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Review

# Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic β-cells

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**Abstract.** Mitochondrial metabolism is crucial for the coupling of glucose recognition to the exocytosis of the insulin granules. This is illustrated by in vitro and in vivo observations discussed in the present review. Mitochondria generate ATP, which is the main coupling messenger in insulin secretion. However, the subsequent $Ca^{2+}$ signal in the cytosol is necessary but not sufficient for full development of sustained insulin secretion. Hence, mitochondria generate ATP and other coupling factors serving as fuel sensors for the control of the exocytotic process. Numerous studies have sought to identify the factors that mediate the amplifying pathway over the $Ca^{2+}$ signal in glucose-stimulated insulin secretion. Predominantly, these factors are nucleotides (GTP, ATP, cAMP, NADPH), although metabolites have also been proposed, such as long-chain acyl-CoA derivatives and glutamate. Hence, the classical neurotransmitter glutamate receives a novel role, that of an intracellular messenger or co-factor in insulin secretion. This scenario further highlights the importance of glutamate dehydrogenase, a mitochondrial enzyme well recognized to play a key role in the control of insulin secretion. Therefore, additional putative messengers of mitochondrial origin are likely to participate in insulin secretion.

**Key words.** Mitochondria; glutamate dehydrogenase; glutamine; insulin secretion.

## Introduction

Blood glucose control depends on the normal regulation of insulin secretion from the pancreatic β-cells and on insulin action on its target tissues. Most forms of type 2 diabetes display disregulation of insulin secretion combined with insulin resistance. The aetiology of type 2, or non-insulin-dependent diabetes mellitus is still poorly understood and has been characterised in only a limited number of cases. Mitochondrial diabetes, a rare subform of the disease, caused by mutations in mitochondrial DNA, is the consequence of pancreatic β-cell dysfunction. The impact of such mutations on β-cell function reflects the importance of the mitochondria in the control of insulin secretion. β-Cell mitochondria serve as fuel sensors, generating factors coupling nutrient metabolism to the exocytosis of insulin-containing vesicles. The latter process requires an increase in cytosolic $Ca^{2+}$, which depends on ATP synthesised by the mitochondria. This organelle generates other factors, of which glutamate has been proposed as a potential intracellular messenger.

## Glucose recognition by the β-cell

Glucose homeostasis is tightly controlled by insulin secretion from the pancreatic β-cells and by insulin action on muscle and other target tissues. The β-cell is poised to adapt rapidly the rate of insulin secretion to fluctuations in blood glucose concentration (fig. 1). Glucose equilibrates across the plasma membrane through a low-affinity glucose transporter [1]. It is then phosphorylated to glucose-6-phosphate by high-$K_m$ hexokinase IV, or glucokinase, which determines the rate of glycolysis and the generation

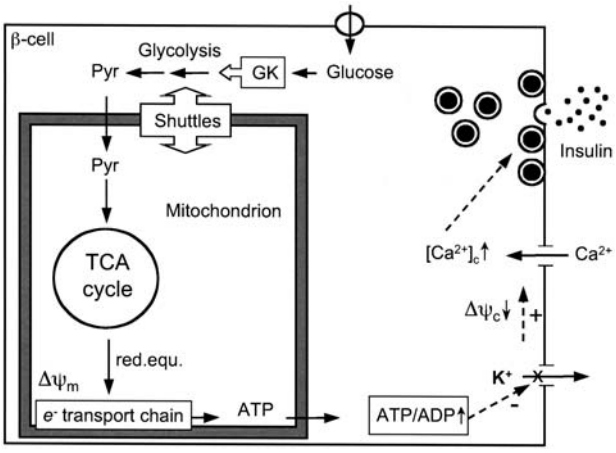


Figure 1. Model for coupling glucose metabolism to insulin secretion in the β-cell. Glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase (GK), which initiates its conversion to pyruvate (Pyr) by glycolysis. Pyr preferentially enters the mitochondria and fuels the TCA cycle, resulting in the transfer of reducing equivalents (red.equ.) to the respiratory chain, leading to hyperpolarisation of the mitochondrial membrane ($\Delta\Psi_m$) and generation of ATP. ATP is then transferred to the cytosol, raising the ATP/ADP ratio. Subsequently, closure of $K_{ATP}$ channels depolarises the cell membrane ($\Delta\Psi_c$). This opens voltage-gated $Ca^{2+}$ channels, increasing the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$), which triggers insulin exocytosis.

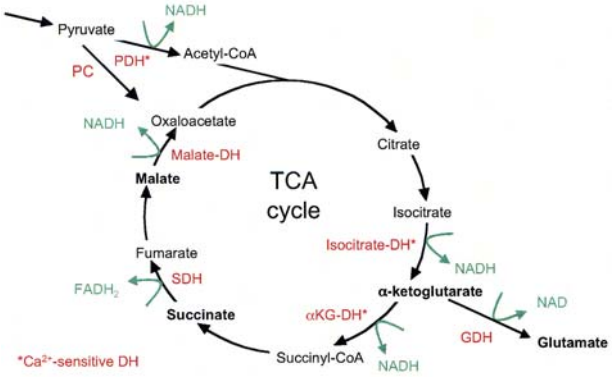


Figure 2. The tricarboxylic acid (TCA) cycle with $Ca^{2+}$-sensitive dehydrogenases (DH). In the mitochondria, pyruvate is a substrate for both pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC). Among the TCA cycle enzymes, succinate-DH (SDH) is also part of the respiratory chain (complex II). In certain conditions (see text), glutamate is produced from α-ketoglutarate by glutamate dehydrogenase (GDH).

of pyruvate [1]. Mutations in the glucokinase gene that result in decreased glucose sensitivity affect blood glucose regulation and are associated with maturity onset diabetes of the young, type 2 (MODY-2) [2]. Conversely, glucokinase mutations resulting in a gain of function of the enzyme cause one form of a syndrome of persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) [3]. Together, these mutations in the glucokinase gene illustrate the role of this enzyme as a gatekeeper determining the flux of intermediates undergoing glycolysis. High rates of glycolysis are maintained through the activity of mitochondrial shuttles, mainly the glycerophosphate and malate/aspartate shuttles [4, 5], which allow the reoxidation of cytosolic NADH. The malate/aspartate NADH shuttle depends on the mitochondrial aspartate/glutamate carrier. Of interest is that the latter has recently been shown to be stimulated by $Ca^{2+}$ [6]. Blockade of these shuttles inhibits glucose-stimulated insulin secretion [5, 7]. Other shuttles generating cytosolic NADPH have also been described [8, 9].

