

Topical Review

Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell

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Glucose-induced insulin secretion is determined by signals generated in the mitochondria. The elevation of ATP is necessary for the membrane-dependent increase in cytosolic Ca^{2+} , the main trigger of insulin exocytosis. Beta cells depleted of mitochondrial DNA fail to respond to glucose while still secreting insulin in response to membrane depolarisation. This cell model resembles the situation of defective insulin secretion in patients with mitochondrial diabetes. On the other hand, infants with activating mutations in the mitochondrial enzyme glutamate dehydrogenase are characterised by hyperinsulinism and hypoglycaemia. We have recently proposed that glutamate, generated by this enzyme, participates in insulin secretion as a glucose-derived metabolic messenger. In this model, glutamate acts downstream of the mitochondria by sensitising the exocytotic process to Ca^{2+} . The evidence in favour of such a role for glutamate is discussed in the present review.

Blood glucose homeostasis depends on the normal regulation of insulin secretion from the pancreatic beta cells and on the biological action of the hormone on its target tissues. Most forms of type 2 diabetes display dysregulation of insulin secretion combined with insulin resistance. Insulin secretion is stimulated not only by glucose but also by leucine and other amino acids (for review, see Wollheim, 2000). 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) (reviewed by Ahren, 2000). These and other factors, including fatty acids, all participate in the physiological stimulation of insulin secretion. For instance, knock-out of the GIP receptor in the mouse leads to glucose intolerance (Miyawaki *et al.* 1999). In addition, insulin exocytosis is under the direct negative control of noradrenaline (norepinephrine) and somatostatin (Lang *et al.* 1995; Sharp, 1996; Ahren, 2000).

Signal generation by glucose

The beta cell is poised to adapt rapidly the rate of insulin secretion to fluctuations in the blood glucose concentration. Glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase, which determines the rate of glycolysis and the generation of pyruvate (see Newgard & McGarry, 1995). High rates of glycolysis are maintained through the activity of mitochondrial shuttles, mainly the

glycerophosphate and malate/aspartate shuttles (MacDonald, 1981; Eto *et al.* 1999), which allow the reoxidation of cytosolic NADH. Other shuttles generating cytosolic NADPH have also been described (MacDonald, 1995; Farfari *et al.* 2000). As demonstrated in isolated purified beta cells, as much as 90% of glucose-derived carbons are oxidized by the mitochondria (Schuit *et al.* 1997). This is favoured by the very low expression of monocarboxylate transporters in the plasma membrane coupled with low activity of lactate dehydrogenase (Sekine *et al.* 1994; Ishihara *et al.* 1999; reviewed in Ishihara & Wollheim, 2000). Pyruvate, once transported into the mitochondria, is a substrate both for pyruvate dehydrogenase and pyruvate carboxylase. These enzymes ensure, respectively, the formation of acetyl-CoA and oxaloacetate. The latter provides anaplerotic input to the tricarboxylic acid (TCA) cycle (Schuit *et al.* 1997; Farfari *et al.* 2000). 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. In addition to pyruvate dehydrogenase, two TCA cycle enzymes, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase, are activated by Ca^{2+} (reviewed by Duchen, 1999). In the consensus model of glucose-stimulated insulin secretion, mitochondrial metabolism increases the cytosolic ATP/ADP ratio. This leads to closure of ATP-sensitive K^+ channels (K_{ATP}) and depolarisation of the plasma membrane. As a

consequence, the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) is raised by the opening of voltage-sensitive Ca^{2+} channels (see Rorsman, 1997). The increase in $[\text{Ca}^{2+}]_c$ is the main trigger for exocytosis, the process by which the insulin-containing secretory granules fuse with the plasma membrane (Lang, 1999). In glucose-stimulated beta cells both the $[\text{Ca}^{2+}]_c$ rises and the insulin secretion are biphasic with a transient first phase and a second, sustained phase (Bergsten, 1995; Kennedy *et al.* 1996).

