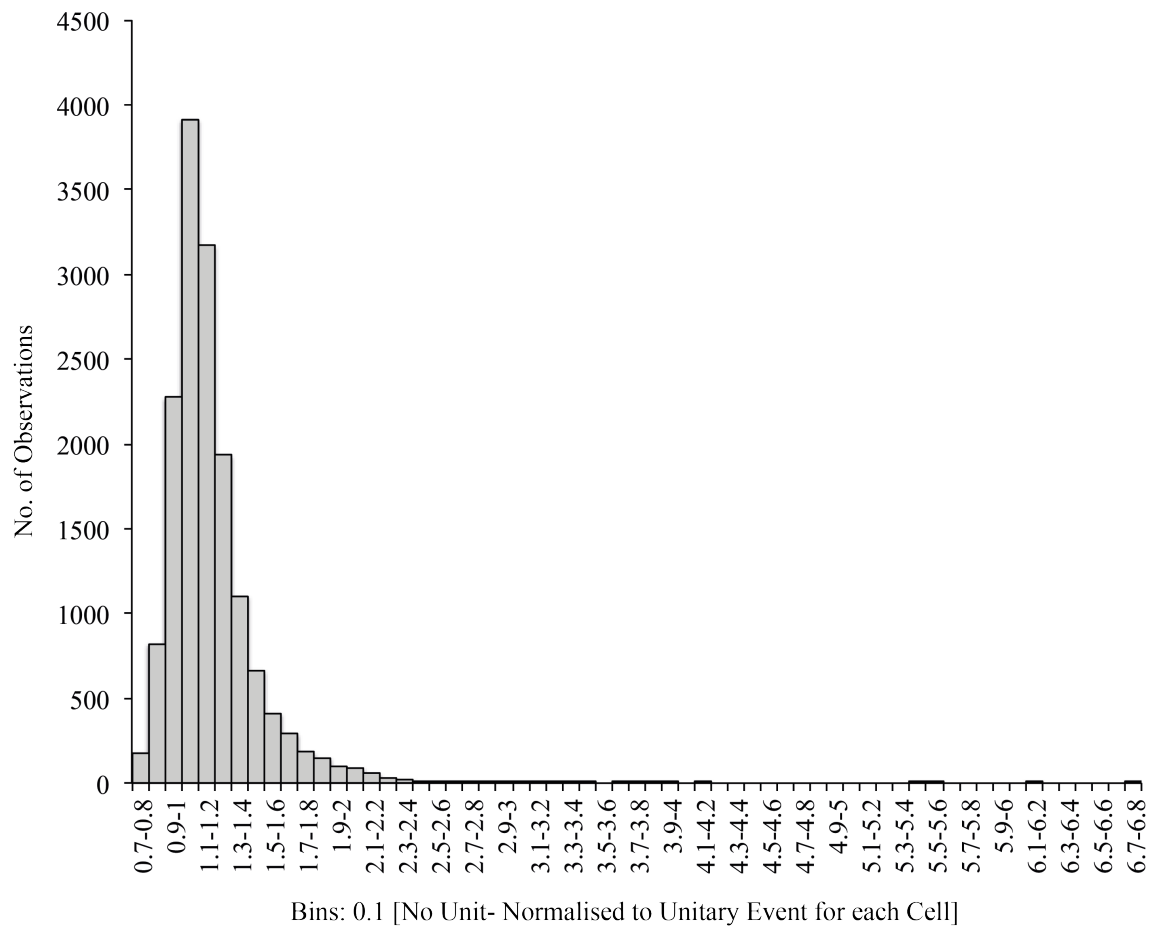


Figure 5.21: Frequency Histogram of Event and Step-level Amplitudes of Calcium Signals. Fluorescence Amplitude Values for each Cell were normalised to the first Mode in its Amplitude Frequency Histogram after Subtraction of its corresponding Baseline (this was assumed to be the Unitary Event); $n=20$.