A particular feature of the β-cell is not only the tight link between glycolysis and mitochondrial oxidative metabolism, but also the extremely high proportion of glucose-derived carbons oxidized in the mitochondria [10]. Indeed, as demonstrated in isolated purified β-cells, as many as 90% of glucose-derived carbons are oxidized by the mitochondria [10]. This is favoured by the very low expression of monocarboxylate transporters in the plasma membrane coupled with low activity of lactate dehydrogenase [11–14]. Moreover, low lactate dehydrogenase and monocarboxylate transporter activities confer selectivity to glucose of insulin release and, for example, may prevent lactate stimulation during exercise [12]. Pyruvate, once transferred into the mitochondria, is a substrate for both pyruvate dehydrogenase and pyruvate carboxylase. These enzymes ensure the formation of acetyl-CoA and oxaloacetate, respectively. Pyruvate carboxylase provides anaplerotic input to the tricarboxylic acid (TCA) cycle and its activity is remarkably high in β-cells [9, 10, 15]. Through activation of the TCA cycle, reducing equivalents are transferred to the electron transport chain resulting in hyperpolarisation of the mitochondrial membrane ($\Delta\Psi_m$) and generation of ATP. Proton export from the mitochondrial matrix, manifested by $\Delta\Psi_m$ hyperpolarisation, has been shown to be rate limiting in the coupling of glucose metabolism to insulin secretion [16].

In addition to pyruvate dehydrogenase, two TCA cycle enzymes, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, are activated by $Ca^{2+}$ [reviewed in ref 17] (see fig. 2), which may reinforce the production of metabolic coupling factors during glucose-stimulated insulin secretion [18]. The ATP formed in the mitochondria by oxidative phosphorylation is transferred to the cytosol. The subsequent increase in the ATP/ADP ratio in the cytosolic compartment causes depolarisation of the plasma membrane by the closure of ATP-sensitive $K^+$ channels ($K_{ATP}$) [19]. In type 2 diabetic patients, sulphonylureas are used to stimulate insulin secretion because they close $K_{ATP}$ channels [20]. Closure of the $K_{ATP}$ channels is the key step by which glucose raises cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$), as it allows the opening of voltage-sensitive $Ca^{2+}$ channels [19, 21], similar to those expressed in other excitable cells (fig. 1). In glucose-stimulated β-cells, both the rise in $[Ca^{2+}]_c$ and insulin secretion are biphasic, with a transient

first phase and a second, sustained phase [22, 23]. The increase in $[Ca^{2+}]_c$ is the main trigger for insulin exocytosis of the secretory granules [21, 24].

## Control of insulin secretion

The $Ca^{2+}$ signal in the cytosol is necessary but not sufficient for the full development of biphasic insulin secretion. By using sulphonylureas, glucose was proposed to evoke $K_{ATP}$-independent stimulation of insulin secretion [25]. This $K_{ATP}$-independent pathway was further characterised in 1992, when glucose was demonstrated to elicit secretion under conditions of clamped, elevated $[Ca^{2+}]_c$ [26, 27]. More recently, knock-out mouse models lacking either of the two functional subunits of the $K_{ATP}$-channel showed a marked reduction, albeit not abolished, in glucose-stimulated insulin secretion [28, 29]. As a consequence of the absence of functional $K_{ATP}$ channels, $[Ca^{2+}]_c$ is already elevated at low glucose concentration. Therefore, noteworthy is that these $\beta$-cells show a partial secretory response to glucose without changes in $[Ca^{2+}]_c$. The $K_{ATP}$-independent pathway is also illustrated by some forms of the PHHI syndrome. This hyperinsulinism is most frequently caused by mutations in one of the two subunits (the sulphonylurea receptor and the KIR 6.2) of the $K_{ATP}$ channel, resulting in uncontrolled $Ca^{2+}$-mediated hypersecretion of insulin [30]. However, PHHI patients often retain some glucose-stimulated insulin secretion above the constitutively increased basal rate [31]. This supports in vitro observations mentioned earlier, which had suggested the existence of a $K_{ATP}$ channel-independent effect of glucose [reviewed in ref. 32]. Glucose is thus capable of eliciting a partial secretory response under conditions of clamped, elevated cytosolic $Ca^{2+}$ concentrations without affecting the plasma membrane potential.

Insulin release is also controlled and modulated by neurotransmitters and hormones. Nutrient-induced secretion is potentiated by the neurotransmitters acetylcholine and pituitary adenylate cyclase-activating polypeptide (PACAP), as well as by the gastrointestinal hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) [33–35]. Conversely, GLP-1 receptor null mice are glucose intolerant [36], while islets isolated from these mice exhibit a well-preserved insulin secretory response to glucose [37]. Knock out of the GIP receptor in the mouse also leads to glucose intolerance [38]. In addition, insulin secretion is subjected to paracrine regulation by glucagon release from the islet $\alpha$-cells [39, 40], although this has been questioned recently from pancreas perfusion experiments [41]. Moreover, insulin exocytosis is under the direct negative control of norepinephrine, somatostatin and circulating epinephrine [reviewed in refs 33, 42–44]. Descriptions of the actions of hormones and neurotransmitters are found in the mentioned reviews.

## Mitochondria in cell function

Mitochondrial metabolism is crucial for the coupling of glucose recognition to insulin exocytosis [45]. ATP generated in the mitochondria is the main coupling messenger in insulin secretion, but other metabolic factors are necessary for the full development of the secretory response. Generation of these mitochondria-derived factors depends on optimal function of this fascinating organelle. Mitochondria derive from the symbiotic association of oxidative bacteria and glycolytic proto-eukaryotic cells [46]. The endosymbiotic model is illustrated by a unique mitochondrial genome in the form of circular DNA (mtDNA) with primitive characters [46]. mtDNA is maternally inherited, because of segregation during early zygote development, and exists in multiple copies in every cell, except erythrocytes. In contrast to nuclear DNA, mtDNA is comprised of only coding sequences and its repair mechanisms are poor. Moreover, it is juxtaposed to the respiratory chain, which generates mutagenic oxygen derivatives [47]. Consequently, mtDNA is particularly sensitive to oxidative stress and is highly susceptible to mutations.

Human mtDNA comprises only 37 genes (16,569 bp), notably those encoding 13 polypeptides, all of which are part of the multi-subunit enzyme complexes responsible for respiration [48]. The vast majority of the enzyme subunits and other mitochondrial proteins are encoded by the nuclear genome (table 1). These proteins are synthesised in the cytosol and imported into the mitochondrion [49]. In

Table 1. Components of the electron transport chain, their enzymatic activity, inhibitors, subunits, and genomic source (encoded by mitochondrial or nuclear genome).

| Complex | Enzyme activity | Inhibitors | Subunits | Mitochondrial subunits | Nuclear subunits |
|---|---|---|---|---|---|
| I | NADH:CoQ oxidoreductase | rotenone | 43 | 7 | 36 |
| II | Succinate:CoQ oxidoreductase | malonate | 4 | 0 | 4 |
| III | CoQ:Cytochrome bc1 oxidoreductase | antimycin | 11 | 1 | 10 |
| IV | Cytochrome c oxidase | cyanide, azide, CO | 13 | 3 | 10 |
| V | ATP synthase : proton translocator | oligomycin | 16 | 2 | 14 |
| ANT | adenine nucleotide translocator | atractyloside | 1 | 0 | 1 |

addition, nuclear DNA controls the transcriptional activity of mtDNA through regulatory proteins such as the mitochondrial transcription factor A (TFAM) [50]. Disruption of the Tfam gene in mice is lethal since homozygous knock-out embryos die at embryonic day (E)10.5 [50]. At E9.5, these knock-out embryos exhibit massive apoptosis [51]. Such in vivo evidence that respiratory chain deficiency predisposes cells to apoptosis contrasts with previous conclusions based on in vitro studies of cultured cells. Indeed, mitochondria have been shown to play a major role in the cascade of apoptosis, or programmed cell death. One of the key events is the release of cytochrome c to the cytosol [52].

