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Local and regional control of calcium dynamics in the pancreatic islet

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Abbreviations: CICR, Ca^{2+} induced Ca^{2+} release; GFP, green fluorescent protein; IP_3, inositol 1,4,5 *tris*phosphate; K_{ATP}, ATP-dependent K^+ channel; MCUa, mitochondrial uniporter a; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+} ATPase; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; T2D, type 2 diabetes; VDCC, voltage-dependent Ca^{2+} channel

Abstract

Ca^{2+} is the key intracellular regulator of insulin secretion, acting in the beta cell as the ultimate trigger for exocytosis. In response to high glucose, ATP-sensitive K^+ channel closure and plasma membrane depolarisation engage a sophisticated machinery to drive pulsatile cytosolic Ca^{2+} changes. Voltage-gated Ca^{2+} channels, Ca^{2+}-activated K^+ channels and Na^+/Ca^{2+} exchange all play important roles. The use of targeted Ca^{2+} probes has revealed that during each cytosolic Ca^{2+} pulse, uptake of Ca^{2+} by mitochondria, endoplasmic reticulum (ER), secretory granules and lysosomes fine-tune cytosolic Ca^{2+} dynamics and control organellar function. For example, changes in the expression of the Ca^{2+} binding protein Sorcin appear to provide a link between ER Ca^{2+} levels and ER stress, affecting beta cell function and survival. Across the islet, intercellular communication between highly interconnected "hubs", which act as pacemaker beta cells, and subservient "followers", ensures efficient insulin secretion. Loss of connectivity is seen after the deletion of genes associated with type 2 diabetes (T2D) and follows metabolic and inflammatory insults that characterize this disease. Hubs, which typically comprise ~1-10 % of total beta cells, are repurposed for their specialized role by expression of high glucokinase (*Gck*) but lower *Pdx1* and *Nkx6.1* levels. Single cell -omics are poised to provide a deeper understanding of the nature of these cells and of the networks through which they communicate. New insights into the control of both the intra- and intercellular Ca^{2+} dynamics may thus shed light on T2D pathology and provide novel opportunities for therapy.

Introduction

A likely role for Ca^{2+} in the control of insulin secretion was first shown by experiments demonstrating that the release of the hormone in response to fuel secretagogues was blocked in the absence of these ions [1] (and references therein). A role for Ca^{2+} as a cardinal trigger for secretion was later enshrined in the "consensus" model of glucose-induced insulin secretion, which involves activation of glycolytic and mitochondrial ATP synthesis, closure of ATP-sensitive K^+ channels (K_{ATP}), plasma membrane depolarisation and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) [2] (Fig. 1). The contributions of intracellular Ca^{2+} transport pathways, and of other intracellular ion channels, in the generation of these signals remains more controversial, and will be discussed later.

Our focus in this contribution will thus be the role and regulation of Ca^{2+} dynamics in and between beta cells. For a more comprehensive review of the role of these ions in the regulation of secretion from other islet neuroendocrine cells, the reader is referred to recent articles, e.g. [3,4].

Key technical developments in Ca^{2+} signalling

A full appreciation of the control and likely importance of glucose-triggered intracellular Ca^{2+} changes in beta cells was significantly enhanced by the development by Tsien and colleagues [5] of chemically-synthesized, low molecular weight and intracellularly-trappable dyes such quin-2 [6] and later fura-2 [7-9]. These optical probes allowed fluctuations in free Ca^{2+} to be reported in real time in single living cells and, subsequently, at the subcellular level, though fluorescence imaging. This technique thus allowed the elaboration of earlier biochemical studies, based on the use of radioisotopes such as ^{45}Ca, and revealed, for example, the importance of Na^+/Ca^{2+} exchange in Ca^{2+} extrusion [10], as well as regionalisation of Ca^{2+} responses to glucose and other stimuli [8].

A limitation of the above class of probes is, however, that they do not usually allow Ca^{2+} to be measured selectively in subcellular organelles. This restricts studies aimed at understanding the molecular mechanisms (transporters, channels etc) which mediate Ca^{2+} flux across the relevant membranes. Alternative probes, developed shortly after the fluorescent reporters, by Pozzan and Rizzuto [11], and deployed by us and others in beta cells [9,12,13], exploited instead natural bioluminescent sensors, such as aequorin from the jellyfish *Aequoria victoria*. Whilst in earlier studies these proteins had been purified and introduced directly into cells, for example after skinning of muscle fibres [14], the development of recombinant technologies meant they could be expressed from the corresponding cDNA. Importantly, after fusion of these cDNAs with those encoding suitable targeting sequences, the bioluminescent probes could be targeted directly to selected subcellular locations. The targeting domains usually involved short peptide sequences or, in some cases, a full length protein [13,15]. Transfection with the corresponding plasmids (or expression from viral constructs), and reconstitution with the prosthetic group coelenterazine, was then coupled to suitable (and usually highly sensitive) detection systems: a simple photomultiplier tube usually sufficed [16], though subsequent developments used in-sequence photocathode arrays for imaging [17].

