Role of mitochondrial calcium in metabolism-secretion coupling in nutrient-stimulated insulin release

KENNEDY, Eleanor, WOLLHEIM, Claes

Abstract

Glucose-stimulated insulin release from pancreatic beta cells involves a complex series of signalling pathways. In many forms of diabetes, lesions in this process cause or aggravate the diabetic phenotype. A common motif in these cascades is the elevation of intracellular Ca2+ both in the cytosolic compartment ([Ca2+]c) and within the mitochondria ([Ca2+]m). These parameters can be effectively monitored using the photoprotein aequorin which can be targeted to subcellular compartments by transfection. It is shown that physiological concentrations of glucose elicit [Ca2+]c oscillations measured with fura-2, which correlate well with oscillatory NAD(P)H fluorescence in the mitochondria. Aequorin measurements of [Ca2+]m, though unable to detect oscillations on a single cell basis, reveal large increases in intraorganellar [Ca2+] in response to glucose, elevated amino acid levels and depolarizing concentrations of KCl. These oscillations, in turn, mirror changes in the insulin secretion profile. Since several of the key mitochondrial dehydrogenases involved in oxidative phosphorylation are exquisitely sensitive to changes in [...]
ROLE OF MITOCHONDRIAL CALCIUM IN METABOLISM-SECRETION COUPLING IN NUTRIENT-STIMULATED INSULIN RELEASE

E.D. KENNEDY, C.B. WOLLHEIM

SUMMARY - Glucose-stimulated insulin release from pancreatic β cells involves a complex series of signalling pathways. In many forms of diabetes, lesions in this process cause or aggravate the diabetic phenotype. A common motif in these cascades is the elevation of intracellular Ca²⁺ both in the cytosolic compartment ([Ca²⁺]ₚ) and within the mitochondria ([Ca²⁺]ₘ). These parameters can be effectively monitored using the photoprotein aequorin which can be targeted to subcellular compartments by transfection. It is shown that physiological concentrations of glucose elicit [Ca²⁺]ₚ oscillations measured with fura-2, which correlate well with oscillatory NAD(P)H fluorescence in the mitochondria. Aequorin measurements of [Ca²⁺]ₘ, though unable to detect oscillations on a single cell basis, reveal large increases in intraorganellar [Ca²⁺] in response to glucose, elevated amino acid levels and depolarizing concentrations of KCl. These oscillations, in turn, mirror changes in the insulin secretion profile. Since several of the key mitochondrial dehydrogenases involved in oxidative phosphorylation are exquisitely sensitive to changes in [Ca²⁺]ₘ, it is proposed that alterations in [Ca²⁺]ₘ lead to increased activity of the tricarboxylic acid cycle and subsequent ATP production, thereby facilitating exocytosis of insulin from secretory granules. The involvement of the mitochondria in these processes is examined, as is the putative role of efficient mitochondrial genome transcription and translation in normal and diabetic states. Diabetes & Metabolism 1998, 24, 7-16.

Key-words: pancreas, β cell, oscillations, calcium, mitochondria, insulin, secretion.

