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Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic β-cells

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In the pancreatic β-cell, insulin secretion is stimulated by glucose metabolism resulting in membrane potential-dependent elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_c$). This cascade involves the mitochondrial membrane potential ($\Delta\psi_m$) hyperpolarization and elevation of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) which activates the Ca^{2+}-sensitive NADH-generating dehydrogenases. Metabolism-secretion coupling requires unidentified signals, other than $[Ca^{2+}]_c$, possibly generated by the mitochondria through the rise in $[Ca^{2+}]_m$. To test this paradigm, we have established an α-toxin permeabilized cell preparation permitting the simultaneous monitoring of $[Ca^{2+}]$ with mitochondrially targeted aequorin and insulin secretion under conditions of saturating [ATP] (10 mM) and of clamped $[Ca^{2+}]_c$ at substimulatory levels (500 nM). The tricarboxylic acid (TCA) cycle intermediate succinate hyperpolarized $\Delta\psi_m$, raised $[Ca^{2+}]_m$ up to 1.5 μM and stimulated insulin secretion 20-fold, without changing $[Ca^{2+}]_c$. Blockade of the uniporter-mediated Ca^{2+} influx into the mitochondria abolished the secretory response. Moreover, glycerophosphate, which raises $[Ca^{2+}]_m$ by hyperpolarizing $\Delta\psi_m$ without supplying carbons to the TCA cycle, failed to stimulate exocytosis. Activation of the TCA cycle with citrate evoked secretion only when combined with glycerophosphate. Thus, mitochondrially driven insulin secretion at permissive $[Ca^{2+}]_c$ requires both a substrate for the TCA cycle and a rise in $[Ca^{2+}]_m$. Therefore, mitochondrial metabolism generates factors distinct from Ca^{2+} and ATP capable of inducing insulin exocytosis

Keywords: aequorin/Ca^{2+}/exocytosis/insulin/mitochondria

Introduction

In the pancreatic β-cell mitochondrial metabolism plays a pivotal role in the generation of signals coupling glucose recognition to insulin secretion (Matschinsky, 1996; Wollheim *et al.*, 1996). The main physiological secretagogue, glucose, is metabolized by the β-cell which causes the closure of ATP-sensitive K^+ (K_{ATP}) channels and depolarization of the plasma membrane potential (Ashcroft and Rorsman, 1989). This leads to Ca^{2+} influx through voltage-gated Ca^{2+} channels and a rise in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) (Hellman *et al.*, 1992; Theler *et al.*, 1992; Wollheim *et al.*, 1996). The increase in $[Ca^{2+}]_c$ is the main trigger of exocytosis, the process by which the insulin-containing secretory granules fuse with the plasma membrane (Bokvist *et al.*, 1995; Wollheim *et al.*, 1996). Ca^{2+}, the universal second messenger, controls several other cellular functions, among them mitochondrial metabolism (Denton and McCormack, 1980; Hansford, 1985). Elevations in $[Ca^{2+}]_c$ are relayed to the mitochondria, the matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) of which is increased with consequent stimulation of Ca^{2+}-sensitive NADH-generating dehydrogenases (McCormack *et al.*, 1990a; Hansford, 1991; Pralong *et al.*, 1994; Hajnóczky *et al.*, 1995; Civelek *et al.*, 1996; Rutter *et al.*, 1996). NADH transfers reducing equivalents to the respiratory chain, thereby ensuring adequate ATP synthesis to balance the augmented energy needs of cell activation (McCormack *et al.*, 1990a). In the β-cells, the initial signal generation by the mitochondria in response to glucose, i.e. the increase in NAD(P)H (Pralong *et al.*, 1990) and in the ATP/ADP ratio (Nilsson *et al.*, 1996), precedes the elevation in $[Ca^{2+}]_c$. Therefore, $[Ca^{2+}]_m$ appears to be involved in the maintenance rather than the initiation of the metabolism–secretion coupling.

Although an increase in $[Ca^{2+}]_c$ is a necessary event in insulin secretion, glucose is also capable of stimulating secretion in a manner not involving K_{ATP} channels and Ca^{2+} influx. This is observed at permissive and constant $[Ca^{2+}]_c$ (Gembal *et al.*, 1993). We hypothesize that this effect could be explained by an activation of the mitochondrial metabolism triggered by an increase in $[Ca^{2+}]_m$. Recently, we have reported that glucose induces $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ rises which are both associated with the stimulation of insulin secretion (Kennedy *et al.*, 1996). A pivotal role of the mitochondria has also been highlighted by the observations that insulin secretion-deficient diabetes mellitus can be associated with mutations in the mitochondrial genome which encodes several subunits of the respiratory chain complexes (Maassen and Kadowaki, 1996). Such mutations have also been linked with other diseases including neurological disorders (Johns, 1996). Moreover, the knock-out of the mitochondrial DNA in a β-cell line was shown to result in the abolition of glucose-induced insulin secretion (Soejima *et al.*, 1996).

To characterize the role of mitochondrial activation in metabolism–secretion coupling, we have monitored two parameters that reflect mitochondrial activation. The first, the mitochondrial membrane potential ($\Delta\psi_m$) drives the second, the $[Ca^{2+}]_m$ whose increase results from hyperpolarization of $\Delta\psi_m$ (Gunter *et al.*, 1994) and leads to the activation of several mitochondrial dehydrogenases (McCormack *et al.*, 1990a; Hansford, 1991). In the present study, we used the glucose-responsive insulin-secreting cell line INS-1 stably expressing the Ca^{2+}-sensitive photo-

protein aequorin, either in the cytosol or in the mitochondria (Kennedy *et al.*, 1996). We also used rat pancreatic islet cells transiently transfected with mitochondrially targeted aequorin.

In order to study the link between $[Ca^{2+}]_c$, mitochondrial activation and the exocytosis of insulin, we have permeabilized cells with *Staphylococcus* α-toxin, generating small holes of only 2–3 nm in the plasma membrane and, therefore, largely preserving cellular integrity (Jonas *et al.*, 1994). This approach renders possible the clamping of $[Ca^{2+}]_c$ at permissive concentrations, the direct stimulation of mitochondrial metabolism by various non-lipophilic substrates, the monitoring of $[Ca^{2+}]_m$ and the measurement of insulin secretion. The present study provides evidence that the activation of mitochondrial metabolism generates factors other than Ca^{2+} and ATP which are capable of inducing insulin exocytosis.

Results

First, the effect of the tricarboxylic acid (TCA) cycle intermediate succinate rendered cell-permeant by the ester binding of a methyl group was tested in intact INS-1 cells. This substrate, which bypasses glycolysis, has been shown to mimic the effect of glucose on insulin secretion (MacDonald and Fahien, 1988). Methyl-succinate induced both a rise in $[Ca^{2+}]_c$ up to 350 nM, and in $[Ca^{2+}]_m$ with a large transient peak reaching 1.2 μM (Figure 1A). These changes were accompanied by a 10-fold stimulation of insulin release (Figure 1B).