The first chimaeric probe to be used in this way in beta cells was an aequorin, linked to the leader sequence of mitochondrial cytochrome *c* oxidase subunit VIII [18], thus localising it to the mitochondrial matrix [9]. Probes targeted to the endoplasmic reticulum (ER) and Golgi [19], as well as secretory granules [19], were subsequently deployed, and will be discussed in more detail below. Although later superseded by more sensitive fluorescent recombinant probes based on green fluorescent protein (GFP) [3,20], much of our current knowledge of organellar Ca^{2+} dynamics in beta

cells was based on the use of the above. GFP-based probes, in contrast, rely on Ca^{2+}-induced changes either in the intrinsic fluorescence of the GFP backbone (created by the introduction of a Ca^{2+} sensing domain onto a circularly-permuted fluorophore) or the Ca^{2+}-dependent change on the interaction between two spectrally-shifted GFP variants (Förster resonance energy transfer; FRET), again controlled by Ca^{2+} binding to peptide linking the pair. Table 1 provides a list of chemical and recombinant Ca^{2+} probes and examples of their use in beta cells (or other tissue where information is not available).

The following section summarises the key findings made using the above targeted probes in beta cells. These are summarized in Figure 1.

Localisation of glucose-induced Ca^{2+} changes in beta cells

Cytoplasmic Ca^{2+} levels in beta cells are maintained *via* a complex interplay between Ca^{2+} entry (predominantly through VDCC), Ca^{2+} uptake and release from/into intracellular stores, organelles and secretory granules, and Ca^{2+} extrusion *via* plasma membrane pumps (*e.g.* Ca^{2+} ATPase) and exchangers (*e.g.* Na^{+}/Ca^{2+} exchanger). Alteration at any point in this regulatory cascade – either as a result of changes in energy metabolism and hence K_{ATP} channel closure, or in the expression or post-translational modification of Ca^{2+} channels/pumps/exchangers – will lead to alterations in beta cell Ca^{2+} concentration, with knock-on effects for insulin release.

Mitochondria: Mitochondrial metabolism is critical for glucose-induced insulin secretion [21]. Correspondingly, alternative fates for glycolytically-generated pyruvate catalysed by lactate dehydrogenase (*LDHA*) and the plasma membrane lactate / pyruvate transporter *SLC16A1* (MCT1) are selectively suppressed, or "disallowed", in the beta cell, i.e. these activities are present at unusually low levels in beta cells compared to all other tissues [22].

Although long considered to serve chiefly as buffers and reservoirs of Ca^{2+} in mammalian cells, mitochondria were ultimately recognised as the *targets* of cytosolic Ca^{2+} increases, whose oxidative metabolism is subject to control by these ions, as a result of work in the 1970s and 1980s by Denton, McCormack and Halestrap [23]. Three NAD^{+}-linked intramitochondrial enzymes, pyruvate (PDH), NAD^{+}-isocitrate (ICDH) and 2-oxoglutarate (OGDH) dehydrogenase are strongly stimulated by Ca^{2+}, either through the activation of the associated protein phosphate phosphatase (PDH) or through direct binding and allosteric regulation [24,25]. Other enzymes involved in mitochondrial oxidative metabolism, including the aspartate-glutamate exchanger Aralar (*Slc25a12*), implicated in the control of beta cell glucose metabolism [26], respiratory chain complexes and the mitochondrial F_1F_o ATP synthase [27], as well as the adenine nucleotide translocase (ANT) (which, as a complex with the phosphate carrier, may create the "mitochondrial permeability transition pore" (PTP), associated with cell death) [28], are also subject to regulation by Ca^{2+} (Fig. 2). Finally, glycerol phosphate dehydrogenase (GPDH) [29] is also subject to control by Ca^{2+}, though in this case Ca^{2+} binding is thought to occur on the cytosolic face of the inner mitochondrial membrane. Additionally, mitochondria are the source of putative coupling factors such as glutamate [30,31] which may contribute to the "K_{ATP} channel-independent" actions of glucose on insulin secretion [32]. The regulation of the production of these factors by Ca^{2+} is presently not well understood. Of note, glucagon-like peptide-1 (GLP-1), which appears to engage this pathway through increases in intracellular cAMP and activation of *Exchange protein directly activated by cyclase* 2 (Epac2, Rapgef4) [33,34] was also found to increase ATP/ADP levels in beta cells in our hands [35,36] though not in others' [37].

As observed in extrapancreatic cells [18], increases in mitochondrial free Ca^{2+} (from <100 nM to 1-20 μM), which exceed those observed in the cytosol (~100 nM to ~500 nM), are apparent upon stimulation of clonal beta cells [9,12] by high glucose or KCl-induced depolarisation. We [38] and others [39] have subsequently argued, in line with the "Denton hypothesis" (above), that mitochondrial Ca^{2+} increases following those in the cytosol provide a further stimulus (feed-forward) for ATP synthesis,

> …..mitochondrial Ca^{2+} increases following those in the cytosol provide a further stimulus (feed-forward) for ATP synthesis…..

thus ensuring an amplification of the glucose-induced increase in ATP synthesis for a given increase in extracellular glucose. Direct imaging of ATP/ADP increases with the recombinant sensor Perceval [38] provided further evidence for this view. Moreover, silencing of the mitochondrial calcium uniporter MCUa [40] led to a blockade of the second phase of ATP/ADP increase in beta cells. Likewise, expression of the Ca^{2+} binding protein S100G, molecularly-targeted to the mitochondrial matrix, blocked glucose-induced NAD(P)H and ATP/ADP increases and reduced the second phase of glucose-stimulated insulin secretion [41]. Conversely, silencing of the mitochondrial Ca^{2+}/Na^{+} exchanger NCLX delayed glucose-induced insulin secretion [42]. At present, a demonstration of the impact of genetic inactivation of MCUa in the beta cell in mice is still awaited. Interestingly, mitochondrial Ca^{2+} and ATP/ADP increases display a stronger dependence on the frequency of cytosolic Ca^{2+} spikes, rather than their amplitude, indicating that the mitochondrial Ca^{2+} uptake systems provides a means of achieving "frequency modulation" of the biological response [43].