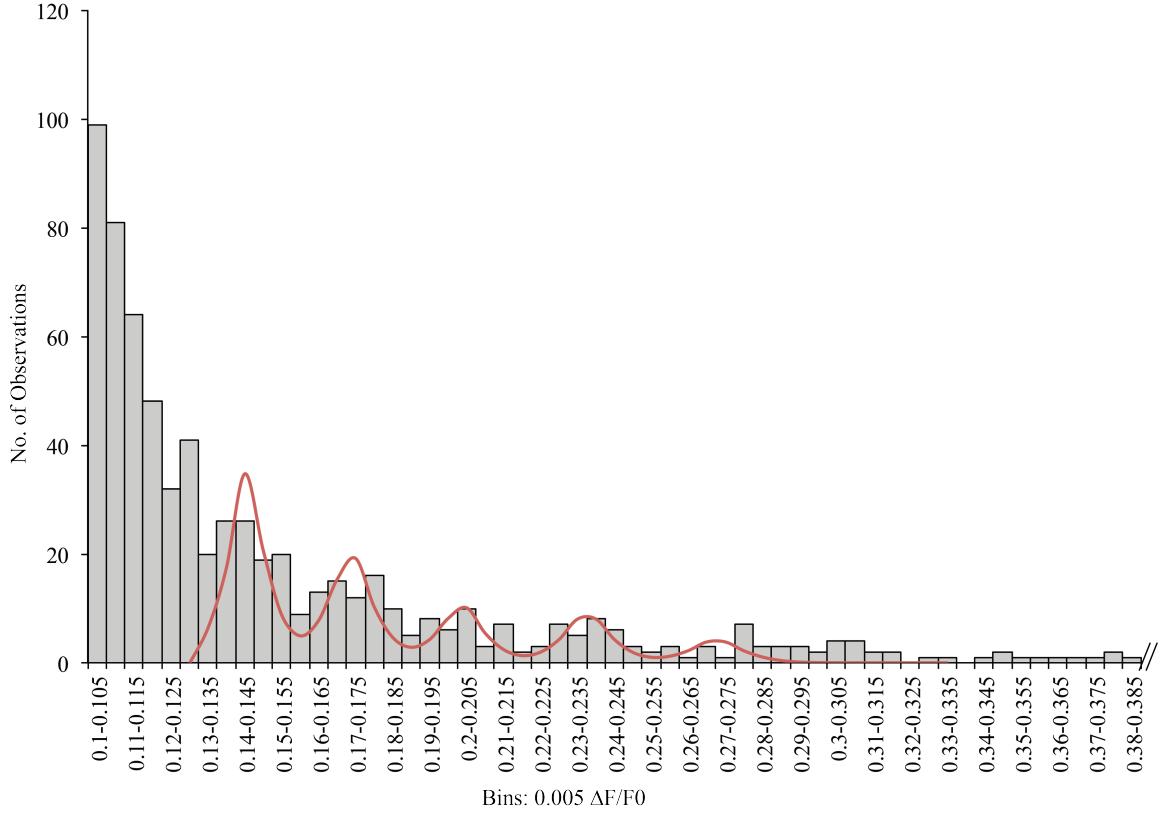


Figure 5.22: *Frequency Histogram of Calcium Event Amplitude across Areas of Interest within a Single Cell ($n=51$). Note: The Histogram was cropped for better resolution. the Actual Maximum Intensity Bin is 2.09-2.095.*

were chosen by hand on the basis of their containing at least one individual calcium event; and these were analysed for their mean amplitudes individually. Analysing a smaller area for its mean, rather than a larger one for its maximum will minimise the effects of individual stray pixels, and will still capture spikes, as they have an area larger than an individual pixel that increases in brightness). The result across 51 areas of interest in one cell after subtraction of baseline can be seen in Fig. 5.22.

Again we see a left-skewed distribution with exponential decay (Fig. 5.22). However, the decay does display modal behaviour akin to that observed in the frequency histogram for all cells (Fig. 5.20). The period between modes is similarly small at $\sim 0.03 \Delta F/F_0$. The five peaks of the putative modal distribution were selected as means for individual Gaussian distributions; and 3 to 4 bins to their left and right

(depending on whether the peak was within one bin or between two bins) used to calculate the respective distribution's standard deviation. The resultant means and standard deviations were used to fit a quintuple Poly-Gaussian function (Sum of five Gaussian functions with Means, SDs: 0.143, 0.007; 0.171, 0.008; 0.201, 0.0089; 0.235, 0.0092; 0.270, 0.0103, respectively) to the data. This is shown as the red line in Fig. 5.22. While this distribution fits; the putative unitary event amplitude is so low at $0.03 \Delta F/F_0$, that the baseline standard deviation, $0.05 \Delta F/F_0$, lies above it. This observation, as well as its order of magnitude is in line with that observed across all cells in Fig. 5.20. It is also in the vicinity of unitary event amplitudes observed by Parker et al. and Thorn et al. for IP_3 calcium blips (Smith and Parker, 2009; Fogarty et al., 2000) as well as Nelson et al. for TRPV4 channels (Sonkusare et al., 2012). So it may be the case that the mean amplitude for NAADP-dependent elementary events is indeed $\sim 0.05 \Delta F/F_0$ (and within this particular cell $\sim 0.03 \Delta F/F_0$); but that the employed experimental data and analysis methods simply do not allow for a visualisation of the modal nature of these events in the early part of the distribution.

5.11 Discussion

It is clear from the above data that NAADP and glucose trigger elementary calcium events in β -cells. Quantal analysis of the events triggered by NAADP (those stimulated by glucose were not used for this as glucose is an unspecific stimulus and may trigger events via multiple pathways) suggests that there exists a unitary event which has a mean amplitude of $\sim 0.05 \Delta F/F_0$. This value is so small that it could represent the calcium release through a single what has been termed calcium release unit (CRU) rather than any larger cluster of channels. Parker even states in his review of elementary calcium events that signals of this magnitude ($0.05 \Delta F/F_0$) could represent release through a single channel (Parker et al., 1996). This means it would be a 'true' unitary event- the atom of calcium signals. While sparks and puffs represented the

smallest discernible release events through RyRs and IP₃Rs for a long time, they have more recently been ‘split’ into their true unitary events using optical quantal analysis with TIRF (Smith and Parker, 2009) or its combination with patch clamping (Wang et al., 2004). As the present study utilised this same advanced technology, which wasn’t available to those initially researching IP₃R- and RyR-dependent events, it seems the equivalent of sparks and puffs were skipped as the smallest discernible events in favour of a blip-like unitary event.

Why is it, then, that the present data show a polymodal distribution only at the very end of the frequency histogram’s ‘tail’; and why does the normalised frequency histogram show no apparent modes?

The latter question is reasonably easily answered. The events were not normalised to the actual unitary event (which is so small that it lies below the distribution mean), but to the first discernible frequency peak 2SDs over the mean. This is likely to be a quintuple or more of the unitary event already, but will also vary from cell to cell. If one now normalises one cell to 5n (where n is the unitary event), one cell to 7n, and one cell to 3n, etc; one is likely to end up with a skewed distribution because practically all events will be located between the first two units (as was indeed seen in Fig. 5.21), when in actual fact they should be providing the different peaks. To normalise events in this way is a remnant of spark and puff analysis, where nothing smaller could be observed, and events were not recorded in nanometer sections; so recruited multiples of sparks were still common enough to create modes on the high amplitude end of the frequency histogram. As it stands, one should normalise to the period between observed modes for each individual cell, as this would represent that cell’s true unitary event amplitude.

None of the above would be an issue, of course, if one could eliminate the large amount of noise that makes the baseline mean and standard deviation (and thus the signal cut-off) so high. If this were not occluding the records, one might well see a reasonable modal distribution all the way through the record and the first discernible

peak in the frequency histogram would actually be the unitary event. The reasons why the present data are inadequate in this way are as follows:

There was a technical problem with the microscope's shutter, causing it to close slightly out of synchrony with the acquisition camera at irregular intervals. This poses a problem for the frame by frame subtraction algorithm as it will lead to the production of a number of artefactual fluorescence change values that were included in the analysis as it was impossible to manually extract those frames.