RÉSUMÉ - Rôle du calcium mitochondrial dans le couplage métabolisme-sécrétion au cours de l'insulino-sécrétion stimulée par les nutriments. La stimulation de l'insulino-sécrétion par le glucose implique une chaîne complexe de signaux. Dans plusieurs formes de diabète, des anomalies de ce processus causent ou aggravent le phénotype diabétique. Un élément commun de ces cascades est l'augmentation du Ca²⁺ intracellulaire dans le cytosol ([Ca²⁺]ₚ) et dans les mitochondries ([Ca²⁺]ₘ). Ces paramètres peuvent être suivis en utilisant l'aequorine, une photoprotéine qui peut être ciblée dans les compartiments subcellulaires par transfection. Des concentrations physiologiques de glucose induisent des oscillations de [Ca²⁺]ₚ, mesurées grâce au fura-2, qui sont bien corrélées à la fluorescence oscillatoire de NAD(P)H dans les mitochondries. Les mesures de [Ca²⁺]ₘ, par l'aequorine, bien qu'incapables de détecter des oscillations au niveau d'une cellule, révèlent des augmentations de [Ca²⁺]ₘ dans les organelles en réponse au glucose, aux acides aminés ou à des concentrations dépolarisantes de KCl. Ces oscillations se superposent aux variations de l'insulino-sécrétion. Comme plusieurs dehydrogénases mitochondriales impliquées dans la phosphorylation oxydative sont extrêmement sensibles aux variations de [Ca²⁺]ₘ, il est proposé que les altérations de [Ca²⁺]ₘ conduiraient à une activité accrue du cycle de l'acide tricarboxylique et donc à une production d'ATP facilitant l'exocytose de l'insuline à partir des granules sécrétories. L'implication des mitochondries dans ces processus est envisagée, comme l'est le possible rôle du génome mitochondrial dans les états normaux et diabétiques. Diabetes & Metabolism 1998, 24, 7-16.

Mots-clés : pancréas, cellule β, calcium, mitochondrie, insuline, sécrétion.
Blood glucose homeostasis is critically dependent on the tight regulation of insulin secretion from the pancreatic β cell [1]. Insulin is stored in secretory granules of the β cell, of which only a small proportion is released, even following strong stimulation [2]. Glucose and leucine are the two main nutrient stimuli of physiological importance capable of initiating insulin release [3]. Their effect is potentiated by a variety of hormones and neurotransmitters, including glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK) and acetylcholine (ACh) [2, 4].

The consensus model of insulin secretion induced by both glucose and leucine allocates a central role to the mitochondria in metabolism-secretion coupling [3, 5, 6]. The pancreatic β cell in the rodent expresses predominantly the GLUT-2 glucose transporter which effectively equilibrates the perceived blood glucose concentration across the plasma membrane [5]. Compelling evidence indicates that the high Km, glucose-phosphorylating enzyme, glucokinase, is the glucose sensor, i.e. the flux-determining reaction of glycolysis [7, 8]. In fact, one of the subtypes of maturity onset diabetes of the young (MODY) has been associated with mutations in the glucokinase gene resulting in impaired glucose-induced insulin release [8, 9]. Likewise, targeted knock-out of β-cell glucokinase in mice led to a defective responsiveness to glucose [10]. Further to its conversion to pyruvate, glucose carbons enter the mitochondria and feed into the tricarboxylic acid (TCA) cycle which generates reducing equivalents in the form of NADH and FADH2 [3, 5]. These are relayed to the electron transport chain situated predominantly on the matrix side of the inner mitochondrial membrane. Through the increase in respiration, ATP is generated by the F1/F0-ATPase and exported to the cytosol [8]. The subsequent increase in the ATP:ADP ratio [11] promotes membrane depolarization following closure of the ATP-sensitive K+ (KATP) channels [12]. It has recently been suggested that glucose not only increases ATP concentration but also decreases ADP concentration in β-TC-3 cells, which may be the determining factor in glucose-triggered closure of KATP channels [8]. Furthermore, stimulatory glucose concentrations increase the GTP:GDP ratio by an as yet poorly defined mechanism [11, 13]. Plasma membrane depolarization gates the influx of Ca2+ via voltage-sensitive L-type Ca2+ channels [14].

The resultant increase in cytosolic Ca2+([Ca2+]c) displays oscillations which correlate with the glucose-evoked rhythmic plasma membrane potential activity [15]. During bursts of Ca2+ action potentials, spikes of stimulated insulin secretion are observed [16, 17]. Moreover, Ca2+ oscillations have been associated with fluctuations in the ATP:ADP ratio, which most probably reflect mitochondrial activation [18]. This contention is borne out by measurements of oscillatory oxygen consumption that denote a concurrent change in islet respiration [19]. This review will deal with the role of Ca2+ in the coupling of the oxidation of glucose and other nutrient secretagogues to the exocytosis of insulin.