The mitochondrial uptake of Ca^{2+}, mediated by a low-affinity uniporter, is driven by the $\Delta\psi_m$, while Ca^{2+} efflux is controlled by both Na^+-dependent and -independent antiporters (Gunter *et al.*, 1994). In INS-1 cells, 5 mM methyl-succinate induced a depolarization of the plasma membrane (Figure 2A) and the hyperpolarization of the mitochondrial membrane (Figure 2B). The latter effect is also observed in response to the physiological stimulus, glucose (12.8 mM final) (Figure 2C). The permeabilization of the plasma membrane renders possible the study of cell-impermeant metabolic substrates such as non-esterified succinate. *Staphylococcus* α-toxin-permeabilized INS-1 cells exhibited a rapid hyperpolarization of the $\Delta\psi_m$ after the addition of 5 mM succinate (Figure 2D). In contrast, and as a control of the mitochondrial integrity, the protonophore FCCP which dissipates the $\Delta\psi_m$ (Gunter *et al.*, 1994), evoked large depolarizations (Figure 2B–E). In the β-cell the respiratory chain is also activated by reducing equivalents produced in the cytosol by glycolysis via the glycerophosphate shuttle (MacDonald and Brown, 1996) providing $FADH_2$ to site II, as is the case for succinate. Accordingly, glycerophosphate caused a hyperpolarization of the $\Delta\psi_m$ in permeabilized INS-1 cells (Figure 2E). (It should be noted that we added glycerophosphate twice concentrated compared with succinate since the racemic mixture was used.) The experiments in Figure 2D and E demonstrate that *Staphylococcus* α-toxin effectively permeabilizes INS-1 cells, while leaving mitochondria functional.

The question then arises as to whether activation of mitochondrial metabolism is necessary only to provide the changes in ATP levels that in turn regulate plasma membrane K_{ATP} channels, or whether other factors are

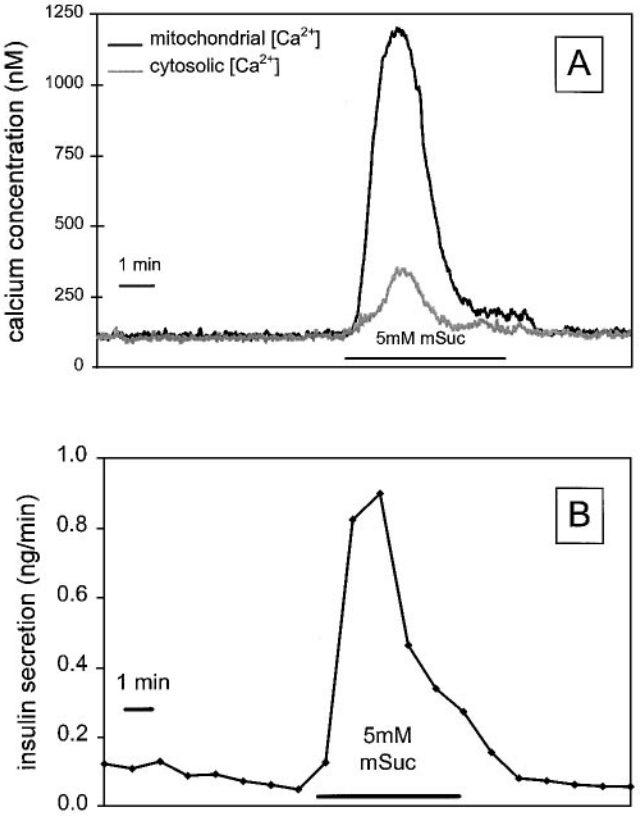

Fig. 1. Rises in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (**A**) are associated with stimulation of insulin secretion (**B**) by methyl-succinate (mSuc). Insulin-secreting INS-1 cells expressing the Ca^{2+}-sensitive photoprotein aequorin in the cytosol or targeted to the mitochondria were perifused with KRBH permitting the simultaneous photon detection for $[Ca^{2+}]$ measurements and effluent collection for insulin assay (see Materials and methods). Insulin secretion was measured with $[Ca^{2+}]_m$ in INS-1/EK-3 cells. Each trace is representative of at least three different experiments.

produced by this organelle that could control insulin secretion. Permeabilized INS-1/EK-3 cells expressing mitochondrial aequorin were perifused with an intracellular-type buffer containing saturating [ATP] (10 mM) and free $[Ca^{2+}]$ clamped at a permissive level (500 nM). The addition of succinate induced a large transient peak in $[Ca^{2+}]_m$, followed by a sustained plateau (Figure 3A). The simultaneous measurement of insulin release demonstrated that succinate stimulated the initial rate of secretion 20-fold preceding a 4-fold sustained rate above baseline (Figure 3B).

Staphylococcus α-toxin offers several advantages over other permeabilization procedures, since while making the plasma membrane leaky to low molecular weight compounds it leaves not only organelles but also cytoplasmic proteins *in situ* (Jonas *et al.*, 1994). In particular, cytosolic aequorin remains trapped in the permeabilized cells, thereby allowing continuous monitoring of $[Ca^{2+}]_c$. In this model of permeabilized cells, succinate did not induce any changes in $[Ca^{2+}]_c$ as demonstrated in INS-1/C-29 cells expressing cytosolic aequorin (Figure 3C). As expected from the low affinity of the mitochondrial Ca^{2+} uptake system (uniporter), succinate did not augment $[Ca^{2+}]_m$ when $[Ca^{2+}]_c$ was clamped at basal 100 nM in permeabilized cells (Maechler *et al.*, 1996). It should be