Additionally, with TIRF, where the field of view is only (at most) 200 nm deep, one does not know whether one is looking at the origin of an elementary event, or some of its 'cloud', if the source of the event is just outside the evanescent field. Especially at the present acquisition rate, where it is impossible to determine the up- and down-stroke of any event, it is impossible to determine whether a particular signal is an in-focus small event or an out-of focus larger one. This again adds to the variability of the data. For this reason, Cheng and colleagues used only artificially triggered sparks in one particular submembrane site (for which they had established the existence of one channel cluster) for their quantal analysis (Wang et al., 2004). The same group had previously demonstrated using mathematical modelling that if events occur in a quantal fashion throughout a sample but are measured without considering their shape and focus, one is unlikely to observe a polymodal distribution (Cheng et al., 1999). Instead, one would observe a left-skewed monotonic decay (see Fig. 5.23). Balke and colleagues produced a similar result (Izu et al., 1998). The authors go as far as claiming that any observation of a poly-Gaussian distribution as described in classical quantal analysis is due to observer bias. And indeed many quantal analysis studies involve a large amount of human selection of events. But even when using a fully automated method, as was done in the present study, and in many others, polymodal distributions can be observed.

Apart from technicalities in data collection and analysis leading to variability in the distribution, there are several probable physiological reasons that would shape the

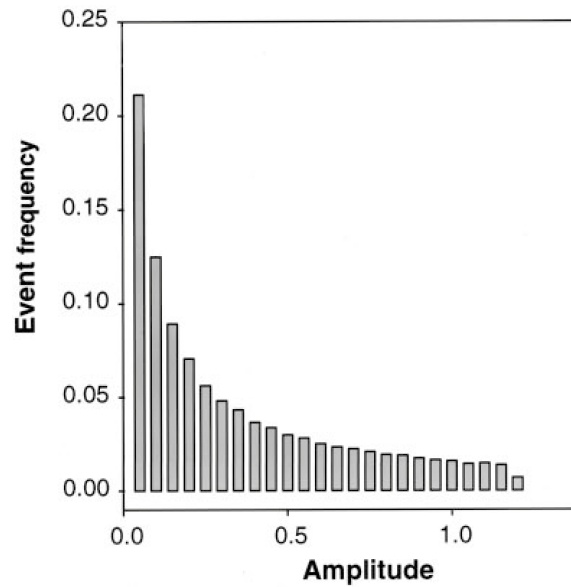


Figure 5.23: Histogram of Predicted Calcium Event Amplitude. Reproduced from Cheng et al. (1999).

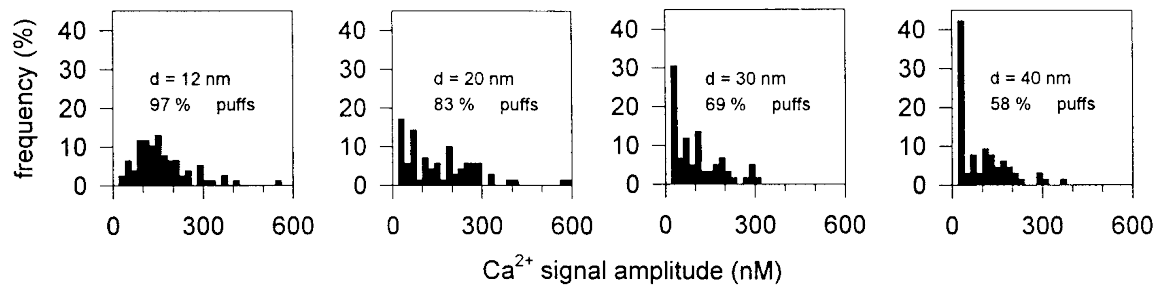


Figure 5.24: Effect of Inter-Channel Distance on Signal Amplitude Distribution. Amplitude Distributions obtained with four virtual 25-Channel Clusters of IP_3 Receptors with a Center to Center Distance of 12, 20, 30, and 40 nm, respectively. With decreasing channel density, the frequency histogram moves from a Gaussian Distribution to a decaying one, where blips are disproportionately more abundant than puffs. Modified from Swillens et al. (1999).

frequency distribution. Firstly, It may be the case that individual release sites within each cell show elementary fluorescence signals of different amplitudes. There may be substantial differences between the fluorescence intensities of unitary events depending on where in the cell they occur. This could be dependent on how fluo-4 is distributed within the cell (Thomas et al., 2000) or the juxtaposition of channels and stores in individual areas. Same-size events could thus lead to different sized fluorescent signals. Another possible explanation why one may or may not observe modal behaviour of events in any given cell, or any given area of interest within a cell, is that TPCs are likely to interact with other calcium release channels. So depending on whether one happens to be observing an area where there are multiple different organelles or multiple different channels present, one may observe events of different amplitudes. If observed spikes are the result of multiple TPC channels opening, or a mixture of TPC, RyR and IP₃Rs, they may vary in size, as the unitary event for each of these channels may be different, and the result of an interaction between the channels different yet again from a linear summation of their individual release quanta. TPCs themselves don't support CICR (Zhu et al., 2010b), so any triggering of larger signals must be via other receptors or via NAADP stimulating multiple TPCs simultaneously. Additionally, it is not clear whether NAADP binds to TPCs themselves, or whether it might act via a binding protein (Lin-Moshier et al., 2012; Walseth et al., 2012b), which in turn may also stimulate channels in addition to TPCs. Interestingly, Swillens and colleagues (Swillens et al., 1999) used mathematical modelling to show that the shape of the frequency distribution of event amplitudes can give insight into the distribution of the channels that give rise to it. They showed that depending on how closely packed channels (in this case virtual IP₃Rs) within a cluster were, their respective amplitude frequency histograms would change in characteristic ways. This is illustrated in Fig. 5.24. Thus the frequency histogram of the present study might suggest a density for sub-membrane clustering of channels in β -cells. In any case the system in itself is so

complex that the observed results are likely to be reflective of this complexity and not in the first instance an artefact of the acquisition or analysis procedures.