The Ca^{2+} signal in the cytosol is necessary but not sufficient for the full development of biphasic insulin secretion. It was first shown in 1992 that glucose elicits secretion under conditions of clamped, elevated $[\text{Ca}^{2+}]_c$ (Gembal *et al.* 1992; Sato *et al.* 1992). The importance of this K_{ATP} -independent pathway is illustrated by mouse models lacking functional K_{ATP} following the knock-out of either of the two subunits of the channel (Miki *et al.* 1998; Seghers *et al.* 2000). The beta cells of these animals show spontaneous increases in $[\text{Ca}^{2+}]_c$ and glucose is still able to cause limited insulin secretion. Thus, both the K_{ATP} -dependent and -independent insulin secretion require mitochondrial metabolism (Detimary *et al.* 1994).

Mitochondrial diabetes

Non-insulin-dependent diabetes mellitus, or type 2 diabetes, is a polygenic disease characterised by impaired insulin secretion (Porte, 1991) often combined with insulin resistance (Kahn, 1998). A subform of type 2 diabetes referred to as maternally inherited diabetes and deafness (MIDD) has been linked to mutations in the mitochondrial genome. These mutations involve deletions or point mutations, e.g. in tRNA^{Leu(UUR)} at position 3243 also giving rise to mitochondrial myoencephalopathy, lactic acidosis and stroke-like episodes (MELAS) (Maassen & Kadowaki, 1996). In MIDD there is a progressive impairment of insulin secretion, which may eventually lead to severe insulin deficiency reminiscent of type 1 (insulin-dependent) diabetes. The clinical picture of these patients illustrates the well-known dependency of glucose-stimulated insulin secretion on mitochondrial function. It was already demonstrated three decades ago that mitochondrial poisons inhibit the effect of glucose (Hellman, 1970).

In vitro models of mitochondrial diabetes

Patients with mutations in mitochondrial DNA (mtDNA) display heteroplasmy, i.e. mitochondria within the same cells harbour both normal and mutated forms of the multicopy mtDNA. Autopsy of a mitochondrial diabetic with the 3243 mtDNA point mutation revealed much higher heteroplasmy (63%) in beta cells compared with other cell types (Kobayashi *et al.* 1997). The mutated mtDNA could be enriched in engineered cell lines by fusing patient-derived enucleated skin fibroblasts with a mtDNA depleted human cell line. After selection, such cybrids carry nearly 100% of either mutated or wild-type mtDNA. These cells with mutated mtDNA exhibited grossly altered morphology of the mitochondria and severely decreased oxygen consumption

(van den Ouweland *et al.* 1999). Unfortunately, the mitochondria of patient origin cannot be introduced into human beta cells for the study of stimulus–secretion coupling, as human insulin-secreting cell lines are not yet available. By contrast, the mtDNA of several rodent beta cell lines has been depleted by chemical treatment resulting in so-called ρ° cells. In rat insulinoma INS-1 cells mtDNA depletion also resulted in altered mitochondrial morphology and inhibition of glucose-stimulated ATP production (Kennedy *et al.* 1998). The latter explains why glucose does not depolarise the plasma membrane potential in INS-1 ρ° cells (Fig. 1B) compared with the depolarisation seen in control INS-1 cells (Fig. 1A). The deficient ATP generation and membrane depolarisation are secondary to the impaired activation of the mitochondrial electron transport chain. This is reflected in the absence of hyperpolarisation of $\Delta\Psi_m$ normally seen in control cells (Fig. 1C and D). Similar results were obtained with the membrane-permeant mitochondrial substrate methyl-succinate (Fig. 1E–H), which mimics the effect of glucose on insulin secretion (Maechler *et al.* 1997). This suggests that mitochondrial metabolism rather than glycolysis is defective in ρ° cells. As expected from these results, glucose does not increase $[\text{Ca}^{2+}]_c$ and insulin secretion in different ρ° beta cell line preparations (Soejima *et al.* 1996; Kennedy *et al.* 1998; Tsuruzoe *et al.* 1998; Hayakawa *et al.* 1998). These ρ° cells still synthesise, store and secrete insulin as demonstrated by insulin secretion in response to the $[\text{Ca}^{2+}]_c$ raising agents KCl and glibenclamide, which do not require mitochondrial metabolism (Kennedy *et al.* 1998; Tsuruzoe *et al.* 1998; Hayakawa *et al.* 1998). Replenishment of MIN-6 ρ° cells with normal mitochondria from mouse fibroblasts completely restored glucose-stimulated insulin secretion as shown in an elegant study by Soejima *et al.* (1996). Taken together, these results emphasise the crucial role of the mitochondria in the generation of metabolic coupling factors in glucose-induced insulin release.