### THE ROLE OF MITOCHONDRIA IN β-CELL ACTIVATION

Extensive studies have demonstrated a key role for mitochondrial metabolism in nutrient-stimulated insulin secretion [5, 6, 20-23]. Glucose and leucine oxidation, as monitored by CO2 production by islets, correlates well with insulin secretion [20, 24]. In addition, inhibitors of the respiratory chain and other mitochondrial poisons prevent the initiation of insulin secretion by nutrients [20, 21, 25]. In the β cell, there is tight coupling between mitochondrial metabolism and glycolysis, the latter being inhibited in response to blockers of oxidative phosphorylation [24, 25]. This distinguishes the β cell from other tissues which display the Pasteur effect, i.e. stimulated glycolysis caused by mitochondrial blockade [24, 25]. The molecular basis for this has been suggested to be the extremely low activity of lactate dehydrogenase (LDH) in the β cell, associated with high levels of mitochondrial, FAD-linked glycerophosphate dehydrogenase [25, 26]. The consequences of this enzymatic imbalance are exploited by the β cell to optimize energy production since the introduction of electrons into the respiratory chain via the glycerophosphate shuttle produces 2 moles of ATP less than that produced by total pyruvate oxidation. The latter is favoured by low LDH levels which are indeed the lowest in any mammalian cell type [25, 27]. Although there are slight discrepancies between the LDH values obtained in β cells, which possibly reflect a difference in the cell-sorting procedure, overall activity remains low as compared to other cell types, in particular the hepatocyte [25, 27]. As a result of low LDH activity, lactate output during glucose stimulation of sorted β cells, but not whole islets, is minimal [20, 25]. This is also observed in INS-1 and β-HC cells, two differentiated cell lines with glucose metabolic profiles similar to those of native β cells [25, 28].

It was suggested almost two decades ago by Denton and colleagues that Ca2+ plays a crucial role in the provision of energy during cell activation [29]. They proposed a model in which the rise in [Ca2+]c was relayed to the mitochondria, causing an increase in the matrix Ca2+ concentration of the organelle ([Ca2+]m). The mitochondria contain three well-characterized NADH-generating dehydrogenases which are Ca2+-sensitive [30]. These are: 1) pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl CoA, Ca2+ sensitivity being conferred by a Ca2+-activated phosphatase; 2) NAD isocitrate dehydrogenase, which turns isocitrate into α-ketoglutarate; and 3) α-ketoglutarate dehydrogenase, which converts α-keto-
Ca²⁺-DRIVEN METABOLIC OSCILLATIONS IN THE β-CELL

Evidence for the role of Ca²⁺ in driving β-cell metabolism has been gathered from experiments in which [Ca²⁺]ᵢ was measured in parallel with NAD(P)H in single, fura-2-loaded β-cells [37]. In this preparation, stimulatory glucose concentrations generate regular [Ca²⁺]ᵢ oscillations in approximately 50% of cells [38], with a periodicity of ~ 1.4 min [37]. Likewise, glucose promotes NAD(P)H oscillations with a similar periodicity (~ 1.3 min) [37]. The [Ca²⁺]ᵢ oscillations are reminiscent of those observed in intact mouse and human islets [15-17,39]. In contrast, glucose-induced increases in NAD(P)H fluorescence were not resolved into oscillations in whole mouse islets [39]. A possible explanation for this apparent discrepancy is suggested from the results shown in Figure 1. Whereas 11.2 mM glucose causes large Ca²⁺ oscillations, the corresponding fluctuations in NAD(P)H are of comparatively small amplitude (Fig. 1A and B). These latter oscillations could easily escape detection during simultaneous recording from a large number of islet cells. It has been shown that, though glucose increases NAD(P)H fluorescence in islet cells, it lowers FAD fluorescence simultaneously [22]. This, in conjunction with other biophysical evidence, strongly favours the notion that the effect of glucose on NAD(P)H fluorescence largely reflects increased NADH generation by the mitochondria [22]. It can therefore be inferred that increased [Ca²⁺]ᵢ stimulates mitochondrial NADH production.