Challenging the above view, it has been suggested [44] that, since increases in mitochondrial NAD(P)H are not observed when Ca^{2+} increases are imposed through cellular depolarisation alone (*i.e.* in the absence of glucose), these ions do not stimulate mitochondrial ATP synthesis. Tengholm and colleagues [45] have similarly proposed that, as cytosolic ATP/ADP levels fell upon stimulation of Ca^{2+} influx and rose when the latter was blocked, Ca^{2+} *per force* is an inhibitor of mitochondrial ATP synthesis. Drews et al [44] have also argued that activation of mitochondrial ATP synthesis would tend to oppose the well-known bursting activity of the beta cell, and associated Ca^{2+} oscillations, by leading to further closure of K_{ATP} channels between bursts. It should be emphasised that non-perturbing experiments of the sort presented by Drews and colleagues are difficult to interpret, measuring as they do steady state levels of intermediates, rather than changes in flux: coincident activation of ATP consumption in the cytosol by Ca^{2+} (e.g. for ion pumping, protein synthesis) may thus drive the ADP re-oxidation of NAD(P)H by the respiratory chain. Of note, modelling studies [46] have demonstrated that Ca^{2+} activation of PDH is required in to provide an adequate fit to experimental observations of oscillatory activity in glycolytic flux and cytosolic Ca^{2+}.

Although early studies revealed heterogeneity in ATP levels between islet regions corresponding to a few cells (resolution in this case being limited by the relatively weak photon production by firefly luciferase, used in these studies) [47], others have shown this not to be the case using different approaches (*i.e.* NADH/NADPH imaging) [48], highlighting possible divergence between NADH/NADPH and ATP/ADP fluxes.

Secretory pathway. Ca^{2+} is present at very high levels in the granule lumen, largely bound to insulin hexamers with Zn^{2+}. Total concentrations of Ca^{2+} are thus ~120 mM in this compartment [49]. By contrast, measurements of intragranular Ca^{2+} achieved using a chimeric Ca^{2+} sensor generated by

the fusion of vesicle-associated membrane protein-2 (VAMP2) and aequorin, indicated free Ca^{2+} levels in secretory granules in the ~40 μM range [50]. Importantly, a fraction of the intragranular pool may be mobilized in response to a glucose-generated increase in intracellular nicotinic acid adenine dinucleotide phosphate (NAADP). Whether this, or a lysosomal [51] pool, is then responsible for two pore channel 2 (TPC2) -dependent and localised Ca^{2+} changes which may control plasma membrane potential (by regulating flux through a non-selective cation channel, possibly TRPM4 or TRPM5) is still under investigation. Of note, TPC2 (*Tpcn2*) deletion from the beta cell in mice had no effect on insulin secretion or glucose homeostasis *in vivo* [52], possibly as a result of compensation by TPC1 (*Tpcn1*); simultaneous inactivation of both channels in the beta cell will be needed to test this hypothesis. Interestingly, highly localised depolarisation-induced Ca^{2+} changes were also apparent at the secretory granule surface [53] and may play a role in catalysing exocytosis though interaction with plasma membrane located channels, as described above, or critical soluble N-ethylmaleimide sensitive factor attachment protein (SNAP) and SNAP receptor (SNARE) proteins (e.g. Synpatotagmin 7) [54] located on the granule itself.

Endoplasmic reticulum: The chief systems through which Ca^{2+} is accumulated by and released from the ER are shown in Fig. 1. The ER is an important site for Ca^{2+} clearance, with modelling studies showing that sarco(endo-)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity accounts for ~ 60% of Ca^{2+} removal [55]. Although proposed in one study to cause a decrease in ER Ca^{2+} due to the activation of Ca^{2+}-induced Ca^{2+} release (CICR) [56], subsequent work [20], using a fluorescent probe (the "cameleon", Ycam-4ER) carefully checked for pH-induced artefacts, as well as studies using a targeted aequroin [12], revealed increases in this compartment following stimulated Ca^{2+} influx in response to depolarisation provoked artificially with K^+ ions, or glucose. It is, of course, difficult to exclude the possibility that CICR, although outweighed by Ca^{2+} uptake to give net Ca^{2+} accumulation, does not play a role to limit the latter. In this context, the role of ryanodine receptors in beta cells has been a source of controversy [57]; the most recent and sensitive RNAseq-based studies have, however, revealed low but detectable levels of expression (at the mRNA level) of this family in beta cells from both mice [58] and man [59], with RyR3 and RyR2 being present, respectively, in islets from these species.

It should also be emphasised that, whilst mobilisation of intracellular stores probably plays only a minor role in beta cell responses to glucose, the response to G_q-coupled receptors, e.g. metabotropic glutamate receptors and muscarinic agonists, certainly involves the generation of inositol 1,4,5-trisphoshate (IP_3) and the opening of ER-located IP_3 receptor channels, which leads to Ca^{2+} release [6]. The role of local domains of Ca^{2+} released in this way, and known to be important for the stimulation of mitochondrial metabolism in other (non-excitable) cell types, appears to be less important in beta cells [3].

Interestingly, ER Ca^{2+} depletion, causing ER stress, is associated with beta cell failure and T2D risk [60]. As recently reported [61], the Ca^{2+} binding protein sorcin forms a link between beta cell lipotoxicity and ER Ca^{2+} stores. Sorcin (also called SRI) is a 22 kDa member of the penta-EF-hand family of Ca^{2+}-binding proteins. In the presence of elevated Ca^{2+}, sorcin undergoes a conformational change that triggers its reversible translocation from the cytoplasm to membranes where it interacts with specific target proteins, including RyR, which are subsequently inhibited (Fig. 1). Mice over-expressing sorcin selectively in the pancreatic beta cell are resistant to diet-induced failure of insulin secretion and glucose intolerance [61].