Mitochondrially driven insulin exocytosis

Numerous studies have sought to identify the factor(s) mediating the K_{ATP} -independent effect of glucose on insulin secretion. Neither protein kinase A nor protein kinase C appeared to be implicated. Malonyl-CoA has been proposed as a metabolic coupling factor in insulin secretion (Prentki *et al.* 1992), being a messenger derived from citrate generated in the mitochondria (Farfari *et al.* 2000). However, disruption of malonyl-CoA accumulation during glucose stimulation did not affect the secretory response (Antinozzi *et al.* 1998), leaving the debate open as to the role of long chain acyl-CoA as a coupling factor (see Corkey *et al.* 2000). In order to study the link between mitochondrial activation and insulin exocytosis, we have established a *Staphylococcus* α -toxin-permeabilised beta cell model permitting the clamping of $[\text{Ca}^{2+}]_c$ and nucleotides such as ATP. This preparation can be directly stimulated with various mitochondrial substrates including succinate, a TCA cycle intermediate. Three mitochondrial dehydrogenases are known to be activated by

Ca^{2+} in various tissues (Denton & McCormack, 1980; Hansford, 1985), including insulin-secreting cells (Sener *et al.* 1990; Civelek *et al.* 1996; Maechler *et al.* 1998). Therefore, the increase in $[\text{Ca}^{2+}]_m$ conveniently reflects mitochondrial activation (Rizzuto *et al.* 1992; Duchen, 1999). To monitor mitochondrial activation, we measured mitochondrial free $[\text{Ca}^{2+}]_m$ using INS-1 cells stably expressing the Ca^{2+} -sensitive photoprotein aequorin (Kennedy *et al.* 1996) with simultaneous assessment of insulin secretion. When the $[\text{Ca}^{2+}]_c$ was clamped at 500 nM, succinate caused a marked biphasic increase in $[\text{Ca}^{2+}]_m$ (Fig. 2A), an effect secondary to hyperpolarisation of $\Delta\Psi_m$ (Maechler *et al.* 1997). This mitochondrial activation resulted in biphasic insulin release

(Fig. 2B). As Ca^{2+} enhances succinate oxidation under these conditions (Maechler *et al.* 1998), it can be concluded that mitochondrial activation directly stimulates insulin exocytosis. The obvious question is whether the increase in $[\text{Ca}^{2+}]_m$ 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 $[\text{Ca}^{2+}]_m$ rise and abolished insulin release induced by succinate (Fig. 2C and D). A $[\text{Ca}^{2+}]_m$ rise is thus necessary for the mitochondrially driven insulin exocytosis, but Ca^{2+} is not sufficient. Indeed, the sole $[\text{Ca}^{2+}]_m$ elevation without provision of carbons to the TCA cycle (anaplerosis) failed to elicit insulin secretion under