This hypothesis is strengthened by the abolition of NAD(P)H oscillations in the presence of EGTA, which blocks Ca²⁺ influx and glucose-stimulated [Ca²⁺]ᵢ rises (Fig. 1C and D). Moreover, K⁺ depolarization promoted increases in both [Ca²⁺]ᵢ and Ca²⁺ influx-dependent elevations in NAD(P)H fluorescence in single rat β cells [37]. Direct evidence that Ca²⁺ generates NADH in mitochondria was recently obtained in permeabilized HIT-T15 cells by a combination of the mitochondrial substrate, pyruvate, with elevated Ca²⁺ concentrations [40].

To gain further insight into the role of Ca²⁺ as a regulator of mitochondrial function, cell lines stably expressing the Ca²⁺-sensitive photoprotein, aequorin, have been established [41]. These clonal lines were derived from highly differentiated, glucose-sensitive INS-1 cells [42]. Glucose-stimulated insulin secretion profiles remained clearly biphasic and occurred in the physiological concentration range [41]. Stable expression of aequorin in the mitochondria is achieved by transfecting cells with a chimera encoding aequorin with an N-terminal targeting sequence comprising the presequence of cytochrome-c oxidase subunit VIII [43]. This INS-1 EK-3 cell line exhibits oscillations in [Ca²⁺]ᵢ when exposed to 10 mM glucose as measured by fura-2, which are similar to those observed in single rat β cells (Fig. 1E and F). To allow comparison of [Ca²⁺]ᵢ fluctuations with corresponding [Ca²⁺]ᵢ changes when the same Ca²⁺ sensor was used, an INS-1 cell line stably expressing cytosolic aequorin was also established [41]. In contrast to more conventional Ca²⁺ indicators such as fura-2, aequorin neither buffers the perceived Ca²⁺ concentration nor undergoes any bleaching, which makes it an ideal tool for measuring [Ca²⁺]ᵢ in a variety of cellular compartments [44].

As shown in Figure 2, increases of glucose in the physiological concentration range between basal 2.8 mM and stimulatory 5 mM and 10 mM cause a graded rise in both [Ca²⁺]ᵢ and [Ca²⁺]ᵢ. In other cell types, [Ca²⁺]ᵢ has been successfully assessed by employing aequorin in transient transfection studies [43,45,46]. This approach can also be applied to both primary rat islet cells [47] and INS-1 cells (Fig. 2B). The results obtained with transient transfection are qualitatively similar to those seen in the INS-1 EK-3 cell line. It should be noted, however, that estimations of basal [Ca²⁺]ᵢ after transient transfection with aequorin are subject to variation depending on differences in expression efficiency. This is particularly pertinent in the monitoring of [Ca²⁺]ᵢ with this method. The cell line stably expressing cytosolic aequorin overcomes this problem and displays [Ca²⁺]ᵢ in the range 100-150 nM, which is in agreement with values reported for [Ca²⁺]ᵢ assessed with fluorescent indicators in β cells and derived cell lines (Fig. 2C, [41]). It is noteworthy that, while resting [Ca²⁺]ᵢ and [Ca²⁺]ᵢ levels are very close, those observed after stimulation are disparate, being higher in the mitochondria than in the cytosol. This applies not only to glucose as seen in Figure 2 but also to the muscarinic agonist, carbachol, and the depolarizing agent,
KCl [41]. A possible mechanism underlying this amplified response of the mitochondria could be that a proportion of these organelles is organized in micro-domains situated close to L-type Ca\(^{2+}\) channels in the plasma membrane and to putative sites of exocytosis. It has already been demonstrated that small changes in

![Graphs A to F](image-url)