This protection is associated with preserved ER Ca^{2+} stores and a lowering of glucose 6-phosphatase C2 (*G6PC2*) expression. Moreover, sorcin overexpression leads to activation of the

unfolded protein response (UPR) sensor *activating transcription factor 6* (ATF6), suggesting that the latter protein may be involved in the protective actions of this protein. Approaches which increase the expression or activity of sorcin may thus provide a means of slowing or preventing beta cell deterioration in T2D (Fig. 3).

> Mice over-expressing sorcin selectively in the pancreatic beta cell are resistant to diet-induced failure of insulin secretion….

Plasma membrane: While their contribution compared to the ER is small, membrane exchangers and pumps still play an important role in Ca^{2+} homeostasis in the beta cell. Recent studies have shown that inhibition of the sodium-calcium exchanger isoform 1 (NCX1) results in glucose-dependent increases in cytosolic Ca^{2+} concentration and insulin secretion [62]. Similarly, heterozygous inactivation of the plasma membrane Ca^{2+} ATPase (PMCA) leads to intracellular Ca^{2+} accumulation, augmented insulin release and, strikingly, improved beta cell proliferation, viability and mass [63].

Beta cell-beta cell communication: intra-islet connectivity

It has been known for many years that intercellular communication is important for normal insulin secretion. Indeed, early studies noted the presence of a right-shifted glucose concentration-response curve, lowered insulin gene expression and impaired insulin release in dissociated cells *versus* their counterparts residing within the islet setting [64,65]. This is likely due to loss of cell-cell coupling, since seminal studies where the predominant gap junction protein connexin 36 was deleted from beta cells showed perturbed population responses to glucose and impaired insulin pulsatility *in vivo* [66]. More recently, ephrins, cilia and paracrine factors (*e.g.* between alpha and delta -> beta cells) have all been shown to contribute to the intra-islet regulation of insulin secretion [67,68].

Critical to the study of islet wide Ca^{2+} dynamics has been the development of high speed Ca^{2+} imaging as a means of providing further evidence for cell-cell communication across the beta cell population. Combined with modelling approaches, this has provided a remarkable level of detail regarding the role of functional beta cell subpopulations in orchestrating insulin secretion. Evidence for "pacemaker" cells within the islet was first obtained in the early 1990s [69]. Subsequently, work from Benninger, Piston and colleagues, who pioneered the application of fast multicellular Ca^{2+} imaging approaches to islets, showed the presence of distinct phase lags between beta cells located within different islet regions [70], building on earlier observations using patch clamp electrophysiology [71,72]. Demonstrating that the organisation of beta cell activity was more complex than originally envisaged, studies using network theory to decode islet-wide Ca^{2+} signals in tissue slices revealed a nodal topology where a minority of cells host the majority of functional connections in terms of coordinated Ca^{2+} rises [73]. Using analogous techniques, it was shown that a small subpopulation of beta cells was responsible for routing information between cells through the islet.

> …..a small subpopulation of beta cells was responsible for routing information between cells through the islet..

These specialised cells, termed "hubs", were shown to be genuine pacemakers *in situ* in islets by Johnston *et al* [74] (Fig. 4). Here, we combined online analyses with optogenetics to map the beta cell population, allowing hubs to be identified and silenced with pinpoint precision using a diffraction-limited laser spot. Parallel experiments were performed to activate the same subpopulation using the light-activated sulfonylurea, JB253 [75,76]. Thus, inactivation of the best-connected hubs - similar to the previously described nodes [73] - led to a near complete, but readily reversible, blockade of Ca^{2+} dynamics. Conversely, reactivation of hubs (at a sub-stimulatory glucose concentration) led, on average, to re-connection to previously-identified follower cells. Using a cell surface-tethered Zn^{2+} probe, which measures Zn^{2+} released from the insulin hexamer [77], silencing of hubs but not followers decreased insulin secretion, demonstrating the fundamental importance of Ca^{2+} dynamics for islet output. Notably, the hubs were targeted by cytokines to induce islet failure, probably due to their lowered expression of the sarco(endo) plasmic reticulum Ca^{2+} ATPase SERCA2 and susceptibility to ER stress (see later).

In a similar manner, we [78] were able to show that beta cell-beta cell contacts also existed in human islets, although coordinated activity seemed to be driven more strongly by incretin compared to glucose, the latter secretagogue instead engaging communication between local clusters [79]. Importantly, this connectivity was disrupted by glucolipotoxic insults *in vitro,* and we were able to infer, through a strong negative correlation with body mass index, a likely association with insulin resistance in man [78]. It should be emphasized that, although we have reported that incretin-enhanced connectivity [78] was unaffected by aging (in contrast to a strong inverse relationship with body mass index), changes in glucose-dependent connectivity have not so far been reported. However, alterations in Ca^{2+} dynamics with age in mice raise the possibility that connectivity may also be targeted [80].

Further supporting a critical role of connectivity in human islets were studies in which the genome-wide identified T2D-associated genes *T-cell factor-7-like 2* (*TCF7L2*) [81] and *Adenylate cyclase 5* (ADCY5) [82] were deleted in rodent islets or silenced in human beta cells, respectively, leading to weakening of local coordination in response to glucose and impaired insulin secretion. Interestingly, deletion of other T2D-associated genes, *SLC30A8* (ZnT8) [83] or *STARD10* (G.A.R. and P.C., unpublished), from mouse islets, had no impact on Ca^{2+} dynamics or intercellular connectivity. Nonetheless, these findings demonstrate the potential for inheritance of altered Ca^{2+} responses as a factor affecting T2D risk.