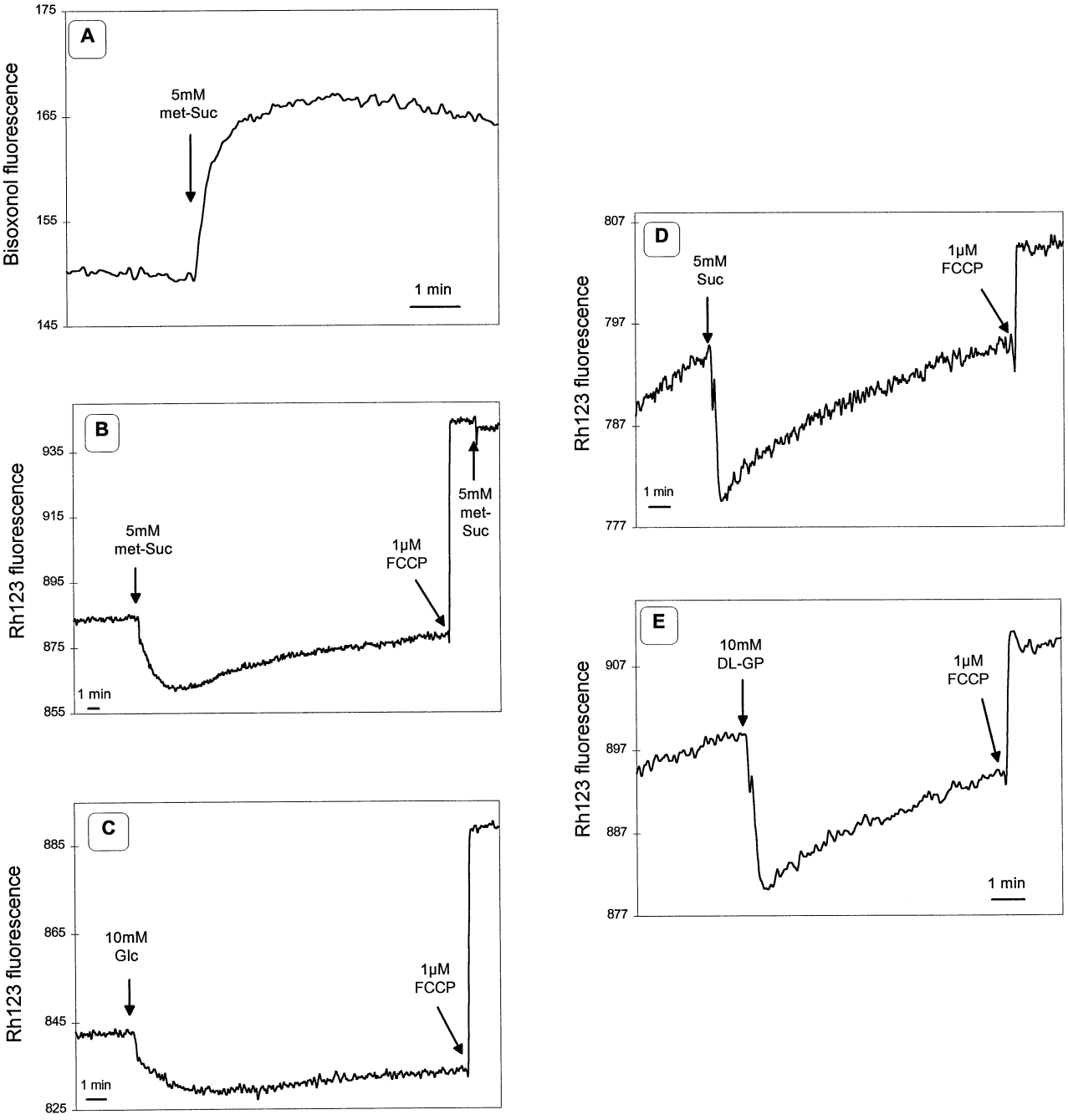

Fig. 2. Plasma membrane and mitochondrial membrane potentials ($\Delta\psi_p$ and $\Delta\psi_m$ respectively) were measured in a suspension of insulin secreting INS-1 cells. $\Delta\psi_p$ was monitored with the fluorescent probe bis-oxonol and the addition of methyl-succinate (met-Suc) induced a depolarization of the $\Delta\psi_p$ observed as a rise in the signal (**A**). Rh123 was used to measure $\Delta\psi_m$ and met-Suc caused a hyperpolarization followed by a slow recovery (**B**). A similar effect was observed in the presence of glucose (Glc) (**C**). When cells were permeabilized with α-toxin and maintained in a buffer containing 500 nM free Ca^{2+}, succinate (Suc) induced a rapid hyperpolarization of $\Delta\psi_m$ (**D**). A very similar effect was observed on $\Delta\psi_m$ with DL-α-glycerophosphate (DL-GP) (**E**). The depolarizing effect of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is shown at the end of each $\Delta\psi_m$ trace as a control of the $\Delta\psi_m$ integrity (B–E). The traces are representative of five (A), five (B), three (C), seven (D) and three (E) experiments.

noted that succinate is ineffective in intact cells (data not shown), and therefore only the response of permeabilized cells is monitored under these conditions.

We next tested the effect of glycerophosphate in order to supply reducing equivalents but not carbons to the mitochondria. In these permeabilized cells, glycerophosphate caused a similar increase in $[Ca^{2+}]_m$ (Figure 3D) and hyperpolarization of $\Delta\psi_m$ (Figure 2E) as succinate, but failed to stimulate insulin secretion (Figure 3E). The measurements of $\Delta\psi_m$ revealed that succinate hyperpolarizes the mitochondrial membrane (Figure 2D), thereby permitting membrane potential-driven influx of Ca^{2+} through the uniporter. Blockade of the uniporter with ruthenium red (Gunter *et al.*, 1994) almost abolished the succinate-induced increase in $[Ca^{2+}]_m$ (Figure 3F). Importantly, this blocker completely inhibited the stimulation of insulin secretion by succinate (Figure 3G). By contrast, CGP37157, which blocks the efflux of Ca^{2+} via