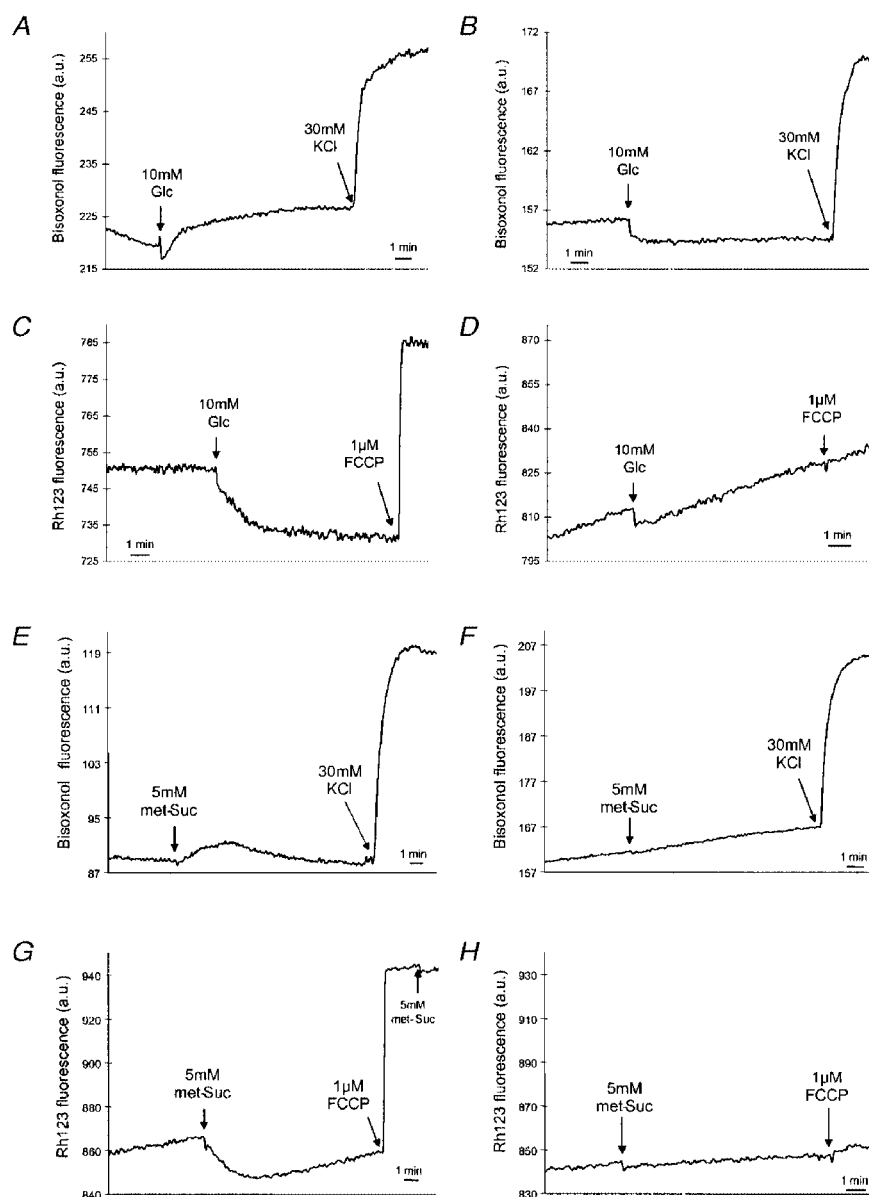


Figure 1. Plasma membrane potential and mitochondrial membrane potential ($\Delta\Psi_m$) in INS-1 and INS-1 ρ^0 cells

Suspensions of cells were loaded either with the fluorescent probe bisoxonol to monitor plasma membrane potential in INS-1 (A and E) or INS-1 ρ^0 cells (B and F) or with Rh123 to monitor $\Delta\Psi_m$ in INS-1 (C and G) or INS-1 ρ^0 cells (D and H). met-Suc, methyl-succinate. Reproduced from Kennedy *et al.* (1998), with permission.

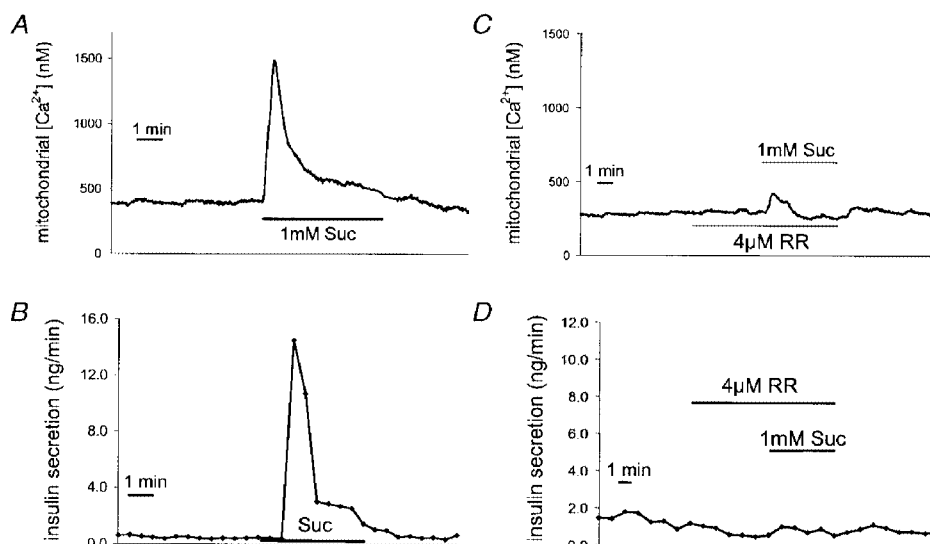


Figure 2. Effects of the TCA cycle intermediate succinate on $[Ca^{2+}]_m$ and insulin secretion in permeabilised INS-1 cells