Transcriptional control of Ca^{2+} dynamics and modifications in T2D

The regulation of Ca^{2+} channels and other players in Ca^{2+} dynamics is still not well understood although the dysregulation of several of these is hinted by studies of islets from T2D versus control subjects. Thus, analysis of the data from Fadista and colleagues [84] reveals a Gene Ontology (GO) group "Regulation of calcium ion transport" as affected with marginal significance (p=0.07). Future studies with larger numbers of subjects will be needed to confirm or refute this finding. Consistent with these observations, recent studies in rodents from Speier and colleagues [85] have shown a gradual degradation of normal glucose-induced islet Ca^{2+} dynamics in islets transplanted into the anterior chamber of the eye, and exposure of the recipients to a diabetogenic diet, compared to animals maintained on a normal diet. Whether similar changes are observed during the course of prediabetes and diabetes in man remains to be ascertained.

It should be noted in this context that there is increasing (though contested) evidence that a loss of beta cell "identity" – involving a loss of expression of beta cell signature genes and the reactivation of "disallowed" genes and genes usually expressed only in beta cell progenitors - contributes to cellular dysfunction in T2D [86]. Of note, two key transcription factors responsible for the establishment and maintenance of beta cell identity, *regulatory factor X6* (RFX6) [87] and *paired box 6* (PAX6) [88], regulate the expression of multiple Ca^{2+} channels, as revealed by targeted inactivation of these factors in the adult mouse beta cell, leading to reduced cytoplasmic Ca^{2+} fluxes. RFX6 has recently been shown to be a new Maturity Onset Diabetes of the Young (MODY) gene by Hattersley and colleagues (http://biorxiv.org/content/early/2017/01/22/101881), suggestive of a possible role for these changes in monogenic diabetes. Moreover, Ca^{2+} channels constitute a Gene Ontology group identified as selectively changing in *Pax6* null islets [88]. Thus, expression of proteins critical for normal Ca^{2+} homeostasis and signalling is a feature of the mature, differentiated beta cell, and may be altered in T2D to affect beta cell responses to glucose.

Interestingly, recent studies (Mitchell et al, under revision) demonstrate that beta cell–beta cell connectivity can persist, and even be enhanced, in the face of marked "de-differentiation" of the beta cells following deletion of *Pax6*. This manoeuvre is associated with drastic decreases in the expression of other key factors such as Pdx1 and MafA, and a > 60% decrease in insulin levels on a per cell basis, and is accompanied by Ca^{2+} increases of lower amplitude but exaggerated connectivity in response to elevated glucose. Intriguingly, levels of *Gck* are increased, whilst *Gcpc2* (which opposes the action of *Gck*) are strongly decreased. These changes are thus remarkably reminiscent of the characteristics described for hub cells [74] (and see above), which were shown to possess lowered PDX1, NKX6.1 and insulin immunoreactivity, and suggest that a significant proportion of beta cells in this model may assume "hub-like" properties favouring glucose metabolism. Nonetheless, overall glucose-induced increases in ATP/ADP are impaired across the islet in this model, at odds with the more energetic status of hubs, although we note that the latter was assessed using mitochondrial membrane potential as a proxy and this may not necessarily reflect ATP synthesis [74]. Formal analysis of hub and spoke numbers, or conversely mosaic rescue experiments where some cells are forced to be mature, will be required therefore to test the above model in future. A tantalizing prospect is that restoration of insulin secretion during T2D, which is associated with frank beta cell de-differentiation (see above and [86,89]), may benefit more from recreating transcriptional and functional heterogeneity rather than uniform maturity. Indeed, recent studies have shown that fasting promotes beta cell regeneration through a backwards step *via* localised expression of *Sox17*, *Pdx1* and *Ngn3* [90].

> …restoration of insulin secretion during T2D … may benefit more from recreating transcriptional and functional heterogeneity.

Critical questions around hub cells and connectivity

Provenance and stability. It remains unclear whether hub cells represent a stable population which forms when each islet develops, or a more transient population subject to complex transcriptional dynamics such as those shown to exist in other endocrine tissue [91]. Whilst we have not observed changes in hub/follower interactions over a period of 2h [74], further developments in

imaging technology will be necessary to explore the nature of these cells over longer time periods (e.g. following the transplantation of islets into the anterior chamber of the eye) [92].

Transcriptional identity. A clutch of recent papers [93-95] has provided transcriptomic data for single beta and other islet cells from both human and mouse using next generation RNA sequencing in single cells (scRNA-seq). Whilst marked heterogeneity was not apparent in rodent islets, this was more evident in beta cells from man. Nonetheless, re-analysis of the data provided by Xin *et al* [94] reveals evidence for a sub-population with hub-like properties (*i.e.* high *Gck*, low *Pdx1* and low *Nkx6.1* expression). This confirms earlier studies by Szabat et al [96], showing the existence of an insulin-low, Pdx1-low population using fluorescence-activated cell sorting. A key future goal will be to perform similar analyses on *bona fide* hub cells identified as described in [74], although a complicating factor may be transcriptional changes expected to occur following dissociation of cells from the tissue environment [97].