Mitochondria constitute the main source of energy, essentially ATP. These organelles are present in most eukaryotic cells, varying in number from hundreds to thousands [48]. In living HeLa cells, mitochondria have been visualised as a continuous network by high-resolution analysis in three dimensions using targeted green fluorescent proteins [53]. This intriguing observation suggested that, in a living cell, mitochondria could be in fact one single mitochondrial network, although this pattern is technically difficult to assess.

Three classes of fuel can activate mitochondria: amino acids, fatty acids and carbohydrates, the latter being of most relevance in β-cells under physiological conditions. In the pancreatic β-cell, ATP and other mitochondrial factors accomplish the coupling of glucose metabolism to insulin secretion. Glycolysis forms pyruvate, the principal mitochondrial substrate, which supplies substrates for oxidation in the TCA cycle. The reducing equivalents of the substrates are transferred to the pyridine nucleotide NADH and the flavin nucleotide $FADH_2$ (fig. 2), which provide electrons to the respiratory chain upon their reoxidation. Electrons can enter the respiratory chain both at complexes I (NADH) and II ($FADH_2$). The latter complex, succinate dehydrogenase, is also an integral part of the TCA cycle. The electron flow drives the extrusion of protons out of the mitochondrial matrix, which establishes the electrochemical gradient across the inner mitochondrial membrane. The mitochondrial membrane potential generated in this way is negative inside and created by complexes I, III and IV. Complex V catalyses the condensation of ADP with inorganic phosphate to yield ATP. The generation of this high-energy bond is powered by the diffusion of protons back into the matrix. Finally, ATP is transferred to the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT). Disruption of the heart/muscle Ant gene in mice results in physiopathological symptoms of mitochondrial myopathy and cardiomyopathy [54].

In the mitochondrial matrix, $Ca^{2+}$ increases the activity of several dehydrogenases. In this manner, increased $[Ca^{2+}]_c$ occurring during cell activation is relayed to the mitochondria via a $Ca^{2+}$ uniporter to cover the energetic

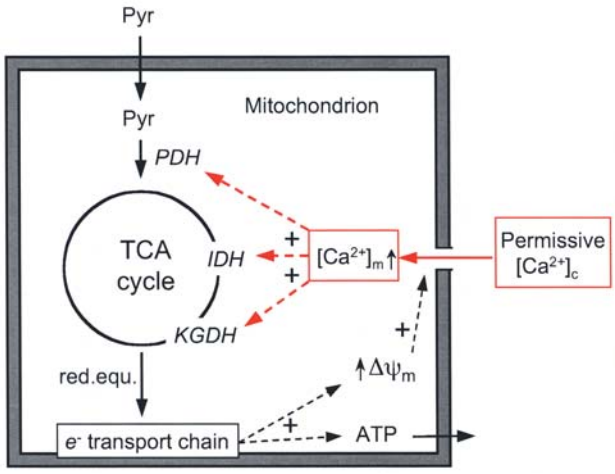


Figure 3. Mitochondrial activation and the feedforward effect of $Ca^{2+}$ on dehydrogenases. The elevation of $[Ca^{2+}]_c$ following cell activation is relayed to the mitochondria via a $Ca^{2+}$ uniporter. Upon permissive $[Ca^{2+}]_c$ (about 300 nM), $Ca^{2+}$ entry is favoured by hyperpolarisation of $\Delta\Psi_m$ secondary to respiratory chain activation. The rise in $[Ca^{2+}]_m$ further activates pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH) and α-ketoglutarate dehydrogenase (KGDH).

requirements of the cell [17, 55]. Such $Ca^{2+}$ entry is favoured by activation of the respiratory chain, for example by glucose in the β-cell. Therefore, hyperpolarisation of $\Delta\Psi_m$ permits the rise in mitochondrial $Ca^{2+}$ ($[Ca^{2+}]_m$) (fig. 3) to reach concentrations sufficient for the activation of NADH-generating dehydrogenases [55, 56]. This feedforward effect of $Ca^{2+}$ depends on permissive levels of $[Ca^{2+}]_c$ and on the availability of substrates for the TCA cycle, ensuring anaplerotic input [57, 58]. Pyruvate dehydrogenase has been shown to be activated by $Ca^{2+}$ in permeabilised HIT-T15 cells, a β-cell line [57], and by glucose in intact rat pancreatic islets [59]. Extracellular $Ca^{2+}$ is recruited for this mitochondrial activation. Indeed, specific blockade of L-type $Ca^{2+}$ channels results in the abolition of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ rises evoked by glucose [23] or by a cell-permeant derivative of the TCA cycle intermediate methyl-succinate [60]. Similarly, blockade of $Ca^{2+}$ influx attenuated the increase of NAD(P)H evoked by glucose in single β-cells [61, 62]. In the β-cell line MIN-6, glucose has been shown to promote ATP elevation both by enhanced substrate supply and by activation of $Ca^{2+}$-sensitive mitochondrial enzymes [63]. Generation of other additive factors derived from glucose metabolism might also be promoted by $[Ca^{2+}]_m$ elevation as discussed later in this review.

## Depletion of mtDNA causes mitochondrial dysfunction in the β-cell

Glucose-stimulated insulin secretion is inhibited by blockade of the respiratory chain, using mitochondrial

poisons or by lowering the oxygen supply to the $\beta$-cell [64]. Another way to impair respiratory chain activity is to create so-called $\rho^\circ$ cells by suppression of those enzyme subunits encoded by mtDNA [65]. Such chemical treatment of $\beta$-cell lines resulted in the depletion of mtDNA with preserved insulin biosynthesis and cell viability, albeit with a reduced proliferation rate [66–69]. In rat insulinoma INS-1 cells, mtDNA depletion also resulted in altered mitochondrial morphology and inhibition of glucose-stimulated ATP production [67]. The latter explains why glucose does not depolarise the plasma membrane potential in INS-1 $\rho^\circ$ cells compared to the depolarisation seen in control INS-1 cells. The deficient ATP generation and membrane depolarisation is secondary to the impaired activation of the mitochondrial electron transport chain. This is reflected by the absence of hyperpolarisation of $\Delta\Psi_m$ normally seen in control cells. Similar results were obtained with the membrane-permeant mitochondrial substrate methyl-succinate [67], which mimics the effect of glucose on insulin secretion [70–72]. This suggests that mitochondrial metabolism rather than glycolysis is defective in $\rho^\circ$ cells. As expected from these results, glucose does not increase $[Ca^{2+}]_c$ and insulin secretion in different $\rho^\circ$ $\beta$-cell line preparations [66–69]. These $\rho^\circ$ cells still synthesise, store and secrete insulin, as demonstrated by insulin secretion in response to the $[Ca^{2+}]_c$-raising agents KCl and glibenclamide, which do not require mitochondrial metabolism [67–69]. Replenishment of MIN-6 $\rho^\circ$ cells with normal mitochondria from mouse fibroblasts completely restored glucose-stimulated insulin secretion [68]. These results emphasise the crucial role of mitochondria in the generation of metabolic coupling factors in glucose-induced insulin release [45].