Cells expressing the Ca^{2+} -sensitive photoprotein aequorin targeted to the mitochondria were permeabilised with α -toxin and perfused with an intracellular buffer containing 500 nM free Ca^{2+} and high ATP (10 mM). The effects of succinate (Suc) on $[Ca^{2+}]_m$ (A) and insulin secretion (B) were measured simultaneously. Influx of Ca^{2+} into the mitochondria via the uniporter was blocked using Ruthenium Red (RR) before the addition of Suc during the measurement of $[Ca^{2+}]_m$ (C) and insulin secretion (D). Modified with permission from Maechler *et al.* (1997).

these conditions (Maechler *et al.* 1997). This strongly suggested the existence of a mitochondrial factor generated through anaplerotic input into the TCA cycle.

Is glutamate the mitochondrially derived coupling factor?

Using the α -toxin-permeabilised cell model, metabolic intermediates of mitochondrial origin were screened for their putative direct action on insulin exocytosis. These and

other experiments suggested glutamate as the coupling factor. Glucose increased the cellular glutamate content in INS-1 cells and human islets (Maechler & Wollheim, 1999). In rat islets Tamarit-Rodriguez *et al.* (2000) reported that glutamate was the only amino acid out of twelve that increased during glucose stimulation, whereas aspartate decreased. Using a similar islet preparation MacDonald & Fahien (2000) did not observe any changes in glutamate levels. In incubated mouse islets, glucose did not augment

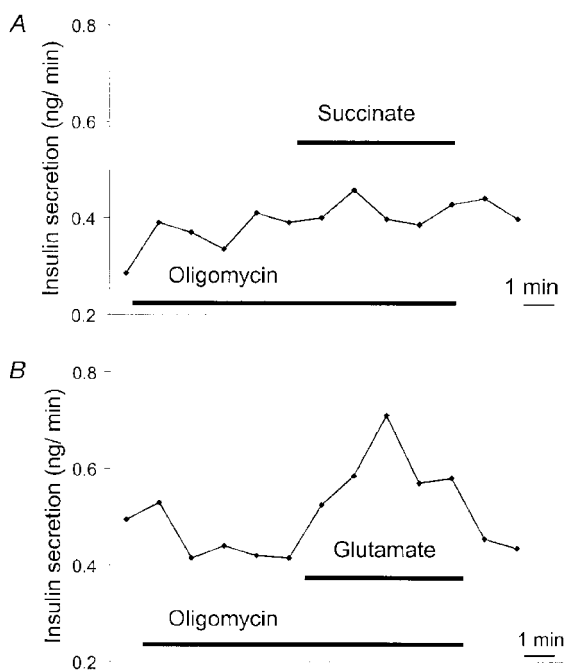


Figure 3. Effects of oligomycin on insulin secretion in permeabilised INS-1 cells

Cells were permeabilised with α -toxin and perfused with an intracellular buffer at 500 nM $[Ca^{2+}]_i$ and 1 mM ATP. Oligomycin ($1 \mu\text{g ml}^{-1}$) suppresses the effect of succinate (1 mM, A) but not of glutamate (1 mM, B) on insulin secretion. Conditions as in Maechler & Wollheim (1999); the traces are representative of three independent experiments.

glutamate (Danielsson *et al.* 1970). Thus, conflicting data emanate from different laboratories regarding the effect of glucose on glutamate levels. This discrepancy might be explained by culture conditions that can substantially increase the background of glutamate, especially in the case of whole islets constituted of a mixture of beta and non-beta cells (Michalik *et al.* 1992). Basal total glutamate levels range from sub-millimolar in INS-1 cells to millimolar in the islet preparations (Danielsson *et al.* 1970; Michalik *et al.* 1992; MacDonald & Fahien, 2000). These data obtained in whole cells do not yield information on changes in the cytosol, the critical compartment for a putative messenger molecule, since mitochondria and possibly secretory granules contain glutamate. In isolated INS-1 cell mitochondria, succinate generates glutamate under conditions in which insulin secretion is stimulated in permeabilised cells (Maechler *et al.* 2000). Using a mannitol–sucrose buffer, unfortunately without a set Ca^{2+} concentration, MacDonald & Fahien (2000) did not observe this effect of succinate in rat islet mitochondria.