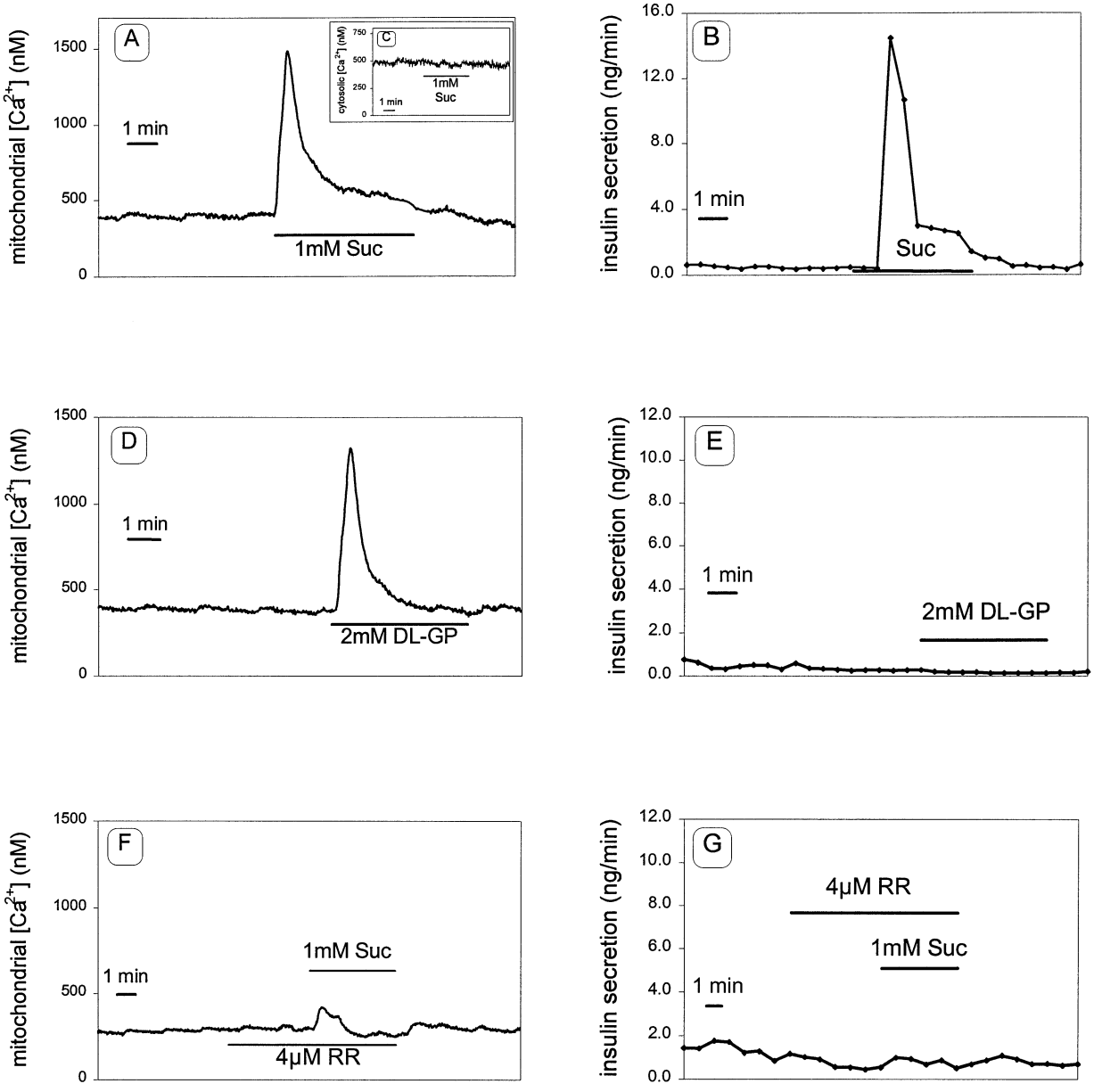

Fig. 3. Effects of the TCA cycle intermediate succinate (Suc) and of the mitochondrial electron donor DL-α-glycerophosphate (DL-GP) on $[Ca^{2+}]_m$ and insulin secretion in permeabilized INS-1 cells. Cells expressing the Ca^{2+}-sensitive photoprotein aequorin targeted to the mitochondria were permeabilized with α-toxin and perifused with an intracellular buffer containing 500 nM of free Ca^{2+} and 10 mM ATP (see Materials and methods). The effects of Suc on $[Ca^{2+}]_m$ (**A**) and insulin secretion (**B**) were measured simultaneously. The stability of $[Ca^{2+}]_c$ was assessed by using INS-1 cells expressing cytosolic aequorin (**C**). The effects of DL-GP are presented in panels (**D**) and (**E**). 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 $[Ca^{2+}]_m$ (F) and insulin secretion (**G**). Each trace is representative of three different experiments except for (A/B), which was performed five times.

the Na^+/Ca^{2+}-antiporter (Cox and Matlib, 1993), did not change the transient peak of $[Ca^{2+}]_m$ evoked by succinate but slightly delayed the return to basal levels (data not shown).

In order to study another mitochondrial substrate under the same conditions, we tested the end product of glycolysis, i.e. pyruvate. The methyl ester form was used since only this derivative activates insulin secretion in islet β-cells (Mertz *et al.*, 1996). This has been suggested to be due to its preferential metabolism in the mitochondrial, rather than cytosolic, compartment (Jijakli *et al.*, 1996). In permeabilized INS-1/EK-3 cells, 5 mM methyl-pyruvate caused a rise in $[Ca^{2+}]_m$ with a transient peak at 1.45 ± 0.53 μM (n = 3) in conditions similar to those of Figure 3A. In static incubations, 5 mM methyl-pyruvate stimulated insulin secretion in permeabilized cells 3.7-fold. This effect was very similar to the stimulation due to 5 mM succinate in the same experiments (Figure 4A).

As succinate and methyl-pyruvate are efficient in both raising $[Ca^{2+}]_m$ and providing carbons to the TCA cycle, we next tried to discriminate between these two effects in the triggering of exocytosis. First, the TCA cycle intermediate citrate was tested alone. Citrate (1 mM) did not raise $[Ca^{2+}]_m$ in permeabilized cells (n = 3) under

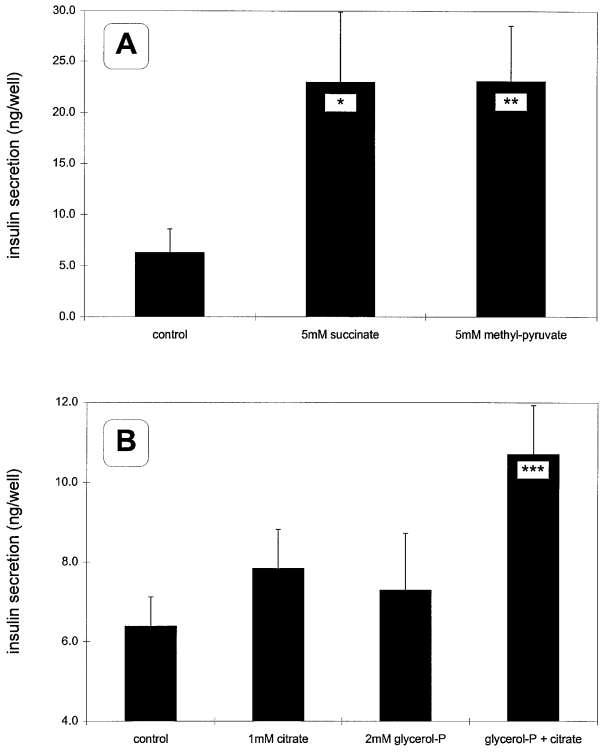

Fig. 4. Effects of mitochondrial substrates (succinate, methyl-pyruvate, citrate) and of the electron donor DL-α-glycerophosphate on insulin secretion in permeabilized INS-1 cells. Cells were permeabilized with α-toxin, preincubated for 30 min in an intracellular buffer containing 500 nM of free Ca^{2+} and 10 mM ATP and then exposed for 10 min to the various agents (see Materials and methods). The results are expressed as mean ± s.e.m. of two independent experiments, each in triplicate. *P <0.05, **P <0.02, ***P <0.01.

the conditions used in Figure 3, nor did it enhance insulin secretion (Figure 4B). Second, when combined with the $[Ca^{2+}]_m$-raising substance glycerophosphate (2 mM), citrate (1 mM) became stimulatory for insulin secretion (Figure 4B).

To ensure that the effect of succinate is not peculiar to insulinoma cells, similar experiments were conducted in normal pancreatic rat islet cells. In a suspension of islet cells, 5 mM of methyl-succinate hyperpolarized the $\Delta\psi_m$ and the protonophore FCCP caused depolarization (Figure 5A). In primary cells transiently transfected with the cDNA encoding mitochondrial aequorin, methyl-succinate also caused a rise in $[Ca^{2+}]_m$ (Figure 5B). Because the cells efficiently transfected were in the minority (~10–15%), a correct calibration of photons was not possible and therefore the $[Ca^{2+}]_m$ was not calibrated in terms of Ca^{2+} concentrations. These changes in $\Delta\psi_m$ and $[Ca^{2+}]_m$ induced by methyl-succinate in primary cells correlated with the stimulation of insulin secretion, observed as a first transient peak 4-fold above baseline followed by a sustained plateau 2-fold above baseline (Figure 5C).

In α-toxin-permeabilized primary pancreatic islet cells, succinate promoted hyperpolarization of $\Delta\psi_m$ and a 5-fold stimulation of insulin secretion under conditions of clamped $[Ca^{2+}]_c$ (Figure 6). Thus, the effects of succinate on insulin secretion are qualitatively similar in INS-1 cells and primary β-cells.