As discussed above, expression of mtDNA is controlled by a nucleus-encoded transcription factor, TFAM, and disruption of this gene in the mouse is lethal [50]. The $\beta$-cell-specific deletion of the Tfam gene caused a diabetic phenotype [73]. The islets of these mice exhibited attenuated hyperpolarisation of $\Delta\Psi_m$ upon glucose stimulation and a diminished secretory response to glucose. These transgenic animals represent the first model of human mitochondrial diabetes and further highlight the pivotal role of mitochondria in stimulus-secretion coupling.

## Mitochondrial disfunctions and their impact on $\beta$-cell metabolism

A specific maternally inherited form of diabetes mellitus has been linked to mutations in the mtDNA [74, 75]. Often associated with neurosensorial deafness, it is also called maternally inherited diabetes and deafness (MIDD). The most frequent mutation encountered is the A3243G mutation in the tRNA (Leu, UUR) gene [75, 76], and together they account for approximately 1% of all diabetic cases [77]. Diabetic patients with a mtDNA mutation have been treated with coenzyme Q10, a component of the respiratory chain, but despite improved insulin secretion there was no effect on diabetic complications [78]. Gene therapy by mitochondrial transfer cannot be envisaged in the near future due to technical limitations, although introduction of mtDNA in mice has been reported. The authors successfully introduced mtDNA with large-scale deletion in mouse embryos and obtained germline transmission of this mutated mtDNA through three generations [79]. The phenotype of these 'trans-mitogenic' mice was essentially limited to kidney failure, lethal before 200 days of age, indicating important species differences in mtDNA segregation.

The diabetic state is generally characterised by accelerated tissue ageing perhaps related to mitochondrial dysfunction. Accumulation of point mutations in mtDNA has been reported to occur in an age-dependent manner in humans [80]. The mitochondria are the principal source of reactive oxygen species (ROS) resulting from imperfect electron transport. Normally, only 0.1% of total oxygen consumption leaks to ROS generation, but the percentage becomes more pronounced in ageing tissue [47]. This deleterious process is amplified by diminishing natural enzymatic defences (e.g. catalase and superoxide dismutase). The low expression of these protective enzymes [81] renders the $\beta$-cell particularly susceptible to ROS actions [82]. In addition to their acute effects, ROS may also lead to enhanced mutations in mtDNA, worsened by the limited repair capacity. Taken together, these observations suggest that ROS may participate in the impairment of glucose-induced insulin secretion seen in both ageing and type 2 diabetes [83].

Different forms of MODY represent monogenic forms of diabetes with autosomal dominant transmission. They are characterised by $\beta$-cell disfunction due to mutations in nuclear genes [84]. MODY1 and MODY3 have been linked to mutations in the transcription factors hepatocyte nuclear factor HNF-4$\alpha$ and HNF-1$\alpha$ respectively [84]. MODY3 is the most common form of this inherited disease and explains about 2% of diabetic cases. Suppression of the HNF-1$\alpha$ gene in mice results in diabetes and impairment of glucose-induced insulin secretion in vitro [85]. $\beta$-cell-targeted expression of a dominant-negative mutant of HNF-1$\alpha$ induced a MODY3-like phenotype in transgenic mice with $\beta$-cell damage and mitochondrial swelling [86]. In cellular model systems, the molecular basis of the defect has been attributed to deranged mitochondrial metabolism [87, 88]. In particular, the defective respiratory chain activation correlated with down-regulation of the TCA cycle enzyme $\alpha$-ketoglutarate dehydrogenase accompanied by an up-regulation of uncoupling protein 2 (UCP2) [88].

UCP2 is an inner mitochondrial membrane protein that tends to diminish the proton gradient generated by the res-

piratory chain. Its overexpression in β-cells attenuates ATP generation and insulin secretion in response to glucose [89]. Conversely, deletion of the UCP2 gene in mice enhances islet ATP generation and insulin secretion during glucose stimulation [90]. Chronic exposure of β-cells to fatty acids induces UCP2 expression, which correlates with reduced glucose-evoked insulin secretion [91]. This may be part of an adaptive mechanism protecting the β-cell against oxidants. Indeed, in in vitro experiments using clonal β-cells, oxidative stress induced UCP2 expression [92] and increased proton conductance in isolated mitochondria [93]. The UCPs could therefore play a role in decreasing the levels of ROS inside the mitochondria.

## Signals and messengers for insulin exocytosis

Intracellular ATP is required for insulin exocytosis [94–96]. A higher ATP/ADP ratio is needed for the closure of $K_{ATP}$ channels compared to the requirement of the exocytotic process itself [95]. ATP is a major permissive factor for insulin secretory vesicle movement and for priming of exocytosis [24, 96]. This is distinct and complementary to the aforementioned action on the $K_{ATP}$ channel. However, at non-stimulatory $Ca^{2+}$ concentrations, ATP does not cause insulin secretion in permeabilised cells [94]. In the presence of stimulatory $Ca^{2+}$, ATP enhances the process [94, 96, 97]. Conversely, glucose-induced ATP elevation does not promote insulin release in the absence of extracellular $Ca^{2+}$ [98]. There was, however, a correlation between the generation of ATP and the $K_{ATP}$-independent insulin secretion evoked by glucose or by the combination of glutamine plus leucine. Therefore, ATP produced from nutrient metabolism could be involved in the $K_{ATP}$-independent secretion. Glucose also generates GTP, which could trigger insulin exocytosis via GTPases [99, 100]. GTP is formed in the mitochondria by the TCA cycle but it is trapped in the organelle. In the cytosol, GTP is mainly formed through the action of nucleoside diphosphate kinase. In contrast to ATP, GTP is capable of initiating insulin exocytosis in a $Ca^{2+}$-independent fashion, which qualifies it as a messenger molecule [94, 100, 101]. Not known is whether GTP acts via a monomeric or heterotrimeric G-protein directly controlling exocytosis [24, 102].

We have known for more that three decades that cAMP potentiates glucose-stimulated insulin secretion. GLP-1, GIP, PACAP and glucagon increase cAMP levels in β-cells [33, 34, 103]. Glucagon has been shown to render β-cells glucose responsive through the generation of cAMP [39]. This paracrine effect of glucagon was also recently demonstrated in human islets [40]. cAMP exerts at least three actions which may render the β-cell glucose competent and enhance insulin secretion: (i) the $Ca^{2+}$ current through L-type $Ca^{2+}$ channels is increased [104]; (ii) β-cells refractory to glucose depolarisation become responsive, showing $K_{ATP}$ channel closure [105]; (iii) the secretory machinery is sensitized to $Ca^{2+}$ [94, 104, 106, 107]. All these actions are mediated by cAMP-dependent protein kinase A. A direct protein kinase A-independent enhancement of insulin exocytosis involving the cAMP-GEFII protein has been described [108]. Recently, protein kinase A was shown to phosphorylate phogrin (phosphatase homologue in granules of insulinoma) [109] and could therefore be a link between cAMP and the exocytotic machinery through the action of protein kinase A. Phogrin is a transmembrane protein of secretory granules, which is phosphorylated upon secretagogue stimulation [110]. Although glucose has been found to increase cAMP levels in some studies, such an effect is not observed in purified β-cells [39]. Therefore, the role of cAMP in glucose-stimulated insulin release is that of a potentiator rather than a mediator.