5.12 Conclusions

The above experiments demonstrate that there are elementary calcium release events in pancreatic β -cells, which are likely quantal in nature. These can be visualised in the immediate vicinity of the cell membrane using TIRF microscopy. They can be triggered by NAADP and glucose. When using glucose at experimental concentrations, thapsigargin is necessary to unmask the events; when using physiological level glucose stimuli, events can be seen without thapsigargin. The observed calcium spikes are not the result of influx through the cell membrane, as they persist in the absence of extracellular calcium and in the presence of high EGTA. It is unlikely that the primary source of calcium for the events is the ER, as they occur after pre-incubation with thapsigargin. The calcium spikes occur on top of a steady calcium ramp, which may be important for two-phase insulin secretion in its own right. Elementary calcium release can occur spontaneously in the presence and absence of extracellular glucose. The complexity of the system makes it difficult to isolate individual channels or calcium stores pharmacologically to assess their roles. However, it is likely that multiple stores and channels; notably acidic stores and TPCs play an important part in this process. The fact that the spiking events are most striking when triggered with NAADP and the fact that they are localised just beneath the membrane (the primary location of insulin granules, the bulk of the acidic organelles in the β -cell; Orci, 1985) is a strong suggestion that these are the stores involved. More research is needed to determine the precise origin of the spikes as well as that of the underlying ramp. It will be crucial to determine the spatial and temporal resolution of individual events to allow for better quantal analysis. Once characterised in elementary terms, it will be fascinating to explore which ‘recruitment options’ the unitary events use to build multiquantal and

ever larger calcium signals, how the calcium spikes affect exocytosis, how inhibiting cell metabolism affects them, whether desensitising concentrations of NAADP can inhibit them, whether IP_3 , tolbutamide, insulin, ACh or depolarisation can trigger them. Furthermore it would be interesting to see if these events occur in the same form in human β -cells, and whether they do in β -cells of diabetics.

Chapter 6

General Discussion

6.1 Summary

This thesis initially presents a characterisation of glucose-induced calcium responses of single dispersed mouse pancreatic β -cells using TIRF microscopy. Responses are classified into four main types and their relative frequency of occurrence assessed. The nature of spontaneous activity in β -cells is examined. Whole cell calcium responses as imaged by epifluorescence microscopy are compared to sub-membrane calcium changes measured by TIRF microscopy. This is followed by a pharmacological confirmation, using TIRF, of the involvement of acidic stores and NAADP receptors in these responses. The recently generated TPC knockout mice are assessed to investigate the role of TPCs in glucose-induced β -cell calcium responses. Furthermore, this thesis documents the existence in pancreatic β -cells of NAADP-dependent localised elementary calcium release events in analogy to calcium sparks and puffs mediated by RyRs and IP₃Rs, respectively. These events were found to occur not only through stimulation with NAADP-AM, but also following a rise in extracellular glucose concentration from 3 G to 6 G. When high glucose concentrations were used for stimulation (16.5 G), the increased activity of SERCA masked the observed events. Pretreatment with thapsigargin allowed for their un-masking in those circumstances. The removal of extracellular calcium not only failed to affect the events; it was necessary for their visualisation. The experiments were repeated in the presence of 5 mM EGTA to

demonstrate unequivocally that they are not the result of extracellular calcium influx. Optical quantal analysis revealed a mean unitary event amplitude of $\sim 0.05 \Delta F/F_0$ for these events; which is consistent with previous reports on elementary calcium events in other cell types through RyRs and IP₃Rs (Parker et al., 1996; Smith and Parker, 2009).

6.2 β -Cells as Models for the Study of Calcium Signalling

Unlike the classic model cell used in the investigation of NAADP signalling, the sea urchin egg (see for example Genazzani et al., 1997), β -cells do not have a resting state (or not unless they are forced into experimental conditions so artificial that one may not conclude much from resulting activity; Islam, 2010). β -cells, by virtue of controlling insulin secretion in response to a constantly fluctuating and never subsiding parameter, blood glucose, always show a certain level of activity. As was seen in the present study, this is reflected in their cytosolic calcium levels. In addition to being spontaneously active, β -cells are variable in their calcium responses to insulin (Johnson and Misler, 2002), and glucose (shown in the present study, amongst many others). Reaction times to glucose for example seem idiosyncratic to particular cells, islets, animals, and as presently demonstrated, genetic backgrounds of animals (Pakhtusova et al., 2003; Gustavsson et al., 2006).

In light of this high level of individuality among β -cells' responses to glucose it may be time to reintroduce the concept of a β -cell's 'calcium fingerprint', as coined by Prentki in 1988 (Prentki et al., 1988). This could be seen as something of a 'calcium phenotype'- the result of a particular expression pattern of channels and receptors that allow for particular patterns of calcium responses to take place. More recently it was shown that patterns of calcium responses from whole islets are varied between

animals, but islets from one animal tended to show a similar pattern that was linked to matching insulin release oscillations in these animals (Nunemaker et al., 2005).

Just as β -cells are often forced into artificial resting states during experiments; they are also forced into artificial activation states by stimulation with exaggerated concentrations of stimulatory glucose, compared to what would occur in the β -cells' physiological environment. The present study demonstrated that stimulation with high glucose concentration and consequently raised SERCA activity (Roe et al., 1994) obscure more subtle changes taking place within the cell. These were unmasked when cells were stimulated with glucose concentrations in the physiological range, or when the consequence of high glucose stimulation (very high SERCA activity) was inhibited using thapsigargin. This was the protocol that led to the discovery of the elementary calcium events described in the present study.

β -cells also do not exist in isolation- the combined responses of groups of cells, islets, and finally the whole pancreas determine insulin secretion into the portal vein. All these levels of organisation are important, and it should not be assumed that a single β -cell's response will, or should, mimic that of the whole organ (a criticism often put forward against single cell study; see for example Rorsman et al., 2011). Single cells represent a different level of organisation to studying whole islets or whole perfused pancreata (in the same way that studying neurotransmission is not made redundant by the possibility of studying brain scans). However, it appears that working with β -cell clusters might be a better approach when using TIRF than working with single cells. Not only do they provide a larger, coherent area within the same focal plane in TIRF as demonstrated above; they also show less spontaneous activity (Pakhtusova et al., 2003); possibly due to cell-cell interactions being intact (Jacques, 2009).