Irrespective of the glutamate levels measured in glucose-stimulated cells, the principal observation is that glutamate directly stimulates insulin exocytosis in permeabilised cells at permissive $[Ca^{2+}]_c$ independently of mitochondrial activation (Maechler & Wollheim, 1999). This was corroborated by addition to permeabilised cells of oligomycin, an inhibitor of

the mitochondrial ATP synthase. Oligomycin abolished insulin release in response to succinate without affecting glutamate-induced exocytosis (Fig. 3A and B). These results suggest that glutamate, in contrast to succinate, acts downstream of mitochondrial metabolism. Finally, addition of the cell-permeant derivative diethyl-glutamate to intact beta cell preparations only stimulates insulin secretion at intermediate but not at basal or optimal glucose concentrations (Sener *et al.* 1994; Maechler & Wollheim, 1999). Similarly, glutamine, which forms glutamate in the beta cell (Michalik *et al.* 1992), enhances insulin release only in the presence of other nutrient stimuli (Malaisse *et al.* 1980). The converging evidence favours the contention that intracellular glutamate requires other glucose-triggered signals, such as an increase in $[Ca^{2+}]_c$.

Role of glutamate in beta cell function

We thus postulate an intracellular messenger role for glutamate in stimulus–secretion coupling. Previously, extracellular glutamate was reported to cause a transient stimulation of insulin secretion in the perfused rat pancreas (Bertrand *et al.* 1992) and to elicit a small secretory response in isolated rat islets (Inagaki *et al.* 1995). It is noteworthy that only approximately 25% of rat beta cells express glutamate receptors (Weaver *et al.* 1996) and that glutamate does not elicit insulin release in intact rat islets (Fahien *et al.* 1988) or INS-1 cells (authors' unpublished observations). We

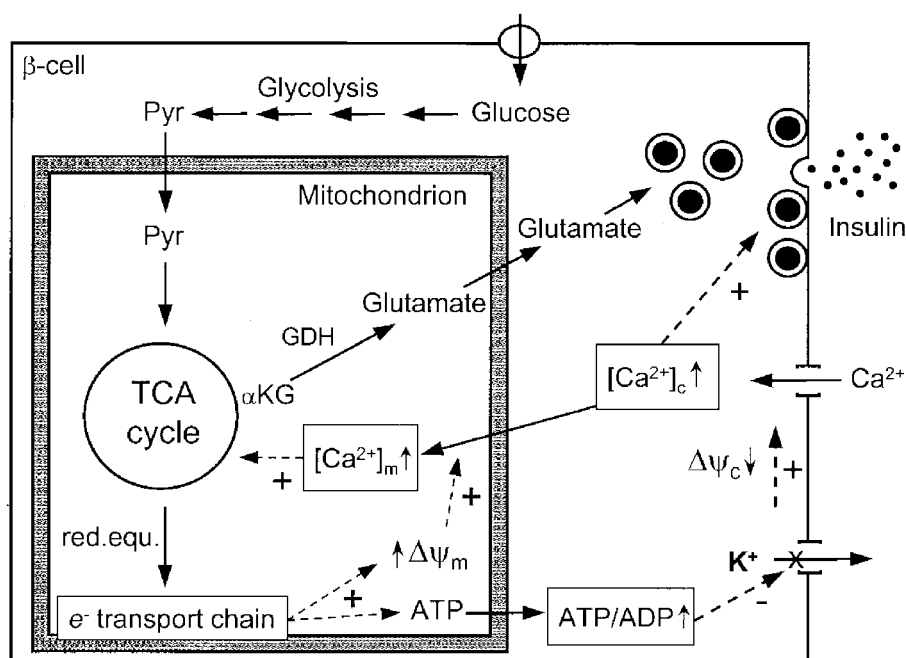


Figure 4. Proposed model for coupling of glucose metabolism to insulin secretion in the beta cell