Among other putative nucleotide messengers (fig. 4), NADH and NADPH are generated by glucose metabolism [for a review see ref. 58]. Single β-cell measurements of NAD(P)H fluorescence have demonstrated that the rise in pyridine nucleotides precedes the rise in $[Ca^{2+}]_c$ [111] and that the elevation in the cytosol is reached more rapidly than in the mitochondria [112]. Cytosolic NADPH is generated by glucose metabolism via the pentosephosphate shunt [113] and by mitochondrial shuttles [9]. An action of NADPH on insulin secretory granules has been proposed from experiments on toadfish islets [114].

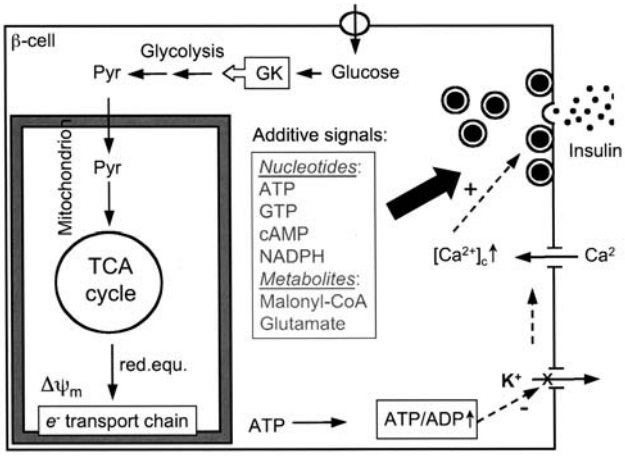


Figure 4. Metabolism-secretion coupling in the β-cell and additive signals of exocytosis. Glucose is phosphorylated by glucokinase (GK) and converted to pyruvate (Pyr) by glycolysis. Pyr enters the mitochondria and fuels the TCA cycle resulting in the transfer of reducing equivalents (red.equ.) to the respiratory chain, leading to hyperpolarisation of $\Delta\Psi_m$ and ATP generation. Closure of $K_{ATP}$ channels depolarises the cell membrane leading to $[Ca^{2+}]_c$ elevation and subsequent insulin exocytosis. Several putative messengers, or additive signals, proposed to participate in the metabolism-secretion coupling are indicated (see text).

In glucose-stimulated $\beta$-cells, the TCA cycle intermediate citrate is exported from the mitochondria. In the cytosol, citrate carbons are transferred to coenzyme A (CoA) to form acetyl-CoA. Subsequently, acetyl-CoA carboxylase catalyses the synthesis of malonyl-CoA, which is a lipid precursor. Malonyl-CoA prevents fatty acid transport into the mitochondria by inhibition of carnitine palmitoyl transferase I (CPT-I) [115]. Consequently, fatty acid oxidation is reduced, favouring the synthesis of long-chain acyl-CoAs in the cytosol. This metabolic switch is at the origin of the proposal that malonyl-CoA acts as a metabolic coupling factor in insulin secretion [116]. The long-chain acyl-CoA hypothesis was substantiated by the observation that palmitoyl-CoA enhances $Ca^{2+}$-evoked insulin exocytosis [117]. However, disruption of malonyl-CoA accumulation during glucose stimulation did not attenuate the secretory response [118], even under conditions in which only the $K_{ATP}$-independent pathway is operative [119]. In view of the inhibition of metabolism-secretion coupling in lipid-depleted $\beta$-cells [120, 121], a permissive role of long-chain acyl-CoAs in insulin release cannot be excluded. Moreover, overexpression of CPT-I in INS-1E cells results in reduced glucose-stimulated insulin secretion [122]. Relevant here is that impaired fatty acid beta-oxidation has recently been associated with a hyperinsulinism syndrome [123]. The patient presented a defect of the mitochondria enzyme short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) due to homozygous mutation. This clinical case again suggests links between fatty acid metabolism and the control of insulin secretion. In conclusion, the role of long-chain acyl-CoA derivatives in metabolism-secretion coupling requires further investigation.

## Mitochondrially driven insulin exocytosis

Numerous studies have sought to identify the factor(s) mediating the $K_{ATP}$-independent effect of glucose on insulin secretion. In the previous paragraphs, we have listed several factors thought to be involved in the potentiation and/or modulation of glucose-stimulated insulin release (fig. 4). Predominantly, these molecules are nucleotides but also metabolites such as the aforementioned long-chain acyl-CoA, which are still debated as putative coupling factors. To study the link between mitochondrial activation and insulin exocytosis, we have established a *Staphylococcus* $\alpha$-toxin-permeabilised $\beta$-cell model permitting the clamping of $[Ca^{2+}]_c$ and nucleotides such as ATP. This preparation can be directly stimulated with various mitochondrial substrates including succinate, a TCA cycle intermediate. As discussed above, three mitochondrial dehydrogenases are known to be activated by $Ca^{2+}$ in various tissues [124, 125], including insulin-secreting cells [57, 72, 126]. Therefore, the increase in $[Ca^{2+}]_m$ conveniently reflects mitochondrial activation [17, 127] (see fig. 3). To monitor mitochondrial activation, we measured mitochondrial free $[Ca^{2+}]$ using INS-1 cells stably expressing the $Ca^{2+}$-sensitive photoprotein aequorin [23] with simultaneous assessment of insulin secretion. When the $[Ca^{2+}]_c$ was clamped at 500 nM, succinate caused a marked biphasic increase in $[Ca^{2+}]_m$, an effect secondary to hyperpolarisation of $\Delta\Psi_m$ [71]. This mitochondrial activation resulted in biphasic insulin release. As $Ca^{2+}$ enhances succinate oxidation under these conditions [72], we can conclude that mitochondrial activation directly stimulates insulin exocytosis. The obvious question is whether the increase in $[Ca^{2+}]_m$ is required for the action of succinate on insulin secretion. To test this, ruthenium red, an inhibitor of $Ca^{2+}$ uptake through the mitochondrial uniporter, was applied. Ruthenium red attenuated the $[Ca^{2+}]_m$ rise and abolished insulin release induced by succinate. A $[Ca^{2+}]_m$ rise is thus necessary for the mitochondrially driven insulin exocytosis, but $Ca^{2+}$ is not sufficient. Indeed, the sole $[Ca^{2+}]_m$ elevation without provision of carbons to the TCA cycle (anaplerosis) failed to elicit insulin secretion under these conditions [71]. This strongly suggested the existence of a mitochondrial factor generated through anaplerotic input into the TCA cycle.