6.3 How do the observed Elementary and Global Calcium Responses add to our Knowledge of Calcium Signalling?

With the recent discovery of TPCs as the target for NAADP, and the present discovery of elementary calcium release events triggered by NAADP-AM; it appears that a ‘set’ of signalling tools is being completed in analogy to the two existing sets: IP₃, the IP₃R, and blips; cADPR, the RyR, and quarks, respectively. It was already suggested by Genazzani et al. (1996) that NAADP-mediated calcium release was quantal on the basis of its graded release from sea urchin homogenate. 16 years on, there is some evidence that this might be what is happening. And in the not too distant future we should be certain that it is. Detecting sparks amidst background in the current study was difficult. Optical blurring, which is minimal with TIRF, but still present; out of focus sparks and random sampling lead to the frequency distribution shown in Chapter 5. However, with the use of sophisticated two-dimensional spark detection software (Banyasz et al., 2007), and a higher temporal resolution in the recordings, a much better quantal profile of these events should be achieved (Cheng and Lederer, 2008). In future, with the development of ever more powerful software for the detection and analysis of sparks (without the observer bias if conducted by hand) as well as the development of better fluorescent dyes, one could reach a level of detecting the flux through an individual ion channel. If a pioneer like Heping Cheng states that ‘*we are only beginning to appreciate the unique biophysics and physiology of these unitary calcium events.*’ (Cheng and Lederer, 2008), it is clear that the field of sparkology is very much in its developing stages, and many more calcium release events will be added to the table below in the near future. Table 6.1 revisits the various elementary calcium signals introduced in Chapter 1 with their amplitudes (Niggli and Shirokova, 2007). Note that the *Blink*, the ‘shadow’ of a calcium spark, in ventricular myocytes

is of the precise order of magnitude as the presently observed calcium release events. In his 1996 review of IP_3 -induced sparks, Parker states that the mean blip amplitude was $0.022 \Delta F/F_0$ (Parker et al., 1996). The results of the present investigation are in line with these previous amplitude estimations for other calcium channels.

Event	Tissue	Cell Type	Amplitude
Spark	Heart	Ventricular Myocyte	$100 - 200nM, \sim 2\Delta F/F_0$
	Skeletal Muscle	Muscle Fibre	$\sim 2\Delta F/F_0$
	Vessel	Smooth Muscle Cell	up to $\sim 4\Delta F/F_0$
	Ileum		
	Ureter		
Quark	Heart	Ventricular Myocyte	30 nM
Ember	Skeletal Muscle	Muscle Fibre	$\sim 0.2\Delta F/F_0$
Spike	Heart	Ventricular Myocyte	$\sim 0.5\Delta F/F_0$
Sparklet	Heart	Ventricular Myocyte	$\sim 0.5\Delta F/F_0$
Puff	Ovary	Oocyte	$100 - 200nM$
	Epithelium	HELA	
Blip	Ovary	Oocyte	$10 - 30nM$
	Endothelium	CPAE	
	Epithelium	HELA	
Mark	Heart	H9C2	$\sim 0.6\Delta F/F_0$
Scrap	Heart	Ventricular Myocyte	$\sim 25\%$ reduction
Blink	Heart	Ventricular Myocyte	~ 0.05 reduction in $\Delta F/F_0$
Skrap	Skeletal Muscle	Muscle Fibre	$\sim 7\%$ reduction

Table 6.1: *Sparkology: Events in different Cell Types and their Peak Amplitudes (Abbreviated from Niggli and Shirokova, 2007).*

The discovered protocol for visualising elementary events is intriguingly simple. Pre-incubation with, but experimentation without, calcium and thapsigargin. As suggested in the introduction, the majority of experimenters did this neither with calcium nor with thapsigargin; which is why these events were missed by protocols that otherwise would have revealed them. The moment a cell is ‘washed’ with calcium-free medium for 10 min (or probably less), all calcium responses to glucose apart from the SERCA ‘dip’ are eliminated. This strongly suggests that the spikes observed are the result of calcium release from thapsigargin-insensitive intracellular stores that would run empty if pre-incubated with calcium-free medium, and are reversibly blocked by high concentrations of thapsigargin. Some of the observed events might likely be insulin containing vesicles moving in and out of the evanescent wave of the TIRF

microscope; but they are very different in nature from the rapid fluxes that are the spiking calcium events (though they would certainly add another ‘layer’ of noise to the spark analysis). It will be interesting to see if events of this nature occur in other species or tissues, also.

6.4 What can TPC Knockout Mice tell us about β -Cell Physiology?

In principle, knockout mice are an excellent way of elucidating the roles of individual proteins, such as ion channels or receptors, in cellular processes. This method of investigation has been used most successfully in the β -cell previously. Sur 1 and Kir 6.2 (K_{ATP} channel subunit) knockout mice, as well as TRPM5 knockout mice have been most useful in the examination of β -cell physiology (Miki et al., 1998; Seghers et al., 2000; Colasoul et al., 2010). In the case of TPCs it appears that firstly there is significant compensation between the two TPCs: In TPC2 KO, TPC1 is over-expressed as a consequence, and vice versa (Chuang, 2011). Further to this it is still not entirely certain whether NAADP binds to the TPCs themselves, or whether it acts through a binding protein (Walseth et al., 2012b). If the latter were the case; all it would take is for the binding protein to also connect with targets other than TPCs, for the knockout effects to look vastly different from what is expected. Additionally, it was demonstrated in the present study that the genetic background on which a knockout is generated may cause a significant change in the phenotypical presentation of that knockout. The difference after rederiving the TPC2 KO is the most plausible explanation of the disparate results between the present investigation and previous studies on TPC2 KO mice (Arredouani et al., submitted for publication).