**Fig. 1.** Effects of increased glucose concentration on \([\text{Ca}^{2+}]_i\) (A and C) and NAD(P)H fluorescence (B and D) in single rat β cells loaded with fluro-2. \([\text{Ca}^{2+}]_i\) measurements (nM) and NAD(P)H fluorescence (% basal values) were recorded in parallel experiments using dual excitation microspectrofluorimetry. Basal glucose concentrations were 4 mM. Figure 1E and 1F are two representative traces of \([\text{Ca}^{2+}]_i\), measurements in fluro-2-loaded INS-1 EK-3 cells exposed to increasing concentrations of glucose, in which basal glucose is 2.8 mM. Reproduced with the kind permission of the authors: A-D [37] and E and F [41].
the extracellular K⁺ concentration elicits rises in [Ca²⁺]ₘ without detectable changes in [Ca²⁺]₀, which points to a preferential link between Ca²⁺ influx and [Ca²⁺]ₘ elevations [41]. This mechanism is further substantiated by the results shown in Figure 2A. The chelation of extracellular Ca²⁺ with an excess of EGTA causes a rapid decrease in [Ca²⁺]ₘ to basal values. As in the case of glucose-stimulated insulin secretion, the increases in both [Ca²⁺]ₘ and [Ca²⁺]₀ in cell lines stably expressing equorin are biphasic.

The influx of Ca²⁺ into the mitochondria is regulated by a low-affinity Ca²⁺ uniporter with a threshold value of approximately 500 nM [48]. Efflux depends on Na⁺/Ca²⁺ and H⁺/Ca²⁺ antiporters [48]. Ca²⁺ uptake is favoured by hyperpolarization of the mitochondrial membrane potential, which increases the driving force for the ion since it is very negative inside (approx. -180 mV). Glucose has been shown to hyperpolarize the mitochondrial membrane potential in both primary β cells and INS-1 cells [22, 47]. The mechanism underlying the amplification of [Ca²⁺]ₘ in comparison with [Ca²⁺]₀, during glucose stimulation may therefore be two-fold. Firstly, any agent which depolarizes the plasma membrane potential, such as KCl, will cause an increase in [Ca²⁺]ₘ because of the favourable localization of the mitochondria. Secondly, nutrient secretagogues, through their stimulation of the respiratory chain and resultant hyperpolarization of the mitochondrial membrane potential, will increase Ca²⁺ uptake by the organelle.

Dissipation of the mitochondrial membrane potential with the potent protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP), caused a lowering of [Ca²⁺]ₘ to approximately 100 nM. This was expected, since the effective collapse of the membrane potential in these conditions results in an equilibration between [Ca²⁺]ₘ and [Ca²⁺]₀. In the presence of 1 μM CCCP, 10 mM glucose failed to elicit any increase in [Ca²⁺]ₘ (Fig. 3A). This is in agreement with the marked attenuation of the response to 20 mM KCl [41]. It should be noted that, under the same experimental conditions, KCl-induced [Ca²⁺]ₘ rises were largely unaffected [49]. The action of CCCP is rapidly reversible since glucose can raise [Ca²⁺]ₘ after a recovery period of only 5 min (Fig. 3A). The effect of CCCP on insulin secretion has also been studied in rat islets. While the secretory response to glucose was significantly but not completely reduced, the corresponding effects of CCCP on glucose oxidation were only marginally altered, whereas the ATP : ADP ratio remained unchanged [20]. This would indicate a fundamental role for a functional mitochondrial membrane potential in the generation of metabolic coupling factors other than ATP since mitochondrial glucose metabolism appears unaffected by the mitochondrial poison.