## Glutamate as a metabolic coupling factor

Thus, the TCA cycle intermediate succinate enhances insulin secretion at the permissive concentration of 500 nM $Ca^{2+}$ and at 10 mM ATP in $\alpha$-toxin-permeabilised INS-1 cells [71]. The magnitude of the response is similar to a rise in cytosolic free $Ca^{2+}$ from 500 nM to 1.3 µM [128]. Other TCA cycle intermediates, such as $\alpha$-ketoglutarate, malate [128] or citrate [71] were inefficient. Stimulation of insulin exocytosis requires not only the provision of carbons to the TCA cycle but also an increase in $[Ca^{2+}]_m$, both requirements achieved by succinate [71, 129]. Exposure of isolated INS-1 cell mitochondria to succinate results in a pronounced production of glutamate [130]. Glutamate can be generated through several biochemical pathways including transamination reactions [reviewed in ref. 131]. In mitochondria, glutamate dehydrogenase (GDH) forms glutamate from the TCA cycle intermediate $\alpha$-ketoglutarate [132]. In permeabilised INS-1 cells, glutamate stimulates insulin secretion, reproducing the effect of succinate, both at 10 and 1 mM ATP [129]. In contrast to succinate, the secretory response to glutamate does not require activation of mitochondrial metabolism [129]. Important in this regard is that oligomycin abolished insulin release in response to succinate without affecting glutamate-induced exocytosis [18]. These results suggest that glutamate, in contrast to succinate, acts downstream of mitochondrial metabolism. As the effect of glu-

tamate is similar at low and high ATP concentrations, ATP is most unlikely to mediate the glutamate-evoked exocytosis.

## What is the action of glutamate downstream of mitochondria?

An effect of glutamate on insulin exocytosis downstream of mitochondria was first proposed in 1999 [129] and recently confirmed by another group [133]. A link between glutamate and the long-chain acyl-CoA derivatives could also be envisaged. Indeed, malonyl-CoA synthesis is catalysed by acetyl-CoA carboxylase, an enzyme that is activated by glutamate and $Mg^{2+}$-sensitive protein phosphatase type 2A [134]. Such an effect has recently been demonstrated in islet β-cells [135], suggesting mechanisms of complementary metabolic signals acting synergistically [133].

Alternatively, glutamate formed in the mitochondria and transferred to the cytosol might be taken up by the insulin-containing granules. This idea is supported by the finding that, in agreement with glutamate transport properties in synaptic vesicles [see ref. 136], collapse of the granule membrane potential and application of an inhibitor of glutamate uptake blocked glutamate-induced insulin exocytosis in permeabilised INS-1 cells [129]. However, glutamate uptake by insulin-containing secretory granules remains to be demonstrated. Of interest is that clonal pancreatic α-cells, secreting glucagon, have recently been shown to accumulate glutamate in their vesicles and to release it upon stimulation [137]. The same group further demonstrated that pancreatic α-cells express one of the two so far identified isoforms of the vesicular glutamate transporters [138].

Vesicular glutamate transporter was first cloned as a plasma membrane inorganic phosphate transporter in 1994 with the name BNPI (for brain $Na^+$-dependent Pi transporter I) [139]. However, only in 2000 did two groups simultaneously recognize this protein as a genuine vesicular glutamate transporter [140, 141], which was hence renamed VGLUT1. More recently, DNPI (for differentiation $Na^+$-dependent Pi transporter I), which is closely related to VGLUT1, was raised to the rank of vesicular glutamate transporter and is now referred to as VGLUT2 [142–145]. DNPI/VGLUT2 has been shown to be present in pancreatic α-cells but not in β-cells [138]. In β-cells, another alternative vesicular glutamate transporter might transport glutamate. One can speculate that glutamate has a general effect to sensitize secretory vesicles to the action of $Ca^{2+}$ in exocytosis. Interestingly, glutamate is usually used as the main anion in experiments employing permeabilised cells or the patch-clamp technique for the monitoring of exocytosis. Churcher and Gomperts [146] used $Cl^-$ as the main anion in permeabilised mast cells and in fact observed that glutamate was required for $Ca^{2+}$-induced exocytosis. At present, the mechanism underlying the permissive action of glutamate in the secretory process is unknown.

## Provision of glutamate to the cytosol

We thus postulate an intracellular messenger role for glutamate in stimulus-secretion coupling (fig. 4), although the precise site of glutamate action downstream of mitochondria remains to be defined. Previously, extracellular glutamate was reported to cause a transient stimulation of insulin secretion in the perfused rat pancreas [147] and to elicit a small secretory response in isolated rat islets [148]. Of note is that only approximately 25% of rat β-cells express glutamate receptors [149] and that glutamate does not elicit insulin release in intact rat islets [150] or INS-1 cells [unpublished observations]. Therefore, in our model, glutamate would act as an intracellular rather than an extracellular messenger in insulin exocytosis.

The principal observation is that glutamate directly stimulates insulin exocytosis in permeabilised cells at permissive $[Ca^{2+}]_c$ independently of mitochondrial activation [129]. Moreover, insulinotropic action of the cell-permeant derivative dimethyl-glutamate in intact β-cell preparations is restricted to permissive conditions, e.g. at intermediate glucose levels or in the presence of a sulphonylurea [129, 151]. A role for glutamate in the amplifying pathway of the nutrient-induced secretory response has recently been substantiated in the model of in situ pancreatic perfusions. In this sensitive model, supplementation of dimethyl-glutamate potentiated the second phase of glucose-stimulated insulin secretion [152]. On the other hand, the importance of intracellular glutamate in the amplifying pathway has been questioned using dimethyl-glutamate. Indeed, in the presence of depolarising concentrations of $K^+$, dimethyl-glutamate was shown to exhibit only minor effects on insulin secretion in isolated rat islets [153]. This could be the consequence of dimethyl-glutamate failure to generate ATP [129], probably because of poor conversion of glutamate to the TCA cycle intermediate α-ketoglutarate [154]. However, the same group, using a very similar approach, subsequently reported that cytosolic glutamate accumulation partially reconstituted signaling beyond mitochondrial metabolism in the β-cell upon glucose stimulation [133]. Taken together, these results demonstrate that intracellular glutamate itself is not sufficient to elicit insulin secretion but participates in the sustained secretory response evoked by glucose.

Glucose increased the cellular glutamate content in INS-1 cells and human islets [129, 155]. In rat islets, glutamate was the only one of 12 amino acids that increased during glucose stimulation, whereas levels of aspartate, a possible $NH_2$ donor, decreased [156]. In other reports, glucose did not change glutamate levels in islets isolated

from rats [157] or mice [158]. The lack of increase in glutamate concentrations reported by MacDonald and Fahien [157] led these authors to conclude that glutamate is not a messenger in insulin secretion, although they did not address the crucial question of a messenger function [157]. Indeed, measurements of total glutamate contents do not reflect fluctuations of the putative co-factor of insulin exocytosis in the cytosolic compartment [18]. Limitation resides in the lack of methods allowing determination of glutamate levels in the relevant cellular compartment, i.e. the cytosol. Substantiation of the role of glutamate requires modulation of its cytosolic levels.