6.5 What Relevance do Elementary Calcium Signals and TPCs have for Human β -Cell Physiology and Diabetes?

6.5.1 Ignition: TPCs in Stimulus-Secretion Coupling

One of the reasons why the K_{ATP} channel hypothesis is so attractive (apart from its beautiful simplicity) is the fact that current anti-diabetic drugs of the sulfonylurea class, which are efficacious in the clinic, are known to act via this route; see Ashcroft and Rorsman (2012) for review. However, as has been the case with many other drugs; their therapeutic success, mediated through a particular pathway, does not necessarily bear resemblance to the molecular pathway underlying the aetiology of the disease in question. It is suggested here that stimulus-secretion coupling is not primarily dependent on the K_{ATP} channel; but on calcium signals from intracellular stores; CICR, and L-type calcium channel dependent influx leading to complex calcium oscillations. The present investigation suggests that the elementary trigger events for this pathway may be mediated by NAADP. The K_{ATP} channel is powerful and necessary; but it is suggested here that it resembles the ‘handbrake’ of the car that is the β -cell; rather than its ignition. K_{ATP} channels are likely to control the responses that take place at the extreme ends of the blood glucose spectrum, but not necessarily those that occur within the normal range in a non-diabetic patient (Islam, 2010). This is why it is an excellent drug target, and why its malfunction has devastating consequences (Ashcroft, 2010).

As glucose uptake by β -cells is not limited by GLUT; the rate-limiting step in its metabolism is glucokinase. If there were a stimulus-secretion pathway triggered by glucose which bypassed its metabolism, it would be important that this pathway involves a rate-limiting step, also; a form of self-desensitisation. NAADP with its bell-shaped concentration-response curve is ideally placed as the messenger for such a

pathway; being self-limiting by nature (Patel, 2003). This self-desensitisation could also underlie the phenomenon that a second glucose-induced calcium response can be difficult to elicit in β -cell experiments, and is usually smaller in amplitude than the first (see Pakhtusova et al., 2003, and references therein). This resembles the studies on the self-desensitisation of the NAADP receptor in sea urchin eggs, where a second NAADP stimulus was significantly inhibited, but responses to cADPR and IP_3 were unaffected (Genazzani et al., 1996).

6.5.2 Potential Future Targets for Anti-Diabetic Drugs

If it were the case that stimulus-secretion coupling depended on a pathway other than the K_{ATP} channel dependent pathway; this would provide an array of potential new drug targets, were it to be even partially elucidated. A particular advantage of targeting molecules other than the K_{ATP} channel might be that effects could still be controlled by the current environment of the β -cells in question. Many of the intracellular store calcium channels for example have dual gating (by calcium and pH, by calcium and various agonists, by calcium and voltage). In principle, such targets would be attractive, as a compound interfering with only one of these gating mechanisms would provide a use-dependent agonism, which, in the case of diabetes, would mean a drug that were unlikely to potentially cause dangerous hypoglycaemia, as can happen with insulin and drugs of the sulfonylurea class (Rodbard et al., 2009). Naturally, this is all purely speculative. But it will be very interesting to see how this field of research develops and what pharmaceuticals might arise from it in the future.

6.6 Recommendations for Future Work

6.6.1 Spark Detection and Quantal Analysis

It will be very important to record the observed elementary calcium events at higher temporal resolution. Further, they must be analysed with a specialised two-dimensional spark detection software which considers this temporal dimension as well as the spread of a spark in space. Spark detection algorithms that use functions akin to a point-spread or Gaussian function to detect sparks in two dimensions could be used (Banyasz et al., 2007).

More research is needed into the origin of these events- which agonists can trigger them? Which calcium stores are involved, and in which order? How does glucose bring this about? How are the observed events linked to exocytosis and insulin release?

It could be useful to record elementary events in parallel with electrophysiological recordings of the same cell (Misler et al., 2009); while possibly simultaneously imaging vesicle exocytosis (Hoppa et al., 2009; Li et al., 2011), or, as recently described, intracellular glucose concentration using a fluorescent glucose sensor (Kaminski et al., 2012).

6.6.2 Knockout Mice

For future experiments it will be important to transfer the TPC2 KO onto the same pure background as the DKO. This will allow for direct comparison between the two types of knockout. It might also be useful to generate an organ-targeted knockout and/or an inducible knockout (see Piret and Thakker, 2011, for review). The advantage of the former being its localised effect, rather than globally affecting the animal's physiology; the latter being that it is likely to prevent compensatory over-expression of other genes during development of the animal.

6.6.3 Pharmacology and Imaging

It would be interesting to investigate whether the localised calcium increases are by themselves enough to trigger exocytosis without calcium influx across the membrane. One could attempt, for example, to trigger exocytosis of insulin in calcium free medium by stimulating directly with NAADP. This could be visualised using an acidic store marker such as LysoTracker, or a classic exocytosis imaging tool such as FM 1-43 used in combination with TIRF (Ivannikov et al., 2012). To avoid any possible effect that the cell membrane visualised in TIRF being adhered to glass by L-lysine might have, one could direct the laser of the TIRF microscope in such a way as to reflect off the cell-water interface on the upper side of the cell. It would certainly be worthwhile to combine patch clamp with TIRF imaging for parallel recordings of calcium elevation and membrane potential. This set-up would have the added advantage of being able to infuse agents such as NAADP and Ned-19 directly into the cell. This would circumvent the uncertainty encountered in the present study of whether a compound is reaching its intracellular target at all, and at what concentration; because it might be relying on transporters in the cell membrane to do so. Certainly, the effect of cis Ned-19 ought to be investigated. The effect of a mitochondrial uncoupler on the glucose-induced elementary events would also be an interesting experiment to conduct. It would provide insight into the upstream or downstream placement of NAADP production in the stimulus-secretion pathway with respect to metabolism. Finally it would be exciting to investigate these processes in human β -cells and see whether they have any bearing on their stimulus-secretion coupling and malfunction thereof in diabetes mellitus.

