Methyl pyruvate initiates membrane depolarization and insulin release by metabolic factors other than ATP

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The role of mitochondria in stimulus–secretion coupling of pancreatic β-cells was examined using methyl pyruvate (MP). MP stimulated insulin secretion in the absence of glucose, with maximal effect at 5 mM. K^+ (30 mM) alone, or in combination with diazoxide (100 μM), failed to enhance MP-induced secretion. Diazoxide (100 μM) inhibited MP-induced insulin secretion. MP depolarized the β-cell in a concentration-dependent manner (5–20 mM). The sustained depolarization induced by 20 mM MP was not influenced by 100 μM diazoxide, but the continuous spiking activity was suppressed by 500 μM diazoxide. Pyruvate failed to initiate insulin release (5–20 mM) or to depolarize the membrane potential. ATP production in isolated β-cell mitochondria was detected as accumulation of ATP in the medium during incubation in the presence of malate or glutamate in combination with pyruvate or MP. There was no difference in ATP production induced by pyruvate/malate or MP/malate in isolated β-cell mitochondria. ATP production by MP/glutamate was higher than that induced by pyruvate/glutamate, but it was much lower than that induced by α-ketoisocaproate/glutamate. Pyruvate (5 mM) or MP (5 mM) had no effect on the ATP/ADP ratio in whole islets, whereas glucose (20 mM) significantly increased the whole islet ATP/ADP ratio. It is concluded that MP-induced β-cell membrane depolarization or insulin release does not relate directly to mitochondrial ATP production. Instead MP may exert a direct extramitochondrial effect, or it may stimulate β-cell mitochondria to produce coupling factors different from ATP to initiate insulin release.

Key words: islets of Langerhans, membrane potential, mitochondrial metabolism.

INTRODUCTION

Mitochondria are considered to play a central role in stimulus–secretion coupling of pancreatic β-cells by initiating insulin secretion through production of ATP [1–3]. Inhibition of plasma membrane K^+_{ATP} channels initiates depolarization and triggers Ca^{2+} influx, followed eventually by insulin release. Mitochondria may also modulate insulin release by production of various metabolites, which act through poorly defined mechanisms [4,5].

Pyruvate and methyl pyruvate (MP) are equally well decarboxylated in isolated islets; thus both substrates accumulate in the cytosol with similar concentrations [6]. However, only MP stimulates insulin secretion *in vitro* [7–10] and *in vivo* [11], whereas pyruvate, the main glycolytic intermediate of glucose metabolism, is not insulinogenic [12,13].

Insulin release by MP is accompanied by inhibition of K^+_{ATP}-channel activity and an increase in cytosolic and mitochondrial Ca^{2+} concentrations [7,14]. The insulinogenic effect was attributed to an increased mitochondrial ATP production induced by MP. It was speculated that MP might non-specifically penetrate the inner mitochondrial membrane due to its lipophilic nature. Accumulation of MP in the mitochondrial matrix would stimulate a supranormal mitochondrial ATP production to initiate insulin release. There is, however, no direct experimental evidence for that hypothesis.

This study aims at further elucidating the mode of action of MP by comparing the effects of glucose, pyruvate or MP on insulin secretion, β-cell membrane potential and mitochondrial ATP production.

MATERIALS AND METHODS

Microbial collagenase P (EC 3.4.24.3), ADP (potassium salt), ATP (potassium salt), diadenosine pentaphosphate (trilithium salt), phosphoenol pyruvate and electrophoretically homogenous lyophilized BSA were purchased from La Roche Biochemicals (Mannheim, Germany). BSA (fraction V) was from BDH Chemicals (Poole, Dorset, U.K.). Firefly luciferase (EC 1.13.12.7) and D-luciferin were purchased from Biothema AB (Dalarö, Sweden). Pyruvate kinase (EC 2.7.1.40), L-glutamate, malate, MP, α-ketoisocaproate (KIC), diazoxide and antimycin A were from Sigma. Pyruvate was from Aldrich (Steinheim, Germany). Hepes was obtained from Calbiochem (La Jolla, CA, U.S.A.). NaCl, KCl, KH_2PO_4, $MgSO_4$ and KOH (Suprapur) was from Merck. $MgCl_2$ was from BDH Chemicals (Poole, Dorset, U.K.). Quartz bi-distilled water was used throughout.

Assay of insulin release

Fed NMRI mice of both sexes were used for the determination of insulin release. Islets were isolated by collagenase digestion. Batches of five islets were incubated with the indicated substrates for 1 h at 37 °C. The incubation medium (1 ml) was Krebs–Ringer buffer supplemented with Hepes (10 mM) at pH 7.4. After incubation, an aliquot of 100 μl was taken for determination of insulin by RIA, using rat insulin as standard and rat insulin antibodies.