Nature of the connections between network-connected cells. Whilst connections driven by inter-islet neurons remain possible, the loss of these during isolation and culture makes this an unlikely mechanism. Cilia also represent a possible means of conducting signals and these have been shown to contact ~ 25% of the beta cell population [67]. Of note, disruption of the *Bbs4* gene in mice leads to basal body/primary cillary perturbations [67] and is associated with impaired first phase insulin release both *in vivo* and in isolated islets.

Proximity to other islet types. An intriguing possibility is that hubs may be located within a particular niche, for example in close proximity to blood vessels, to other islet cell types (delta or alpha cells?) or to nerve endings. We note here that differences between the innervation of human and mouse islets [98] may be of relevance, although even in human islets rare connections are still seen between neurons and beta cells [98]. It must be emphasised that the studies reported up to now have used cultured islets wherein both interneurons and blood vessels have largely collapsed. Studies are therefore underway to investigate these questions, including through imaging islets implanted in the anterior eye chamber [92] and directly in the pancreas [99] where neural and vascular supplies are rewired or maintained.

Role of gap junctions. The demonstrated requirement for Cx36 (*Gjd2*) [74] indicates that gap junctions are essential for transmission. Our current strategies have relied on imaging across a layer of cells in a single plane, and it is conceivable that out-of-plane connections, involving a "train" of cells which transmit signals exists. Alternative imaging modalities, which capture further cell layers, and higher rates of image capture, will be important to explore this possibility. Although the highly-scattering nature of the islet prevents truly deep imaging at cellular resolution, light-sheet approaches in relatively opaque zebrafish may provide a useful alternative to investigating 3D Ca^{2+} dynamics in the islets.

Clinical relevance. Great strides have been made in recent years to develop beta-like cells from human embryonic stem (hES) and induced pluripotent stem cells (iPS) [100]. Intriguing questions remain as to whether these resemble any of the islet cell types described by our functional imaging studies described above [74,78], as well as those defined by Grompe and colleagues [101]. It will equally be of interest to know whether "hubbiness" can be recapitulated in the "synthetic" islet-like organoids generated through these routes *in vitro* or *in vivo*. Moreover, whether beta cell re-programming *in vivo* properly recapitulates a heterogeneous landscape is unknown, and this may be relevant for T2D treatment considering the requirement for subtle cell-cell differences in insulin release, as shown by functional and transcriptomic studies.

Human islet hubs. Although human islets have dissimilar architecture to mouse islets [102], cell-cell coordination does occur within local cell clusters [74], as well as globally in response to incretin [78]. Whilst we have identified hub cells in human islets, it has not been possible with current strategies to prove directly their role as pacemakers for glucose responsiveness due to the inefficient expression of halorhodopsin (NpHR) in human beta cells. The use of alternative optogenes alongside photopharmacological tools should resolve this question in the future. Likewise, recent developments in GLP-1R pharmacology including light-activated incretin-mimetics and GLP-1R positive allosteric modulators [103] will help to delineate whether incretin-regulated connectivity is driven by a subpopulation of cells, or whether it is the consequence of a more global phenomenon.

Why are hubs so fragile? An interesting observation is that hubs express low levels of SERCA2, a serendipitous finding from attempts to examine ER content (later confirmed to be normal using the marker protein disulfide isomerase) [74]. The reason for this is unknown, but may underlie the susceptibility of these cells to cytokine insults due to ER stress mechanisms [104]. Conversely, the lowered insulin expression found in hubs may offset this vulnerability, since genetically reducing insulin output during beta cell compensation to high fat diet has recently been shown to protect against ER stress [105].

Summary, conclusions and perspectives

Since the elucidation of its importance as key controller of insulin secretion in the 1970s and 80s, interest in Ca^{2+} as a potential target for therapeutic intervention in T2D has remained high. The recent description of coordinated Ca^{2+} signals across the islet as vital for normal secretion has further emphasized this role. Recent transcriptomic analyses at the single level, and the generation of targeted knockouts in mice, will be vital for understanding how these changes occur and perturb normal islet function, and in particular affect the interaction between beta cell hubs and followers. The extent to which changes in the machinery controlling Ca^{2+} dynamics also acts in other islet cell types to influence T2D susceptibility remains a further important area for investigation

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Table 1. **Comparison of Ca^{2+} indicators used in beta cells**

Probe	Advantages	Disadvantages	Example
Fura 2	• Ratiometric • Easy to calibrate • Quantitative • Large Stokes shift • Kd close to resting Ca^{2+} levels • Difficult to load	• UV excitation • Dual excitation • Complex optical setup • Difficult to load • Medium dynamic change • Incompatible with confocal microscopy • Can compartmentalise to organelles	[9]
Fluo3/4/8	• Very bright • Large dynamic range • Compatible with most filter sets/lasers • Compatible with confocal microscopy • Reduced phototoxicity (as laser power can be reduced due to brightness). • Easy to load	• Single wavelength • Quantitation requires careful calibration • Small Stokes shift	[106]
Rhods/X-Rhods	• Red-shifted • Allow mitochondrial Ca^{2+} measures • Easy to load	• Single wavelength • Cytoplasmic Ca^{2+} measures are difficult due to rapid mitochondrial sequestration • Relatively low affinity makes assessment of basal Ca^{2+} difficult • Poor dynamic range	[74]
Fura-Red	• Red-shifted • Ratiometric • Can be used with Fluo3/4/8 to allow confocal ratiometric imaging • Difficult to load	• Low quantum yield • High concentrations need to be loaded; hence, risk of Ca^{2+} buffering • Very poor dynamic range	[38]
CaSIRs	• Far-red- to NIR-shifted • Reduced autofluorescence/background • Very large dynamic range • Easy to load	• Single wavelength • Lysosomal accumulation • Relatively low affinity makes assessment of basal Ca^{2+} difficult	[107]