However, when glucose metabolism is severely restricted by the presence of inhibitors of the respiratory chain, insulin secretion is also reduced, indicating that the two driving forces are equally important [20]. Perfusion of rat islets with amytal, a potent inhibitor of site I of the respiratory chain, in the presence of elevated glucose concentrations led to complete abolition of the insulin secretory profile [21]. In addition, as can be seen in Figure 3B, 10 mM glucose fails to elicit an increase in [Ca²⁺]ₘ in the presence of amytal. As expected, the response to depolarizing concentrations of KCl is largely unaltered by the inhibition of oxidative phosphorylation since its ability to increase [Ca²⁺]ₘ is largely independent of the integrity of the mitochondrial respiratory chain. As with CCCP, the
effects of amyntal appear to be completely reversible. After comparatively short recovery periods, glucose can again increase \([Ca^{2+}]_m\) to levels routinely observed in the absence of any prior manipulations. Taken together, these data suggest a close relationship between rises in \([Ca^{2+}]_m\) and insulin secretion in response to nutrient. The obvious role of ATP, or perhaps more precisely the ATP:ADP ratio, should not be overlooked. In rat islets, both amyntal and antimycin A, another potent inhibitor of respiration, caused severe reductions in the measurable levels of ATP and abolished glucose-stimulated insulin secretion [20,21]. It has been calculated that glucose, to achieve insulin secretion, must increase ATP production above a critical threshold [28]. It has been suggested that this is due mainly to a mass action effect of glucose, the rate of which is determined by glucokinase, the glucose sensor of the \(\beta\) cell, and ultimately by mitochondrial \(F_1/F_0\)-ATPase [8].

It is well-known that agents which interfere with \(K_{ATP}\) channel closure by nutrient metabolism inhibit the stimulation of insulin secretion [12]. Diazoxide, the most studied of these agents, completely inhibits the glucose-mediated rise in \([Ca^{2+}]_m\) (Fig. 4A). Leucine and its deamination product KIC, which are metabolized directly by the mitochondria, also depolarize plasma membrane potential and cause closure of the \(K_{ATP}\) channels [12,50]. Leucine was found to elicit \([Ca^{2+}]_m\) rises similar to those of glucose (Fig. 4B). Likewise, its effects were abolished by the presence of diazoxide, which again emphasizes the important role of a permissive rise in \([Ca^{2+}]_m\), relative to a rise in \([Ca^{2+}]_m\) since leucine also raises \([Ca^{2+}]_m\) [51, 52].

Work from several laboratories has provided evidence that glucose is also capable of eliciting insulin secretion in a \(K_{ATP}\) channel-independent manner [53, 54]. As initially demonstrated by Gembal et al. [53], glucose can still cause insulin secretion in the presence of diazoxide, provided that \([Ca^{2+}]_m\) is raised to a permissive level by membrane depolarization with KCl, thereby allowing an influx of \(Ca^{2+}\) through L-type \(Ca^{2+}\) channels. Under these conditions, leucine and D-glyceraldehyde also promote insulin secretion, which indicates that mitochondrially generated coupling factors are involved in the exocytotic process [55].

Fig. 3. Effects of amyntal (1 mM) (A) and CCCP (1 \(\mu\)M) (B) on \([Ca^{2+}]_m\) measurements in INS-1 \(\beta\)-cells in response to 10 mM glucose.

Fig. 4. Effects of diazoxide (250 mM) on \([Ca^{2+}]_m\) induced by the nutrient secretagogues glucose 10 mM (A) and leucine 10 mM (B).
The generation of such metabolic coupling factors is shown schematically in Figure 5. Pyruvate is formed from glycolysis and enters the mitochondria preferentially due to the low LDH levels of the β cell and glucose-responsive cell lines [25,28]. In the mitochondria, pyruvate is decarboxylated to acetyl CoA, a reaction catalyzed by the Ca\(^{2+}\)-sensitive enzyme complex PDH (denoted 1 in Fig. 5) or carboxylated to oxaloacetate by pyruvate carboxylase [56]. The latter reaction provides anaerobic input to the TCA cycle [3]. Leucine also feeds into the TCA cycle via acetyl CoA production. As previously mentioned, NAD isocitrate dehydrogenase and α-ketoglutarate dehydrogenase also constitute Ca\(^{2+}\)-sensitive reactions (denoted 2 and 3 respectively in Fig. 5) [30]. Through the production of reducing equivalents, electrons are fed into the respiratory chain and ATP is synthesized. Glucose metabolism also stimulates the α-glycero-phosphate (GP) shuttle [34]. This is one of the shuttles which transports reducing equivalents from the cytosol to the mitochondria. Its mitochondrial component, the FAD-linked GP dehydrogenase, responds to fluctuations of [Ca\(^{2+}\)]\(_{e}\) in view of its location on the outer surface of the inner mitochondrial membrane [26, 31]. The nutrient secretagogues hyperpolarize the mitochondrial membrane potential through increased activity of the respiratory chain [22, 47]. As a result of stimulated mitochondrial metabolism, the cytosolic ATP:ADP ratio increases, which is instrumental in the closure of K\(_{\text{ATP}}\) channels [11, 12]. The resultant depolarization and electrical activity promote gated Ca\(^{2+}\) influx and elevation of [Ca\(^{2+}\)]\(_{m}\) [2].