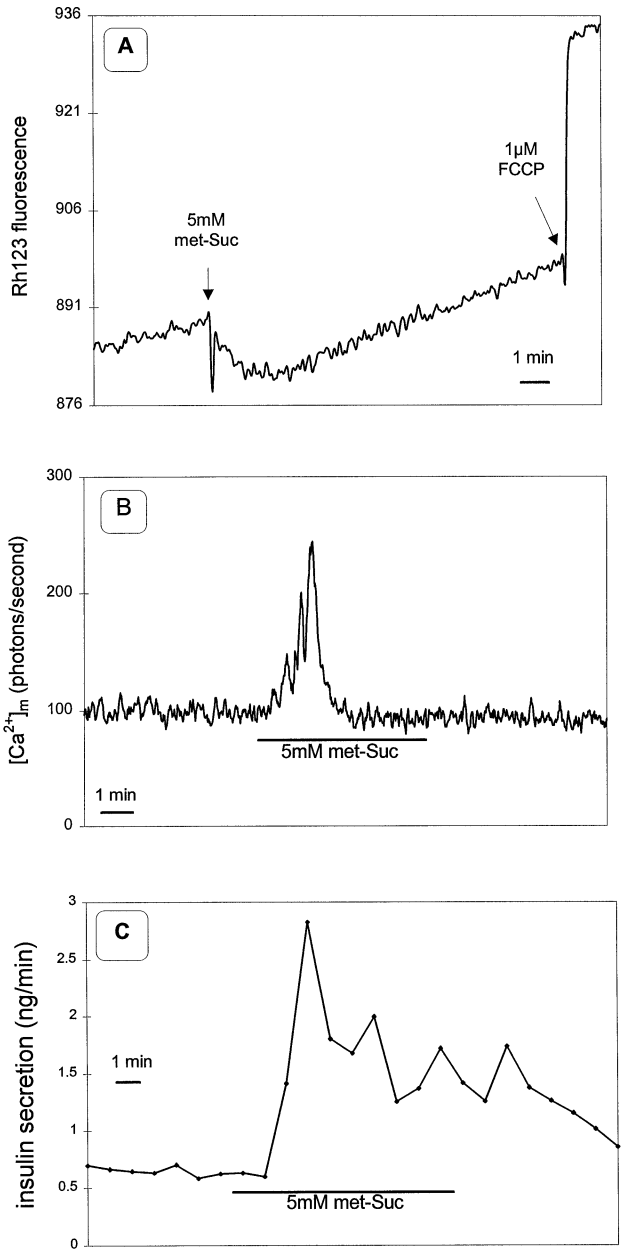

Fig. 5. Methyl-succinate (met-Suc) induced a hyperpolarization of the mitochondrial membrane potential ($\Delta\psi_m$) (**A**), a rise in $[Ca^{2+}]_m$ (**B**) and the stimulation of insulin secretion (**C**) in rat islet cells. Islets were isolated from rats and cultured for 1–4 days before the experiment. For mitochondrial $[Ca^{2+}]$ measurement (B), cells were used 3 days after transfection with the plasmid encoding for mitochondrially targeted aequorin. The met-Suc hyperpolarized and FCCP depolarized $\Delta\psi_m$ (A). Traces are representative of three (A), ten (B) and four (C) experiments.

Discussion

There is abundant evidence for the crucial role of mitochondrial metabolism in glucose-induced insulin secretion (Gerbitz *et al.*, 1996; Matschinsky, 1996; Prentki, 1996). The integrity of the respiratory chain is a prerequisite for the action of glucose, as recently demonstrated by the elimination of the mitochondrial genome encoding subunits of the respiratory chain enzymes (Soejima *et al.*, 1996). The present results substantiate the sequence of events in which the generation of reducing equivalents by

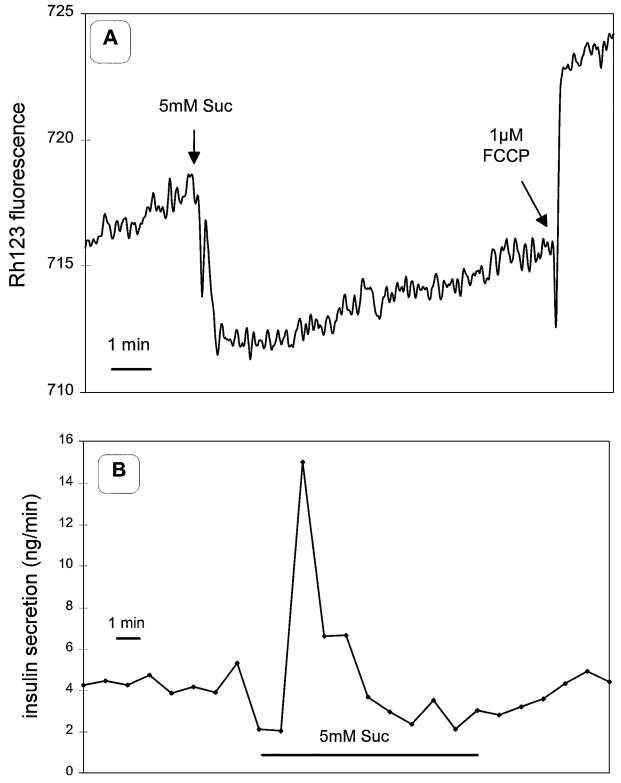

Fig. 6. In permeabilized islet cells, of succinate (Suc) induced a hyperpolarization of the mitochondrial membrane potential ($\Delta\psi_m$) (**A**) and the stimulation of insulin secretion (**B**). Islets were isolated from rats and cultured for 1–4 days before the experiments. Cells were permeabilized with α-toxin and incubated in the intracellular buffer containing 10 mM ATP and ~500 nM free Ca^{2+}. Suc hyperpolarized and FCCP depolarized $\Delta\psi_m$ (A). Traces are representative of three (A) and four (B) experiments.

substrates in the mitochondria and their subsequent transfer to the respiratory chain causes hyperpolarization of the $\Delta\psi_m$. This permits the rise in $[Ca^{2+}]_m$ to concentrations sufficient for the activation of NADH-generating dehydrogenases (McCormack *et al.*, 1990a; Rutter *et al.*, 1996) (Figure 7). This feed-forward 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 (Civelek *et al.*, 1996; Prentki, 1996). Indeed, pyruvate dehydrogenase has been shown to be activated by Ca^{2+} in permeabilized HIT-T15 cells, a β-cell line (Civelek *et al.*, 1996), and by glucose in intact rat islets (McCormack *et al.*, 1990b). Extracellular Ca^{2+} is recruited for this mitochondrial activation. Indeed, the blockade of L-type Ca^{2+} channels by a specific blocker results in the abolition of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ rises evoked by glucose (Kennedy *et al.*, 1996), or by the TCA cycle intermediate methyl-succinate (Maechler *et al.*, 1996). Similarly, blockade of Ca^{2+} influx attenuated the increase of NAD(P)H evoked by glucose in single β-cells (Pralong *et al.*, 1994). The effect was not abolished, however, perhaps due to the proposed mass action of glucose (Matschinsky, 1996) which does not require elevated $[Ca^{2+}]_m$. Simple membrane depolarization by potassium raises $[Ca^{2+}]_c$ and stimulates insulin secretion but does not reproduce the prominent and long-lasting secretory response to glucose (Gembal *et al.*, 1993; Wollheim *et al.*, 1996), implicating unidentified metabolic coupling factors distinct from Ca^{2+}.

In the present study we have demonstrated that when permeabilized insulin-secreting INS-1 cells were perifused with a fixed permissive level of $[Ca^{2+}]_c$ (500 nM), mitochondrial substrates, i.e. succinate or methyl-pyruvate, not only raise $[Ca^{2+}]_m$ but also stimulate the exocytosis of insulin. In contrast, when $[Ca^{2+}]_c$ is clamped to the level observed in intact resting cells (~100 nM, see Figure 1A), succinate enhances neither $[Ca^{2+}]_m$ nor insulin secretion (data not shown).