GCamPs	• Genetically encoded • Can be targeted to cell compartments • Conditional expression is possible in *e.g.* beta or alpha cells • Slow, fast and medium variants available	• Requires viral vectors or mouse genetics for expression • Single wavelength • Dynamic range lower than Fluo3/4/8 • Kinetics slower than Fluo3/4/8	[108]
GECOs and RCamPs	• Red-shifted • Genetically encoded • Can be targeted to cell compartments • Conditional expression is possible in *e.g.* beta or alpha cells	• Requires viral vectors or mouse genetics for expression • Single wavelength • Dynamic range lower than GCamPs and Fluo3/4/8 • Kinetics slower than Fluo3/4/8 • Early versions localise to the nucleus	[109]
Aequorins	• Bioluminescent • Minimal phototoxicty as excitation not required • Genetically encoded • Can be targeted to cell compartments • Large dynamic range • Quantitative	• Requires viral vectors . • Requires continuous addition of the substrate coelenterazine. • Requires specialised imaging setups	[9]

Conflict of interest

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FIGURE LEGENDS

Figure 1: **Systems controlling subcellular Ca^{2+} dynamics in the pancreatic islet beta cell**. Glucose entry via Glut1 or Glut 2 leads to an increase of intracellular ATP/ADP ratio which closes K_{ATP} channels. The resulting decrease of membrane polarization allows the activation of voltage-dependent Ca^{2+} channels (VDCC). Acceleration of glucose metabolism stimulates the activity of plasma membrane Ca^{2+}-ATPase (PMCA) and Sarco(endo-)plasmic reticulum Ca^{2+}-ATPase (SERCA). The ER then serves as a Ca^{2+} reservoir. Mitochondria take up Ca^{2+} through the Mitochondrial Calcium Uniporter (MCU) and release it by a Na^{+}/Ca^{2+} exchanger (NCLX) or via the permeability transition pore (PTP) under conditions of metabolic stress. A plasma membrane Na^{+}/Ca^{2+} exchanger is present at the plasma membrane (NCX). SG, secretory granule, Lyso, lysosome. For further details see the text.

Figure 2: **Calcium regulation of mitochondrial metabolism.** Pyruvate is transported into the mitochondria and is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) which then enters the tricarboxylate (TCA) cycle. Calcium entry into mitochondria is facilitated by the mitochondrial calcium uniporter (MCU) which accelerates substrate movement through the TCA cycle via Ca^{2+}-dependant activation of isocitrate dehydrogenase (IDH) and α-ketoglutarate dehydrogenase (-KG). NADH drives respiratory chain complexes to generate a H^{+} gradient and hyperpolarisation of the mitochondrial membrane potential. H^{+} gradient dissipation subsequently stimulates ATP production by ATP synthase (complex V/F_1F_o ATPase), the activity of which is also enhanced by Ca^{2+}. ATP is transported out of the mitochondrial matrix via adenine nucleotide translocase (ANT) which is also stimulated by a rise in mitochondrial Ca^{2+}. Key: aconitase (ACON), citrate synthase (CS), fumarase (FH), malate deydrogenase (MDH).

Figure 3: **Potential roles of sorcin in the pathogenesis of T2D**. See the text and [61] for further details.

Figure 4: **Hubs and Followers**. A) Hub cells (blue) direct the activity of follower cells (grey) via presently undefined mechanisms, leading to coordinated population behaviour and insulin release. B) Optical silencing of hubs using halorhodopsin (NpHR) leads to more stochastic population behaviour and impaired insulin secretion. C) Hubs are more energetic, more metabolic, less mature and less secretory than followers, and are vulnerable to insults (free fatty acid and cytokines).