The actions of the cytosolic Ca\(^{2+}\) signal are two-fold:

1) it triggers exocytosis of the insulin-containing secretory granules, a process also requiring ATP and GTP [2, 13]; and

2) it raises [Ca\(^{2+}\)]\(_{m}\), which is favoured by the aforementioned hyperpolarization of the organelar membrane potential.

At the present time, it cannot be definitively established which of the two actions is more dominant in the K\(_{\text{ATP}}\) channel-independent stimulation of insulin secretion by nutrients. There is now evidence to suggest that the increase in [Ca\(^{2+}\)]\(_{m}\) stimulates the generation of novel mitochondria-derived coupling factors capable of promoting insulin exocytosis when [Ca\(^{2+}\)]\(_{e}\) is clamped to permissive levels (<1 μM) in the presence of saturating ATP concentrations [47]. These conditions were imposed in INS-1 cells permeabilized with Staphylococcus α-toxin which creates small holes solely in the plasma membrane [57]. Only agents such as succinate which are capable of both fueling carbons into the TCA cycle and raising [Ca\(^{2+}\)]\(_{m}\) were found to induce exocytosis of insulin. Accordingly, α-glycero-phosphate, which hyperpolarizes the mitochondrial membrane potential and causes a marked increase in [Ca\(^{2+}\)]\(_{m}\), failed to enhance insulin secretion. Although the nature of the putative mitochondrial coupling factor(s) remains to be defined, it would appear most likely that products of the TCA cycle or compounds synthesized downstream thereof mediate the secretory effect. Acyl CoAs such as malonyl CoA and palmitoyl CoA have been proposed to be

![Fig. 5. Schematic representation of the nutrient-stimulated signalling-secretion pathways in the pancreatic β cell.](image-url)
coupling factors during nutrient stimulation of insulin secretion [3]. Since the latter opens rather than closes \( K_{\text{ATP}} \) channels, the role of these lipid derivatives remains unclear [58]. A relatively small effect of glucose on insulin secretion is also observed when both protein kinases A and C are stimulated under conditions in which \( [\text{Ca}^{2+}]_c \) remains at basal levels [59]. Whether this response involves the same coupling factor(s) as the \( \text{Ca}^{2+} \)-dependent metabolism-secretion process is as yet unknown.