Results obtained with a transgenic mouse model have indirectly highlighted the putative role of intracellular glutamate in insulin secretion. In these mice, targeted overexpression of the glutamate decarboxylating enzyme GAD65 in $\beta$-cells resulted in glucose intolerance without any sign of insulitis or loss of $\beta$-cells. Their islets showed impaired glucose-stimulated insulin secretion, while the response to the $Ca^{2+}$-raising agent KCl was preserved [159]. Although the authors did not measure glutamate levels in the pancreatic islets of these transgenic mice, they discussed reduced cellular glutamate levels as one possible explanation for the diminished response to glucose. Hence, the decrease in cellular glutamate levels can theoretically be achieved by overexpression of glutamate decarboxylase. Upon appropriate expression, this cytosolic enzyme decarboxylates glutamate produced by the mitochondria after its release into the cytosol. Consequently, cytosolic glutamate could be specifically reduced, even during glucose stimulation without affecting major metabolic pathways. The gamma-aminobutyric acid (GABA) thus formed is not believed to affect insulin secretion [160]. In this context, the smaller isoform of glutamate decarboxylase, GAD65, predominantly expressed in rat pancreatic islets [161], has been overexpressed in clonal INS-1E $\beta$-cells and rat pancreatic islets, using recombinant adenovirus [155]. The study demonstrates that overexpression of GAD65 in $\beta$-cells results in reduced glutamate levels and impaired glucose-stimulated insulin secretion, showing a positive correlation between cellular glutamate levels and glucose-induced insulin secretion [155].

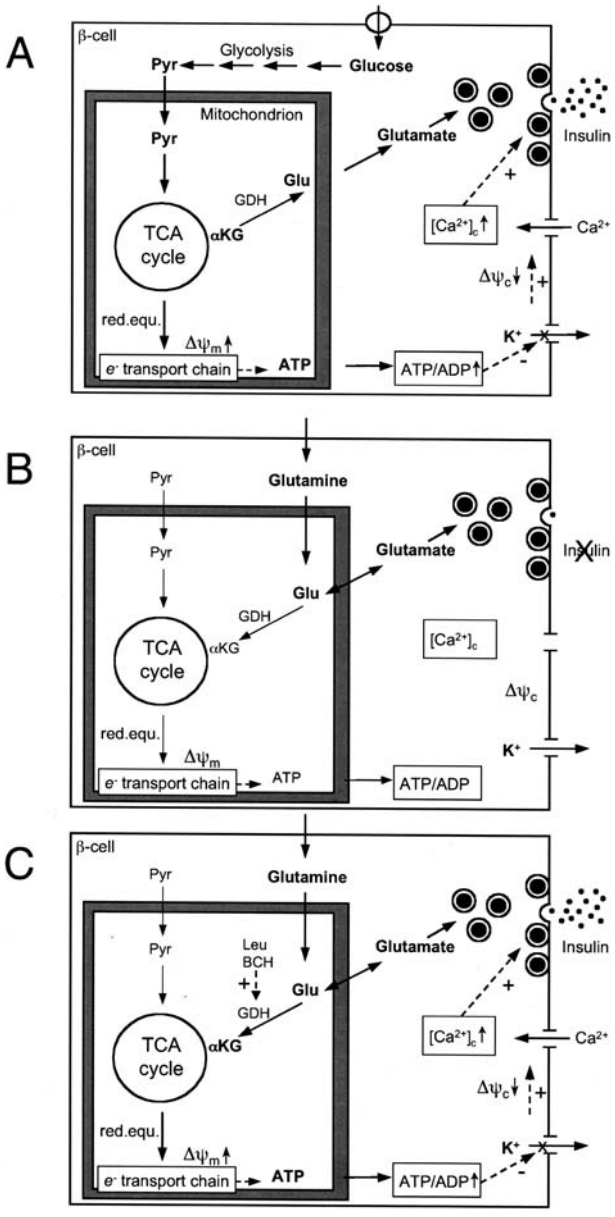


Figure 5. Proposed model for the role of glutamate dehydrogenase (GDH) in metabolism-secretion coupling in the $\beta$-cell. (*A*) Glucose undergoes glycolysis and forms pyruvate (Pyr) which fuels the TCA cycle resulting in respiratory chain activation and generation of ATP. Depolarisation of $\Delta\Psi_c$ leads to $[Ca^{2+}]_c$ elevation and insulin exocytosis. In the mitochondria, GDH forms glutamate (Glu) from the TCA cycle intermediate $\alpha$-ketoglutarate ($\alpha$KG). Glutamate acts downstream of the mitochondria and potentiates the effect of $Ca^{2+}$ on insulin secretion. (*B*) Glutamine is deaminated to glutamate but poorly converted to $\alpha$-KG resulting in deficient ATP generation without stimulation of insulin exocytosis. (*C*) GDH, once activated by leucine (Leu) or its non-metabolisable analogue BCH increases glutamine oxidation and insulin secretion, restoring the effect of glucose.

## Importance of GDH

GDH is a homohexamer located in the mitochondrial matrix, which predominantly forms glutamate from the TCA cycle intermediate $\alpha$-ketoglutarate (fig. 5A). GDH is encoded by a well-conserved 45-kb gene named GLUD1, which is organised into 13 exons [162]. In the brain, this enzyme ensures the cycling of glutamate-glutamine between astrocytes and neurons. Glutamate, the most abundant neurotransmitter, after its discharge by neurons is taken up by astrocytes, protecting against glutamate toxi-

city [163]. Subsequently, astrocytes can use glutamate as a fuel through α-ketoglutarate formation and TCA cycle activation, but the larger part is converted to glutamine [131]. The released glutamine is then used by neurons to generate glutamate, thereby ensuring the cycling. Still in the brain, glutamate is also the precursor of GABA, another major neurotransmitter. Moreover, glutamate plays a crucial role in ammonia metabolism and detoxification, mainly in two organs: the liver, via hepatic ureagenesis, and the kidney, via renal ammoniagenesis and subsequent urinary excretion [131].

In pancreatic β-cells, the importance of GDH as a key enzyme in the control of insulin secretion was recognized more than two decades ago [164]. Later, inhibition of GDH enzymatic activity was shown to result in decreased insulin release [165]. More recently, activating mutations of GDH have been associated with a hyperinsulinism syndrome [166, 167], again revealing the importance of this enzyme. Several studies have addressed the question of the role of GDH in the pancreatic β-cell, but even the preferred direction of the catalytic activity is still debated. The enzyme is allosterically regulated by leucine, pyridine, adenine and guanine nucleotides [168, 169] and catalyses the reaction [132]:

$$\alpha\text{-ketoglutarate} + NH_3 + NAD(P)H \leftrightarrow \text{glutamate} + NAD(P)^+$$

Mutations in GDH associated with a gain of function and hyperinsulinism have been linked in several cases with reduced GTP-mediated inhibition of the enzyme [166, 167, 170]. Missense mutations were found in exons 11 and 12, corresponding to the allosteric domain, resulting, for example, in Lys450Glu modification [170]. More recently, mutagenesis and photoaffinity labeling indeed identified the Lys450 residue as a GTP-binding site on GDH [171]. Previously, the same group used photoaffinity labeling to discover an NADH-binding site of the enzyme in residues Cys270 through Lys289 [172]. GDH was also shown to be regulated by reversible ADP-ribosylation in mitochondria [173]. Inactivation of the enzyme is caused by ADP-ribose association, which is suppressed by NAD(P)H.