We provide evidence that the stimulation of exocytosis of insulin by succinate is not due to changes in $[Ca^{2+}]_c$ or to Ca^{2+} mobilization from the mitochondria, as demonstrated by: (i) the absence of any $[Ca^{2+}]_c$ rise in the presence of succinate in the permeabilized cells (Figure 3C); and (ii) the failure of glycerophosphate to induce insulin secretion despite the large increase of $[Ca^{2+}]_m$ (Figure 3D and E). Indeed, glycerophosphate dehydrogenase is located on the outer surface of the mitochondrial inner membrane, thus feeding electrons into the respiratory chain and producing ATP (Idahl and Lembert, 1995) without providing substrates for the TCA cycle (Figure 7). The increase in $[Ca^{2+}]_m$ evoked by glucose and methyl-succinate (in intact cells), as well as succinate and glycerophosphate (in permeabilized cells) requires hyperpolarization of the $\Delta\psi_m$ which provides the driving force for the Ca^{2+} entry. A similar action of glucose on $\Delta\psi_m$ has been reported previously (Duchen *et al.*, 1993). When the entry of Ca^{2+} was inhibited by the uniporter blocker ruthenium red, both $[Ca^{2+}]_m$ rise and insulin exocytosis were abrogated. Taken together, the present results strongly suggest that an increase in $[Ca^{2+}]_m$ is necessary—but not sufficient—for the activation of mitochondrial metabolism, in turn triggering insulin secretion.

Our permeabilized cell preparation allows the exclusion of coupling factors generated by glycolysis or downstream effectors of ATP such as K_{ATP} channels, as the sole modulators of metabolic stimulation of insulin secretion. Rather, the data suggest that the mitochondria are the source of the putative factor(s) (mt-factor in Figure 7). This factor seems to be derived from the TCA cycle, since insulin secretion was triggered by the anaplerotic substrates succinate and methyl-pyruvate but not by glycerophosphate which transfers reducing equivalents to the respiratory chain without supplying carbons to the TCA cycle (Prentki, 1996). This strongly suggests that an increase in $[Ca^{2+}]_m$ and TCA cycle activity govern the production of the putative factor. This hypothesis is supported by the observation that citrate stimulated insulin secretion only when combined with glycerophosphate (Figure 4B). The messenger molecule could be either one of the TCA cycle intermediates or a derived metabolite such as malonyl-CoA and long chain acyl-CoA. These acyl-CoAs are indeed increased during glucose-induced insulin secretion (Liang and Matschinsky, 1991; Prentki and Corkey, 1996). Nevertheless, the failure of citrate alone to evoke insulin secretion in permeabilized cells (Figure 4B) argues against the latter hypothesis, since citrate is a precursor of malonyl-CoA in the cytosol.

We propose that this putative factor cooperates with ATP and Ca^{2+} to ensure the normal and complete secretory response to glucose, i.e. to optimize the signal transduction from nutrient secretagogues to insulin exocytosis. The defective generation of mitochondrially derived messenger

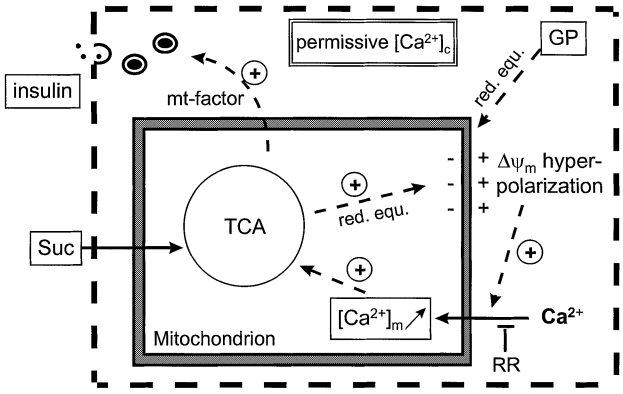

Fig. 7. Model for the control of insulin secretion induced by succinate in the α-toxin-permeabilized β-cell under conditions of permissive $[Ca^{2+}]_c$. TCA, tricarboxylic acid cycle; red. equ., reducing equivalents; GP, glycerophosphate; Suc, succinate; $\Delta\psi_m$, mitochondrial membrane potential; RR, ruthenium red; mt-factor, mitochondrially derived factor.

molecules may explain the impaired insulin secretion in response to glucose and methyl-succinate in islets of a rat model of non-insulin-dependent diabetes mellitus, contrasting with preserved exocytosis due to the non-substrate stimulus, potassium (Katayama *et al.*, 1995).

Whatever the nature of the putative mt-factor (Figure 7), it is proposed that it is released in a microenvironment close to the site of exocytosis. It cannot be determined at present whether the factor acts on the process of exocytosis itself or on one of the preceding steps, such as the interaction of secretory granules with the cytoskeleton (Li *et al.*, 1994). Moreover, the functional link between mitochondria and exocytosis is further indicated by the extreme sensitivity of $[Ca^{2+}]_m$ rises in response to small depolarizations of the membrane potential in INS-1 cells (Kennedy *et al.*, 1996). This concept is probably of general importance, as it is well known that nerve terminals are rich in mitochondria, thereby providing energy fuelling the release of neurotransmitter (vonGersdorff *et al.*, 1996).

Our unprecedented findings demonstrate that the activation of mitochondrial metabolism generates factors other than Ca^{2+} and ATP which are capable of inducing insulin secretion. This crucial role of the mitochondria in metabolism–secretion coupling in the β-cell may explain a phenotype of diabetic patients which has been linked to mutations in the mitochondrial genome (Maassen and Kadowaki, 1996). As such mutations are also associated with neurological diseases (Johns, 1996), mitochondrial function may be crucial not only for the exocytosis of insulin, but possibly also for the regulation of other secretory events such as neurotransmitter release.

Materials and methods

Cell culture

INS-1 cells were cultured in RPMI1640 medium as previously described (Asfari *et al.*, 1992; Kennedy *et al.*, 1996). Stable clones of INS-1 cells expressing either cytosolic aequorin (Brini *et al.*, 1995) or mitochondrial aequorin (Rizzuto *et al.*, 1992) were established as detailed elsewhere (INS-1/C-29 and INS-1/EK-3 respectively; Kennedy *et al.*, 1996) and cultured in the presence of 250 μg/ml G418 (Promega, Madison, USA) for continuous selection of cells expressing the plasmid with the associated neomycin resistance. Pancreatic islet cells were isolated by collagenase digestion from male Wistar rats weighing ~200 g and cultured free-floating in RPMI1640 medium for 5–7 days. On the experimental day the islets were trypsinized (0.025% trypsin, 0.27 mM EDTA) and the resulting single-cell suspension was further maintained under spinner culture conditions in RPMI1640 without glucose (Asfari *et al.*, 1992). Where indicated, suspensions of INS-1 cells were also kept in spinner culture.

Transient transfection of primary cells

Rat pancreatic islet cells were isolated as above, trypsinized and seeded on 13 mm diameter extracellular matrix coated coverslips (Eldan, Jerusalem, Israel) at 4×10^5 cells/ml in RPMI1640 medium. Two days later the cells were washed with phosphate-buffered saline. For each well, 10 μl of the polycationic lipid Lipofectamine (Gibco-BRL, Basel, Switzerland) were diluted in 100 μl of RPMI1640–HEPES (10 mM) medium and 1 μg of plasmid with mitochondrially targeted aequorin inserted into pcDNA1 expression vector (Kennedy *et al.*, 1996) was diluted in another 100 μl of the same medium. The liposome and plasmid solutions were then mixed and the volume was adjusted to 250 μl with RPMI1640–HEPES medium. After 30 min incubation at room temperature, this transfection mixture was added to the well before a 5 h incubation at 37°C in air/5% CO_2. The cells were then washed twice with RPMI1640–FCS (10%) medium and further cultured in the same medium for 72 h before the experiment. This transfection procedure resulted in 10–15% of transfected cells as judged by immunofluorescence of the haemagglutinin tag of the aequorin plasmid.