**β-CELL MITOCHONDRIAL FUNCTION AND IMPAIRED INSULIN SECRETION**

The key role of mitochondrial metabolism in nutrient-stimulated insulin secretion is further highlighted by the well-known capacity of methylsuccinate, the cell-permeant analogue of succinate, to mimic the effects of glucose and leucine [60]. The GK rat, a model of non-insulin-dependent diabetes mellitus (NIDDM), displays impaired glucose-stimulated insulin secretion. Interestingly, the secretion stimulated by methylsuccinate was also deficient in isolated GK rat islets, whereas activation of exocytosis following depolarization with \( K^+ \) was normal [61]. The molecular basis of this defect is poorly understood. The etiology of another diabetic syndrome in an animal model, the BDF\(_2\)NZ rat, has been linked to a mutation in subunit 6 of \( F_1/F_0 \)-ATPase [62]. This mitochondrial encoded protein determines intraorganellar ATP production. The islets from these animals only display impaired glucose-stimulated insulin secretion after culture at high glucose concentrations [8]. This is perhaps to be expected from a single point mutation in a protein which is transcribed and translated in the mitochondria. More severe phenotypes would be expected from a mutation affecting translation in a more global manner. Paramitochondrial DNA (mtDNA) is a highly compacted, maternally encoded circular DNA of 16,569 bp, present in several copies within each mitochondrion [63]. MtDNA undergoes comparatively rapid replication during which deletions and mutations are less efficiently repaired than those encountered in the nuclear genome. A number of diseases have now been associated with such alterations in mtDNA, including myoclonic epilepsy and ragged red fibre disease (MERRF), Leber's hereditary optic neuropathy (LHON) and mitochondrial encephalomyopathy, lactic acidosis and stroke-like syndromes (MELAS) [64]. Patients with MELAS are also often diabetic [65]. Similarly, another newly-defined subtype of maternally inherited diabetes often accompanied by deafness (MIDD) is caused by a mutation in tRNA\(^{\text{Leu(UUR)}}\) at position 3243 of mtDNA [66]. Since this mutation affects the translational efficiency of mtDNA, it may be expected to have more severe consequences than isolated point mutations in the mtDNA-encoded subunits of respiratory chain enzymes. In keeping with this idea, the same tRNA\(^{\text{Leu(UUR)}}\) mutation is observed in patients affected by the disabling neuromuscular disease, MELAS. Defective glucose-stimulated insulin secretion appears to be the crucial feature underlying this diabetic syndrome which, however, often evolves into insulin-dependent diabetes mellitus [65,66]. Mutations in mtDNA may, however, also be implicated in more common forms of NIDDM since a report from Japan has shown that 5\% of NIDDM patients exhibit a point mutation at position 3394 of mtDNA in the mitochondrionally-encoded NADH dehydrogenase subunit 1 [67].

Recently, the importance of mtDNA in the development of this pathophysiological entity has been elucidated in studies on the non-insulin-secreting cell line MIN-6. Following chemical elimination of mtDNA, glucose-stimulated insulin secretion was completely abolished. The pivotal role of the mitochondria in this process was underscored by the restoration of the secretory response through replenishment of mtDNA-deficient cells with functional mitochondria using cybrid fusion techniques [68]. In similar studies in INS-1 cells depleted of mtDNA, we found that insulin release was abrogated in the presence of glucose, while the response to \( K^+ \) depolarization was unaffected [69].

**PERSPECTIVES**

Converging evidence points strongly to the fundamental importance of \( [\text{Ca}^{2+}]_c \) in both the onset and sustained secretory response to glucose in β cells. Initiation of \( \text{Ca}^{2+} \) influx following glucose metabolism will assure the continued generation of coupling factors by the mitochondria. As \( [\text{Ca}^{2+}]_c \) oscillates in response to nutrient stimuli via oscillating membrane potential, it is most likely that these events are relayed to the mitochondria in the form of \( [\text{Ca}^{2+}]_m \) oscillations. Such \( [\text{Ca}^{2+}]_m \) oscillations would lead to metabolic fluctuations, as demonstrated by changes in NAD(P)H fluorescence in single β cells [37]. However, at the present time, the \( [\text{Ca}^{2+}]_c \) signal recorded in cell lines stabilily expressing aequorin in the mitochondria cannot be resolved into single transient spikes in individual cells. The reported measurements were obtained from populations asynchronously responding to the stimuli. To achieve single cell recordings, higher expression levels of aequorin must be obtained. To this end, microinjection techniques have been used to introduce aequorin cDNA directly into the nuclei of CHO cells [70]. Furthermore, it will be important to characterize the nature of the mitochondrially-derived coupling factors generated in a \( \text{Ca}^{2+} \)-dependent fashion during nutrient stimulation of insulin secretion. In particular, it will be essential to delineate whether the \( \text{Ca}^{2+} \)-sensitive step is indeed one of the \( \text{Ca}^{2+} \)-activated mitochondrial NADH-generating dehydrogenases or a hitherto less well-defined meta-
bolic reaction. The characterization of these Ca²⁺-dependent functions in the β-cell should, in turn, help elucidate the impairment of insulin secretion in several subtypes of NIDDM.

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