Preferential directional flux from α-ketoglutarate to glutamate has been suggested in mouse islets in which the incorporation of glucose carbons into glutamate was augmented by glucose stimulation, even without changing cellular glutamate content [174]. In most tissues this is the prevailing direction for the enzyme reaction [131, 132]. One notable exception is the astrocyte, which recycles glutamate, released as a neurotransmitter by the neighboring neurons, as discussed above. This pathway favours glutamate oxidative deamination and energy supply through the TCA cycle as well as generation of lactate used by neurons as an important energy source [175]. Conversely, in other tissues where glutamate is preferentially formed from α-ketoglutarate, the most likely donors of ammonia for glutamate synthesis by GDH are glutamine and aspartate [131]. In the cerebral cortex, glutamate production from glucose has been shown to reflect TCA cycle activity and carbon flux in resting humans and exercising rats [176, 177]. Thus, studies using nuclear magnetic resonance (NMR) spectroscopy have demonstrated the enrichment of glutamate carbons upon glucose stimulation [177, 178]. Corresponding experiments have recently been conducted in insulin secreting cells leading to similar conclusions, i.e. glutamate is a major leak out of the TCA cycle during glucose metabolism [179]. Following a 60-min glucose stimulation period, Brennan et al. [179] measured a marked enrichment of glutamate with labeled carbons derived from glucose as well as an enlarged glutamate pool size. Another recent NMR study also reported enrichment of glutamate with glucose carbons [180]. However, the total glutamate concentration was not elevated following the unusual prolonged 4-h glucose stimulation used in this report. This might indicate that along with its de novo synthesis, glutamate was used or released from the cells. Both studies using NMR spectroscopy in β-cell lines demonstrate an important participation of pyruvate carboxylase [179, 180], which is in agreement with previous analyses using biochemical approaches [10, 15]. The remarkably high anaplerotic activity in β-cells in the course of glucose stimulation suggests the loss of TCA cycle intermediates, which must be compensated in the form of oxaloacetate. This evidence adds weight to the model of mitochondria-derived factors, such as citrate [9] or glutamate [129], participating in the stimulation of insulin secretion upon glucose stimulation.

**Glutamine, a conditional secretagogue?**

Conversely to the effect of glucose, mitochondrial metabolism of glutamine/glutamate and ATP production is only weak in islets [151, 154] (see fig. 5B). As glutamine does not generate ATP, it is capable of neither depolarizing the plasma membrane nor of raising cytosolic $Ca^{2+}$ in native β-cells. In mouse islets, glutamine moderately enhances insulin release in $K_{ATP}$ pathway-independent conditions, i.e. when cytosolic $Ca^{2+}$ is maintained at permissive levels by diazoxide and high $K^+$ [98]. However, this stimulatory effect might be blunted due to the inhibitory action of $NH_4^+$, generated by glutamine in cells, which has been shown to inhibit insulin release in both mouse and rat islets secondary to intracellular alkalinisation [98, 181].

Exposure of islets to extracellular glutamine causes a marked increase in their glutamate levels without any increase in insulin secretion [154, 182]. This is explained by the sluggish conversion of glutamate to α-ketoglutarate [154]. Activation of GDH by L-leucine or its non-metabolisable analogue 2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid (BCH) increases glutamine oxidation and insulin secretion, essentially by enhancing the oxidative

deamination of glutamate [150, 183–186] (fig. 5C). These reactions and pathways are dependent on metabolic fluxes imposed by the relative substrate supply. Accordingly, of note is that glutamine oxidation, stimulated by the presence of an allosteric activator of GDH, is inhibited by glucose [186]. Glutamate can be converted to $\alpha$-ketoglutarate either by GDH-dependent oxidative deamination or, alternatively, by transamination reactions. In mitochondria isolated from pancreatic islets, glutamate transamination has been shown to generate $\alpha$-ketoglutarate in the presence of the aliphatic ketomonocarboxylic acid $\alpha$-ketoisocaproate [187, 188], which is also known to stimulate insulin secretion [189].

Unlike glutamine, glucose, the main nutrient secretagogue, increases not only ATP and cytosolic $Ca^{2+}$, but also glutamate to promote optimal signaling for insulin exocytosis. Therefore, there are two diametrically opposed pathways of GDH activation. (i) Upon glucose stimulation, GDH preferentially works in the direction of glutamate generation (fig. 5B). NMR studies have demonstrated this preferential direction in several tissues including insulin-secreting cells [179]. (ii) Upon glutamine exposure, the thus formed glutamate is elevated but poorly converted to TCA cycle intermediates unless BCH (or leucine) allosterically activates GDH, thereby promoting mitochondrial activation and ATP generation (fig. 5C). Expression of mutant GDH, which is associated with unregulated increased GDH activity and the hyperinsulinism syndrome [166, 167], has been recently examined in a $\beta$-cell line [190]. In control cells, glutamine alone did not stimulate insulin secretion. However, in cells expressing the activating mutation, glutamine became an efficient secretagogue, whereas the glucose dose response was left-shifted [190]. Taken together, these data further establish that in $\beta$-cells, under physiological conditions, GDH is strongly non-permissive for the secretagogue function of glutamine and might be rate limiting for that of glucose. The lack of secretory response to glutamine could be the consequence of defective ATP generation. Glutamine is the most abundant amino acid in muscle and plasma [191]. Accordingly, muscle represents an important reservoir from which the glutamine pool can be mobilized during acute exercise [192], a physiological state in which insulin secretion must be avoided. This is in accordance with the low monocarboxylate transporter and lactate dehydrogenase activities in the $\beta$-cell, which avoid lactate-induced insulin release [12]. Therefore, GDH may play a similar role as a gatekeeper to prevent amino acids from being efficient secretagogues.

## Conclusions

The crucial role of the mitochondria in $\beta$-cell function is now well recognised. However, metabolism-secretion coupling is extraordinarily complex and is still far from understood. One can speculate that not only ATP and glutamate but also other mitochondrially derived factors participate in the overall control of exocytosis. Studies in a permeabilised $\beta$-cell model have shown a direct link between mitochondrial activation and insulin exocytosis. Further studies demonstrated a positive correlation between cellular glutamate concentrations and the secretory response to glucose. At present, the mechanism of glutamate action on exocytosis is unknown. In this scenario, the classical neurotransmitter glutamate is allocated a novel role, that of an intracellular messenger or co-factor in insulin secretion. The elucidation of the mode of glutamate action in the $\beta$-cell should help to define its putative implication in other secretory processes. Greater definition of the role of mitochondrial molecular mechanisms in cell activation will certainly help to target therapeutic interventions in diabetes and other metabolic diseases.

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