Appendix

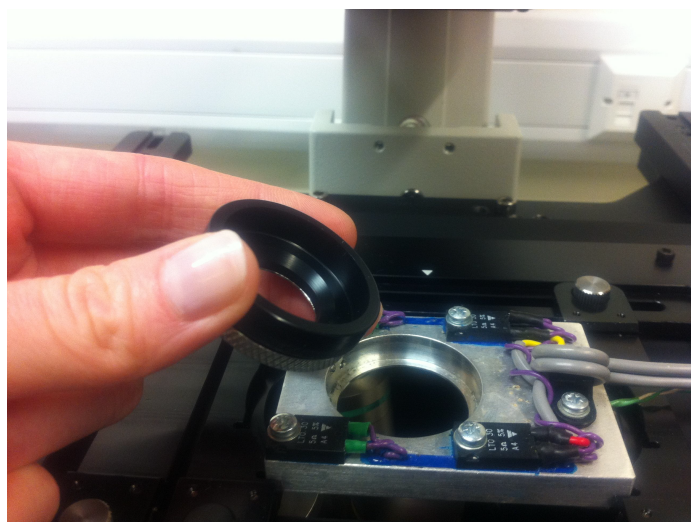


Figure A.1: *Recording Chamber and Heated Headstage used for TIRF Experiments*

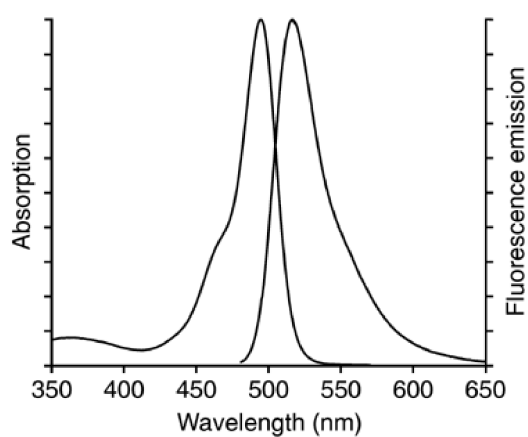


Figure A.2: *Absorption (left) and Fluorescence Emission (right) Spectra of Calcium-saturated Fluo-4 (Reproduced from Invitrogen Life Technologies Corp., 2012)*

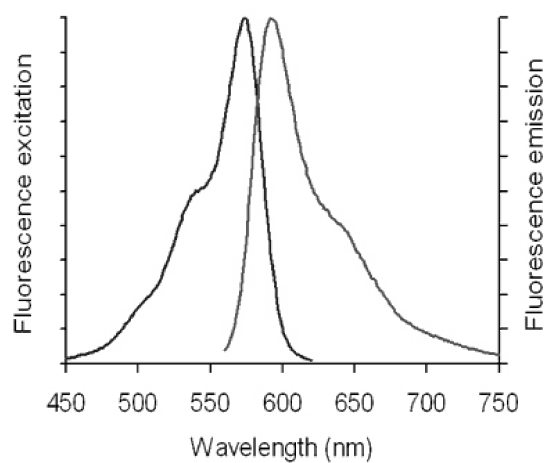


Figure A.3: Absorption (left) and Fluorescence Emission (right) Spectra of LysoTracker Red
(Reproduced from Invitrogen Life Technologies Corp., 2012)

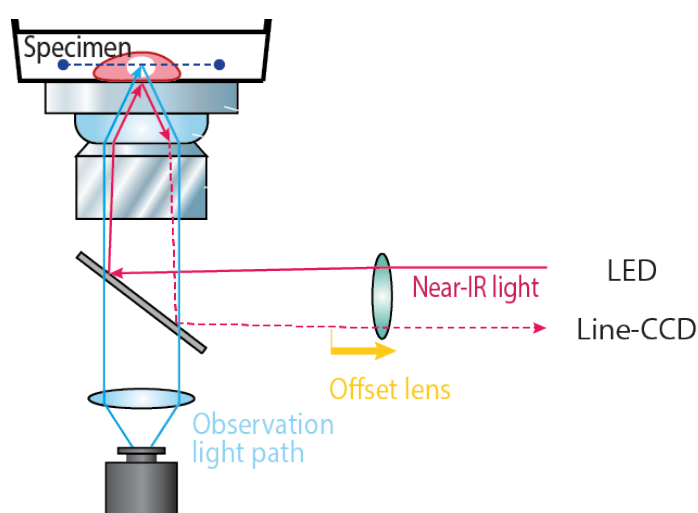


Figure A.4: Perfect Focus System (PFS)
(Modified from Nikon Instruments Inc., 2007)

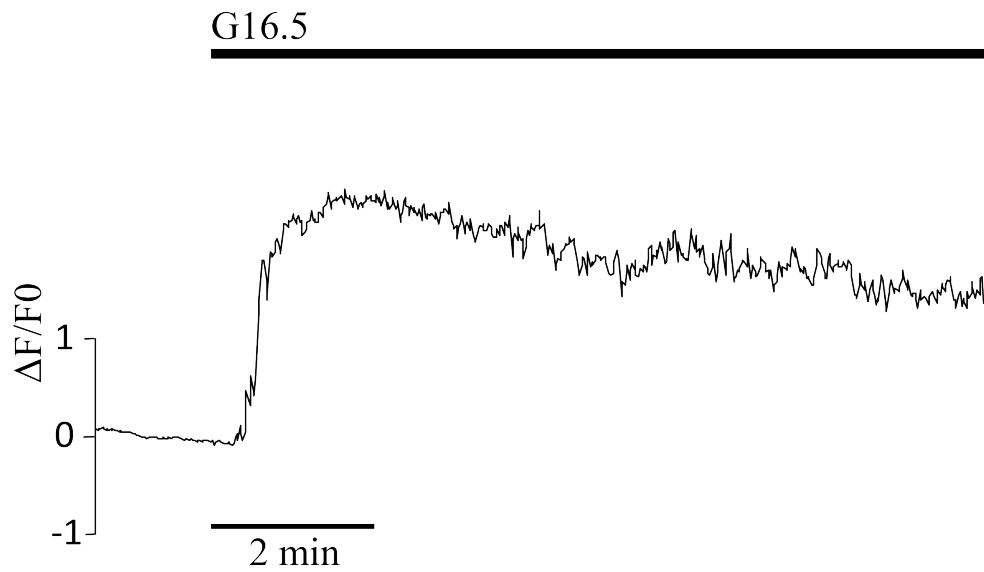


Figure A.5: Representative Trace of a β -Cell Calcium Response to Glucose as imaged with Fluo 5-F.