Permeabilization of cells

Attached INS-1 cells grown on extracellular matrix coated coverslips were permeabilized after a 2–5 day culture period. Cells were first washed with a Ca^{2+}-free HEPES balanced Krebs–Ringer bicarbonate buffer (KRBH) (see below, except no $CaCl_2$, 0.4 mM EGTA). They were then permeabilized with *Staphylococcus aureus* α-toxin (Palmer *et al.*, 1993; Jonas *et al.*, 1994) (1 μg/ coverslip, i.e. per $4–5\times10^5$ cells) at 37°C for 8 min in 100 μl of an intracellular buffer adjusted to ~100 nM free calcium (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM HEPES, pH 7.0, 1 mM ATP, 10.2 mM EGTA, 1.65 mM $CaCl_2$). On some control coverslips, permeabilization was assessed by eosin staining which revealed >95% positively stained cells (Jonas *et al.*, 1994). For $[Ca^{2+}]$ measurement and insulin secretion, perifusion was started with the same low Ca^{2+} intracellular buffer for 2–5 min, and then perifusion was switched to the 500 nM stimulatory intracellular buffer (see below). Cell suspensions of INS-1 or islet cells were permeabilized under similar conditions with 1 μg α-toxin per 1×10^6 cells.

Measurement of luminescence and insulin secretion

Aequorin-expressing cells were seeded on 13 mm diameter coverslips 3–5 days before analysis and maintained in the same medium as above except for G418. For intact cell experiments, cells were seeded on plastic coverslips at a density of 4×10^5 cells/ml, and for permeabilized cell experiments on extracellular matrix-coated coverslips (Eldan) at 2×10^5 cells/ml. Before luminescence measurements, cells were loaded with 2.5 μM of coelenterazine, the prosthetic group of aequorin, in glucose- and glutamine-free RPMI1640 plus 10 mM HEPES for 2–5 h at 37°C (Kennedy *et al.*, 1996). Luminescence was measured by placing the coverslip in a 0.5 ml thermostatically controlled chamber at 37°C at ~5 mm from the photon detector. We used a photomultiplier apparatus (EMI 9789, Thorn-EMI, England), and data were collected every second on a computer (photon counting board, EMI C660) before calibration as described previously (Kennedy *et al.*, 1996). The cells were perifused constantly at a rate of 1 ml/min and, where indicated, 1 min fractions from the effluent were collected for insulin measurement by radioimmunoassay using rat insulin as standard. Suspensions of islet cells were perifused with the same buffers as INS-1 cells using a perifusion apparatus (Kennedy *et al.*, 1996). Intact cells were perifused with KRBH (135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, pH 7.4, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 2.8 mM glucose). During recording, permeabilized cells were perifused with a stimulatory intracellular buffer giving a free Ca^{2+} concentration of ~500 nM (140 mM KCl, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM HEPES, pH 7.0, 10 mM ATP, 10.2 mM EGTA, 6.67 mM $CaCl_2$).

The free Ca^{2+} concentration was calculated by the Maxchelator programme (Bers *et al.*, 1994), and verified experimentally by fura-2 acid fluorescence (Grynkiewicz *et al.*, 1985) as well as with a calcium-selective electrode (Orion, Cambridge, UK). Moreover, permeabilized INS-1/C-29 cells expressing cytosolic aequorin were used in order to estimate the free Ca^{2+} concentration in perifused cells as an *in situ* measurement (Figure 3C). The latter gave an average value of 500 nM, whereas the values were ~400 nM, ~800 nM and ~750 nM for the

calcium electrode, fura-2 fluorescence and the computer programme respectively. Since these values fall in a narrow range, we retained 500 nM as the concentration of the clamped $[Ca^{2+}]_c$.

For insulin secretion in static incubations, the same procedure was used as described above for attached cells on coated coverslips until the wash with the low Ca^{2+} intracellular buffer. Thereafter, cells were preincubated first for 20 min then for 10 min in the 500 nM Ca^{2+}-10 mM ATP stimulatory intracellular buffer before a 10 min stimulation with the test compounds in the same buffer. For all insulin secretion experiments, 0.1% of bovine serum albumin (Sigma, St Louis, USA) was added to buffers as carrier.

Membrane potential

Cell membrane potential was measured in intact cells using the fluorescent probe bis-oxonol (Asfari *et al.*, 1992). Mitochondrial membrane potential ($\Delta\psi_m$) was measured as described (Duchen *et al.*, 1993). Briefly, after the spinner culture, cells were loaded with 10 μg/ml rhodamine-123 (Rh123) for 10 min at 37°C. After centrifugation, the cells were transferred to a fluorimeter cuvette and fluorescence was excited at 490 nm and measured at 530 nm. In some experiments, cells were permeabilized as described above after the Rh123 loading and changes in $\Delta\psi_m$ were monitored in the stimulatory intracellular buffer. All membrane potential measurements were performed at 37°C with gentle stirring in a LS-50B fluorimeter (Perkin-Elmer, Bucks, UK).

Statistical analysis

Where applicable, values are expressed as mean ± s.e.m., and *n* values refer to independent experiments, unless otherwise stated. The significance of difference was calculated by Student's *t*-test for unpaired data.

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References

Asfari,M., Janjic,D., Meda,P., Li,G., Halban,P.A. and Wollheim,C.B. (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology*, **130**, 167–178.

Ashcroft,F.M. and Rorsman P. (1989) Electrophysiology of the pancreatic β-cell. *Prog. Biophys. Molec. Biol.*, **54**, 87–143.

Bers,D.M., Patton,C.W. and Nuccitelli,R. (1994) A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol.*, **40**, 3–29.

Bokvist,K., Eliasson,L., Ämmälä,C., Renstrom,E. and Rorsman,P. (1995) Co-localization of L-type Ca^{2+} channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic β-cells. *EMBO J.*, **14**, 50–57.

Brini,M., Marsault,R., Bastianutto,C., Alvarez,J., Pozzan,T. and Rizzuto,R. (1995) Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration. *J. Biol. Chem.*, **270**, 9896–9903.

Civelek,V.N., Deeney,J.T., Shalosky,N.J., Tornheim,K., Hansford,R.G., Prentki,M. and Corkey,B.E. (1996) Regulation of pancreatic β-cell mitochondrial metabolism: influence of Ca^{2+}, substrate and ADP. *Biochem. J.*, **318**, 615–621.

Cox,D.A. and Matlib,M.A. (1993) A role for the mitochondrial Na^+-Ca^{2+} exchanger in the regulation of oxidative phosphorylation in isolated heart mitochondria. *J. Biol. Chem.*, **268**, 938–947.

Denton,R.M. and McCormack,J.G. (1980) On the role of the calcium transport cycle in heart and other mammalian mitochondria. *FEBS Lett.*, **119**, 1–8.

Duchen,M.R., Smith,P.A. and Ashcroft,F.M. (1993) Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. *Biochem. J.*, **294**, 35–42.

Gembal,M., Detimary,P., Gilon,P., Gao,Z.-Y. and Henquin,J.-C. (1993) Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse B cells. *J. Clin. Invest.*, **91**, 871–880.

Gerbitz,K.-D., Gempel,K. and Brdiczka,D. (1996) Mitochondria and diabetes: genetic, biochemical, and clinical implications of the cellular energy circuit. *Diabetes*, **45**, 113–126.

Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Gunter,T.E., Gunter,K.K., Sheu,S.S. and Gavin,C.E. (1994) Mitochondrial calcium transport: physiological and pathological relevance. *Am. J. Physiol.*, **267**, C313–C339.

Hajnóczky,G., Robb-Gaspers,L.D., Seitz,M.B. and Thomas,A.P. (1995) Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*, **82**, 415–424.

Hansford,R.G. (1985) Relation between mitochondrial calcium transport and control of energy metabolism. *Rev. Physiol. Biochem. Pharmacol.*, **102**, 1–72.

Hansford,R.G. (1991) Dehydrogenase activation by Ca^{2+} in cells and tissues. *J. Bioenerg. Biomembr.*, **23**, 823–854.

Hellman,B., Gylfe,E., Grapengiesser,E., Lund,P.E. and Berts,A. (1992) Cytoplasmic Ca^{2+} oscillations in pancreatic β-cells. *Biochim. Biophys. Acta*, **1113**, 295–305.

Idahl,L.-A. and Lembert,N. (1995) Glycerol 3-phosphate-induced ATP production in intact mitochondria from pancreatic B-cells. *Biochem J.*, **312**, 287–292.

Jijakli,H., Nadi,A.B., Cook,L., Best,L., Sener,A. and Malaisse,W.J. (1996) Insulinotropic action of methyl-pyruvate: enzymatic and metabolic aspects. *Arch. Biochem. Biophys.*, **335**, 245–257.

Johns,D.R. (1996) The other human genome: mitochondrial DNA and disease. *Nature Med.*, **2**, 1065–1068.

Jonas,J.-C., Li,G., Palmer,M., Weller,U. and Wollheim,C.B. (1994) Dynamics of Ca^{2+} and guanosine 5′-[γ-thio]triphosphate action on insulin secretion from α-toxin-permeabilized HIT-T15 cells. *Biochem. J.*, **301**, 523–529.

Katayama,N., Hughes,S.J., Persaud,S.J., Jones,P.M. and Howell,S.L. (1995) Insulin secretion from islets of GK rats is not impaired after energy generating steps. *Mol. Cell. Endocrinol.*, **111**, 125–128.

Kennedy,E.D., Rizzuto,R., Theler,J.-M., Pralong,W.-F., Bastianutto,C., Pozzan,T. and Wollheim,C.B. (1996) Glucose-stimulated insulin secretion correlates with changes in mitochondrial and cytosolic Ca^{2+} in aequorin-expressing INS-1 cells. *J. Clin. Invest.*, **98**, 2524–2538.

Li,G., Rungger-Brändle,E., Just,I., Jonas,J.-C., Aktories,K. and Wollheim,C.B. (1994) Effect of disruption of actin filaments by *Clostridium botulinum* C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets. *Mol. Biol. Cell*, **5**, 1199–1213.

Liang,Y. and Matschinsky,F.M. (1991) Content of CoA-esters in perifused rat islets stimulated by glucose and other fuels. *Diabetes*, **40**, 327–333.

Maassen,J.A. and Kadowaki,T. (1996) Maternally inherited diabetes and deafness: a new diabetes subtype. *Diabetologia*, **39**, 375–382.

MacDonald,M.J. and Brown,L.J. (1996) Calcium activation of mitochondrial glycerol phosphate dehydrogenase restudied. *Arch. Biochem. Biophys.*, **326**, 79–84.

MacDonald,M.J. and Fahien,L.A. (1988) Glyceraldehyde phosphate and methyl esters of succinic acid. *Diabetes*, **37**, 997–999.

Maechler,P., Kennedy,E.D. and Wollheim,C.B. (1996) Mitochondrial Ca^{2+} in insulin-secreting cells. *Experientia*, **52**, A38.

Matschinsky,F.M. (1996) A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes*, **45**, 223–241.

McCormack,J.G., Halestrap,A.P. and Denton,R.M. (1990a) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol. Rev.*, **70**, 391–425.

McCormack,J.G., Longo,E.A. and Corkey,B.E. (1990b) Glucose-induced action of pyruvate dehydrogenase in isolated rat pancreatic islets. *Biochem J.*, **267**, 527–530.

Mertz,R.J., Worley,J.F.,III, Spencer,B., Johnson,J.H. and Dukes,I.D. (1996) Activation of stimulus–secretion coupling in pancreatic β-cells by specific products of glucose metabolism: evidence for privileged signaling by glycolysis. *J. Biol. Chem.*, **271**, 4838–4845.

Nilsson,T., Schultz,V., Berggren,P.-O., Corkey,B.E. and Tornheim,K. (1996) Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca^{2+} in glucose-stimulated pancreatic β-cells. *Biochem. J.*, **314**, 91–94.

Palmer,M., Jursch,R., Weller,U., Valeva,A., Hilgert,K., Kehoe,M. and Bhakdi,S. (1993) *Staphylococcus aureus* α-toxin: production of functionally intact, site-specifically modifiable protein by introduction of cysteine at position 69, 130, and 186. *J. Biol. Chem.*, **268**, 11959–11962.

Pralong,W.F., Bartley,C. and Wollheim,C.B. (1990) Single islet β-cell stimulation by nutrients: relationship between pyridine nucleotide, cytosolic Ca^{2+} and secretion. *EMBO J.*, **9**, 53–60.

Pralong,W.F., Spät,A. and Wollheim,C.B. (1994) Dynamic pacing of cell metabolism by intracellular Ca^{2+} transients. *J. Biol. Chem.*, **269**, 27310–27314.

Prentki,M. (1996) New insights into pancreatic β-cell metabolic signalling in insulin secretion. *Eur. J. Endocrinol.*, **134**, 272–286.

Prentki,M. and Corkey,B.E. (1996) Are the β-cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes*, **45**, 273–283.

Rizzuto,R., Simpson,A.W.M., Brini,M. and Pozzan,T. (1992) Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature*, **358**, 325–327.

Rutter,G.A., Burnett,P., Rizzuto,R., Brini,M., Murgia,M., Pozzan,T., Tavaré,J.M. and Denton,R.M. (1996) Subcellular imaging of intramitochondrial Ca^{2+} with recombinant targeted aequorin. Significance for the regulation of pyruvate dehydrogenase activity. *Proc. Natl Acad. Sci. USA*, **93**, 5489–5494.

Soejima,A., Inoue,K., Takai,D., Kaneko,M., Ishihara,H., Oka,Y. and Hayashi,J.-I. (1996) Mitochondrial DNA is required for regulation of glucose-stimulated insulin secretion in a mouse pancreatic β-cell line, MIN6. *J. Biol. Chem.*, **271**, 26194–26199.

Theler,J.M., Mollard,P., Guérineau,N., Vacher,P., Pralong,W.F., Schlegel,W. and Wollheim,C.B. (1992) Video imaging of cytosolic Ca^{2+} in pancreatic β-cells stimulated by glucose, carbachol and ATP. *J. Biol. Chem.*, **267**, 18110–18117.

VonGersdorff,H., Vardi,E., Matthews,G. and Sterling,P. (1996) Evidence that vesicles on the synaptic ribbon of retinal bipolar neurons can be rapidly released. *Neuron*, **16**, 1221–1227.

Wollheim,C.B., Lang,J. and Regazzi,R. (1996) The exocytotic process of insulin secretion and its regulation by Ca^{2+} and G-proteins. *Diabetes Rev.*, **4**, 276–297.

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