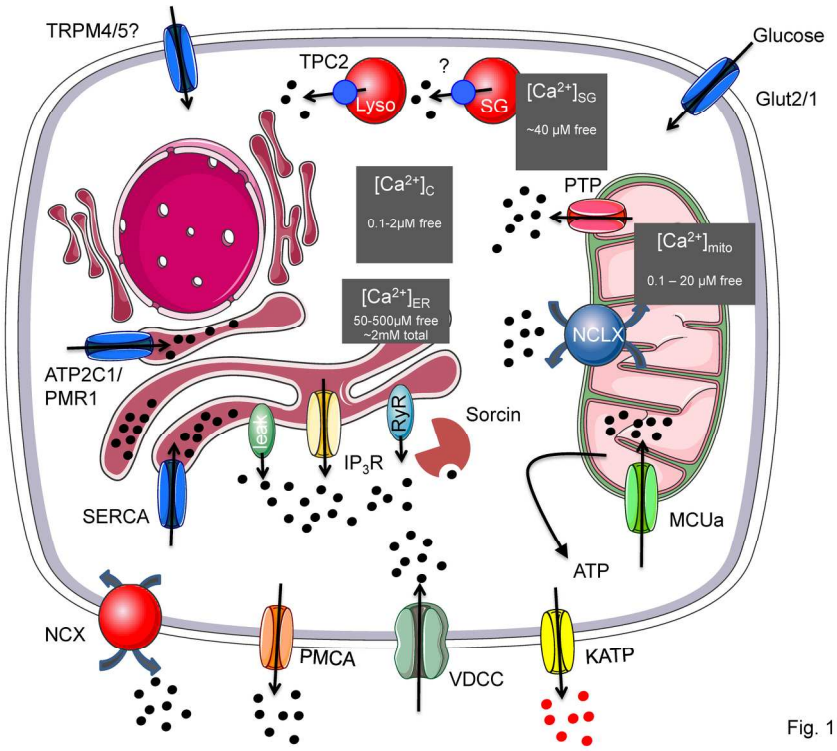

Fig. 1

Figure 1

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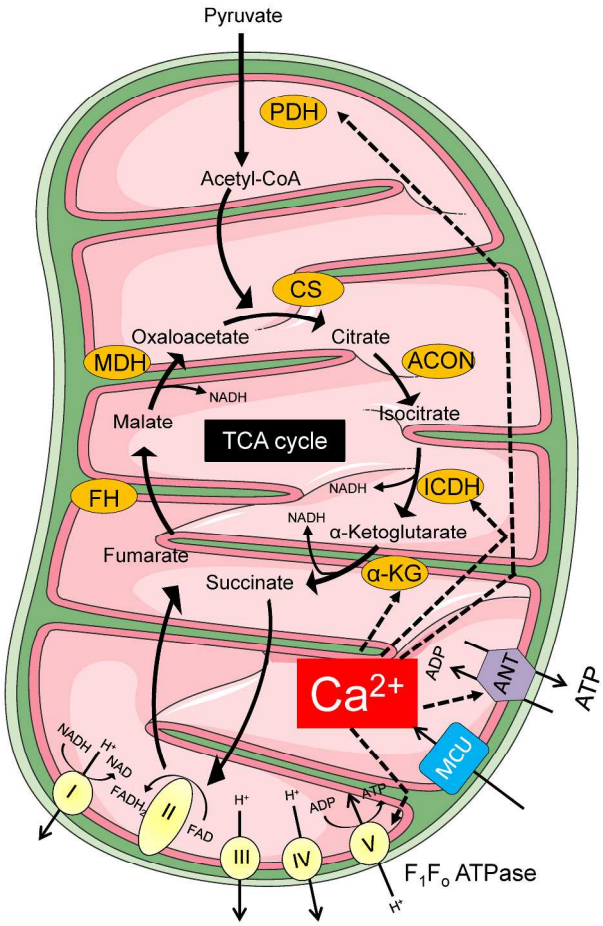

Fig. 2

Figure 2

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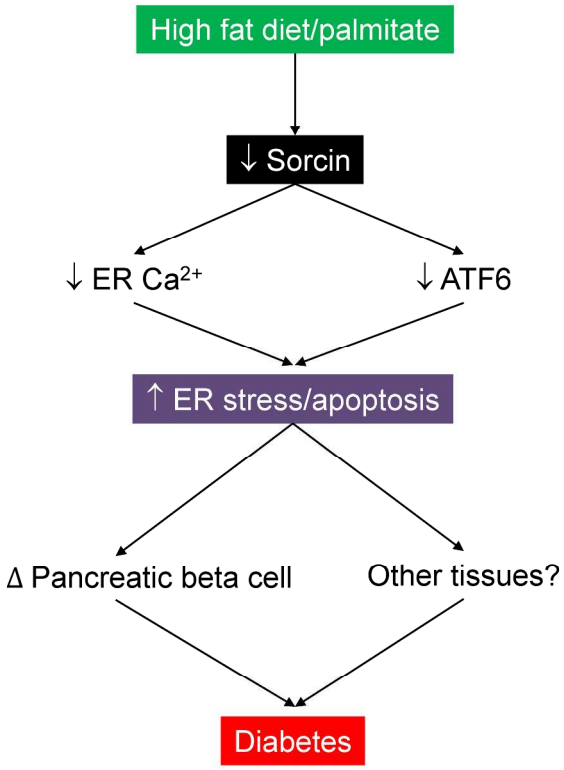

Fig. 3

Figure 3

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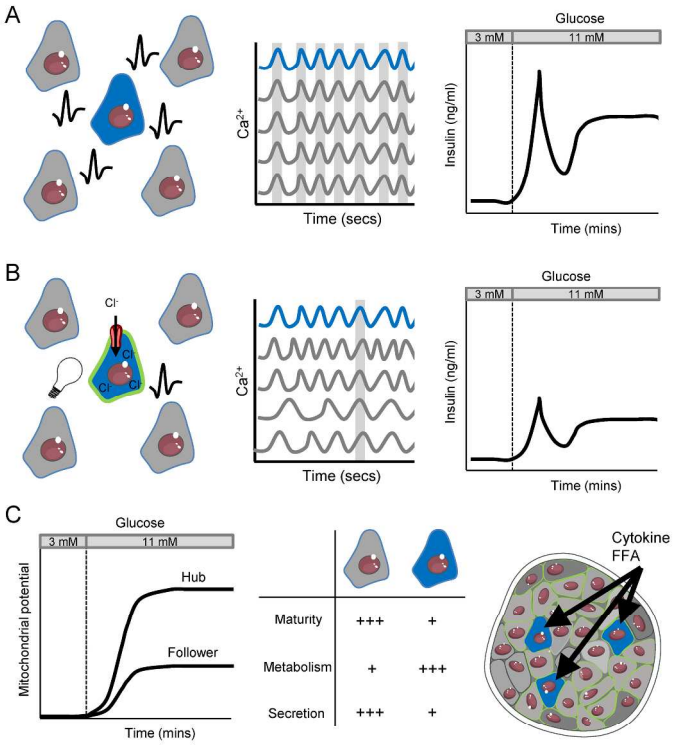

Fig. 4

Figure 4

254x338mm (300 x 300 DPI)