Glycolysis converts glucose to pyruvate (Pyr), which enters the mitochondrion and feeds the TCA cycle, resulting in the transfer of reducing equivalents (red.equ.) to the electron (e^-) transport chain, hyperpolarisation of $\Delta\Psi_m$ and generation of ATP. Subsequently, closure of K^+_{ATP} channels depolarises the plasma membrane which opens voltage-sensitive Ca^{2+} channels, raising $[Ca^{2+}]_c$ and triggering insulin exocytosis. Concomitantly, under conditions of elevated $[Ca^{2+}]_c$, hyperpolarised $\Delta\Psi_m$ promotes $[Ca^{2+}]_m$ rise, further activating the TCA cycle. Glutamate is then formed from α -ketoglutarate (α KG) by glutamate dehydrogenase (GDH). Glutamate uptake by granules would participate in the second phase of insulin secretion. Reproduced from Maechler & Wollheim (1999), with permission.

therefore suggest that glutamate acts as an intracellular rather than an extracellular messenger in insulin exocytosis.

Glutamate can be generated through several biochemical pathways including transamination reactions (see Nissim, 1999). Predominantly, in the mitochondria, glutamate dehydrogenase forms glutamate from the TCA cycle intermediate α -ketoglutarate (Fig. 4). In most tissues this is the prevailing direction for the enzyme reaction with the notable exception of astrocytes (reviewed by Hudson & Daniel, 1993; Nissim, 1999). Several studies have addressed the question of the importance of glutamate dehydrogenase in the pancreatic beta cell. It is noteworthy that glutamine oxidation, stimulated by the presence of an allosteric activator of glutamate dehydrogenase, is inhibited by glucose (Gao *et al.* 1999). The most likely donors of ammonia for glutamate synthesis by glutamate dehydrogenase are glutamine and aspartate (Nissim, 1999). 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 (Hyder *et al.* 1996; Shen *et al.* 1999). The directional flux was also increased in mouse islets since incorporation of glucose carbons into glutamate was augmented by glucose stimulation without a change in cellular glutamate content (Gylfe & Hellman, 1974).

The glutamate formed in the mitochondria is transferred to the cytosol where it is probably taken up by the insulin-containing granules (Fig. 4). In agreement with glutamate transport properties in synaptic vesicles (see Ozkan & Ueda, 1998), collapse of the granule membrane potential and application of an inhibitor of glutamate uptake blocked glutamate-induced insulin exocytosis in permeabilised INS-1 cells (Maechler & Wollheim, 1999). However, glutamate uptake by insulin-containing secretory granules remains to be demonstrated. Glutamate may be transported by the recently identified vesicular glutamate transporter VGLUT1 or BNPI (Bellocchio *et al.* 2000; Takamori *et al.* 2000). It can be speculated that glutamate has a general effect of sensitising 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 & Gomperts (1990) used Cl^- as the main anion in permeabilised mast cells and in fact observed that glutamate was required for Ca^{2+} -induced exocytosis. Our experiments, like those in the mast cells, employed a buffer containing Cl^- as the main anion (Maechler & Wollheim, 1999). At present, the mechanism underlying the permissive action of glutamate in the secretory process is unknown.

Conclusion

Glutamate may not only play a role in the physiological regulation of insulin secretion but may also be implicated in beta cell pathology. It is of interest in this context that a syndrome of hyperinsulinism has been linked to mutations in the glutamate dehydrogenase gene resulting in an increase in enzyme activity (Stanley *et al.* 1998; Yorifuji *et al.* 1999).

The impact of these mutations on insulin secretion has its counterpart in the allosteric stimulation of the enzyme by the amino acid analogue 2-amino-bicyclo[2,2,1]heptane-2-carboxylic acid (Sener & Malaisse, 1980). The putative role of glutamate as a messenger has recently been substantiated in a transgenic mouse model. Those mice overexpressing the glutamate decarboxylating enzyme GAD65 display glucose intolerance without any sign of insulinitis or loss of beta cells. Their islets showed impaired glucose-stimulated insulin secretion, while the response to KCl was preserved (Shi *et al.* 2000).

In conclusion, although the crucial role of the mitochondria in beta cell function is well recognised, the last decade has revealed that metabolism–secretion coupling is more complex than originally expected. It may be speculated that not only ATP and glutamate but also other mitochondrially derived factors participate in the overall control of exocytosis. The elucidation of the mode of glutamate action in the beta cell should help to define its putative implication in other secretory processes.

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