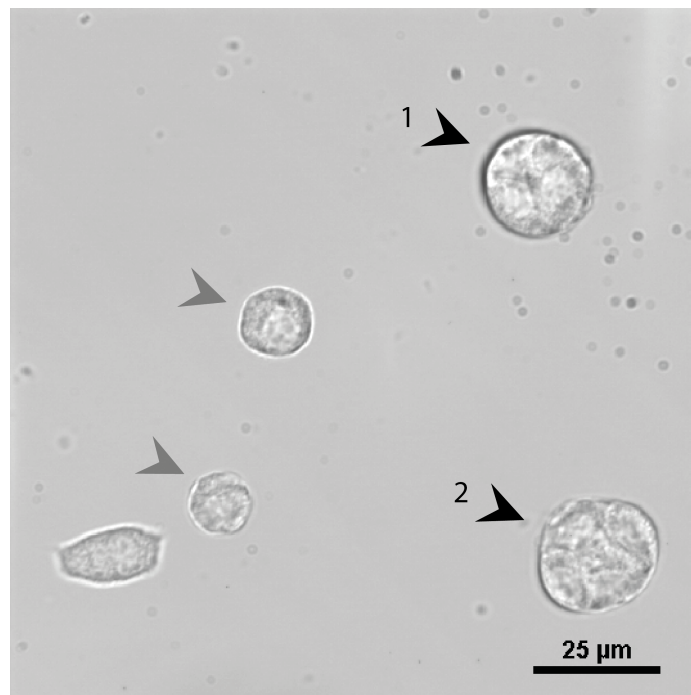


Figure A.6: Brightfield Image of the Cells and Clusters shown in Movie 1 (60x Magnification). Note the two Clusters marked with black Arrows, and two single Cells marked with grey Arrows. In the bottom left Corner is a non- β -Cell. Movie 1 shows the Responses to Stimulation with 16.5 mM Glucose. Note the unsynchronised Calcium Oscillations of Cluster 1; and synchronous Oscillations of Cluster 2.

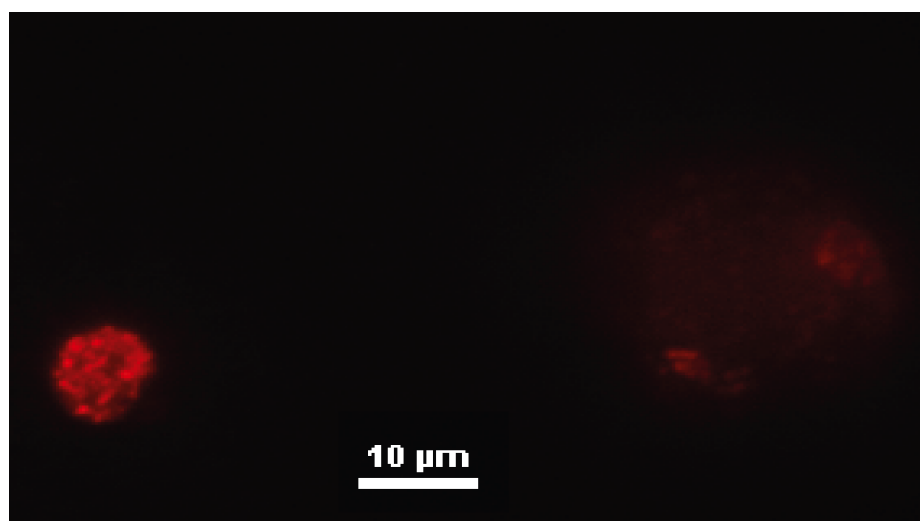


Figure A.7: *LysoTracker* labelling of a single β -Cell (left) and a β -Cell Cluster (right) as viewed with TIRF under 60x Magnification.

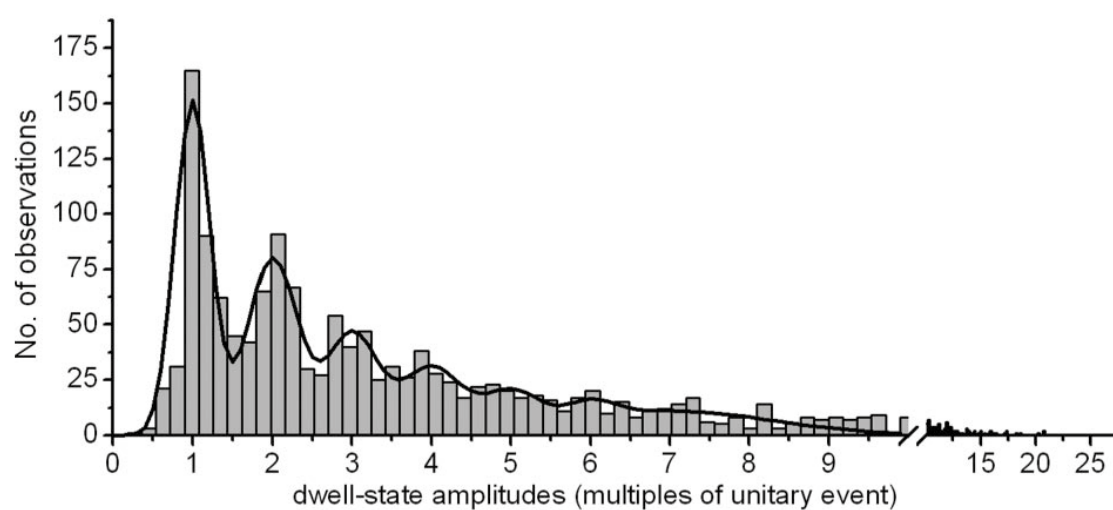


Figure A.8: *Quantal Analysis of Calcium Puffs*
(Modified from Smith and Parker, 2009)

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Statement of Authorship

The following parts of this thesis are not my own work:

The knockout animals used were generated by members of the Department of Pharmacology, University of Oxford, in collaboration with MRC Harwell (Oxfordshire, UK). The generation and genotyping of the animals was complete before the start of the present study. The methodology described in Chapter 2 is a summary of its description in Chuang (2011).

All electron microscopy images presented in this thesis were prepared by Wendy Tynan of the Department of Pharmacology, University of Oxford, from islets which I purified at the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM). This data is presented in Chapter 4; and the corresponding methodology in Chapter 2.

Paula Heister, August 2012