Measurement of membrane potential

Fed NMRI mice of both sexes were used for measurements of the membrane potential of pancreatic β-cells. After decapitation, the excised pancreas was immediately placed in a perifusion chamber. Partly free-dissected islets were impaled with a glass micro-electrode and identified after impalement by the typical appearance of slow waves in the presence of 16.7 mM glucose [14]. Before the addition of test substances, the perfused pancreas was routinely kept for 10 min in the complete absence of glucose. The functional integrity of the impaled β-cell was checked at the end of each experiment by the addition of glucose (16.7 mM). The basal composition of the perifusion medium was 122 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.12 mM $MgCl_2$ and 20 mM $NaHCO_3$. Solutions with substrate were prepared immediately before use. All solutions were equilibrated with O_2/CO_2 (19:1). Evaluation of the electrophysiological data was performed using the Spike 2-software (Cambridge Electronic Devices, Cambridge, U.K.) after on-line digitization of the recordings by the converter device CED 1401 (Cambridge Electronic Devices).

Isolation of mitochondria

Adult female ob/ob mice, starved overnight, were used throughout. Large islets with a high content of β-cells ($> 90\%$; [16]) can be isolated, and the mitochondria obtained may be regarded to represent pure β-cells. Mitochondria were prepared as described in [17]. Comparative studies with mitochondria from lean mice demonstrated a normal function of ob/ob mice mitochondria [17].

Incubation of mitochondria

Cold mitochondrial suspension (5 μl) was added to 995 μl incubation medium prewarmed to 37 °C [18]. Incubation was stopped after 10 min by the addition of a mixture of antimycin A (0.5 μM), D-luciferin (0.4 μM) and luciferase (0.5 nM). The samples were then rapidly cooled to room temperature, and the ATP produced was determined by integrating the light emission over a period of 6 s in a Packard Tricarb scintillation spectrometer, operated out of coincidence.

Quantification of mitochondrial ATP production

Due to the restricted amount of tissue only a minute amount of mitochondria can be isolated. Thus protein assay is not applicable to quantify the amount of intact mitochondria from islets. Isolated mitochondria produce ATP by two different reactions that can be monitored in parallel incubations. Mitochondria produce ATP in the presence of ADP (50 μM) as the sole substrate due to adenylate kinase activity [17]. The accumulated ATP in the medium during incubation of isolated mitochondria with ADP depends on the amount of adenylate kinase and corresponds to the amount of intact mitochondria during incubation. For each mitochondrial preparation the amount of intact mitochondria during incubation is determined by the ATP production by adenylate kinase (nmol ATP/10 min of incubation). Adenylate kinase can be specifically inhibited by diadenosine pentaphosphate [18,19]. When adenylate kinase is blocked during an incubation in the presence of mitochondrial substrates and ADP, the accumulated ATP in the medium corresponds to ATP production induced by oxidative phosphorylation (nmol ATP/10 min of incubation). In the following, all mitochondrial activities are expressed as the unitless ratio of the accumulated mitochondrial ATP production induced by oxidative phosphorylation/mitochondrial adenylate kinase activity, detected in a parallel control incubation. This allows

relating mitochondrial ATP production by oxidative phosphorylation to the amount of intact mitochondria during the incubation and facilitates the comparison of various mitochondrial preparations.

Determination of ATP/ADP ratios in whole islets

Four ob/ob mice islets were incubated for 1 h in 1 ml of Krebs–Ringer–Hepes buffer [130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.6H_2O$, 2.5 mM $CaCl_2$, 20 mM Hepes (pH 7.4)/1% albumin] at 37 °C. The incubation was stopped by rapid transfer of the islets to ice-cold 0.1 M KOH, containing 0.2 mM EDTA, mechanical rupture and heating to 60 °C for 10 min. ATP was determined by the bioluminometric method described above. ADP was determined as the difference in total islet ATP content after converting ADP into ATP with pyruvate kinase.

Data processing

Duplicate samples were carried through the entire experimental procedure for insulin measurements and determination of mitochondrial ATP production. The mean of two samples was entered as one observation.

Statistics

Mean values of two groups were compared using the two-tailed independent Student's t test. Multiple comparison of mean values was performed with ANOVA followed by a Newman–Keuls test at a probability level of $P < 0.05$.

RESULTS

MP stimulated insulin release in the absence of glucose, whereas pyruvate (20 mM) was ineffective (Figure 1). Maximal secretion was observed at a concentration of 5 mM MP. Higher concentrations of MP were less effective in stimulating insulin release. MP potentiated insulin secretion induced by 20 mM glucose in a
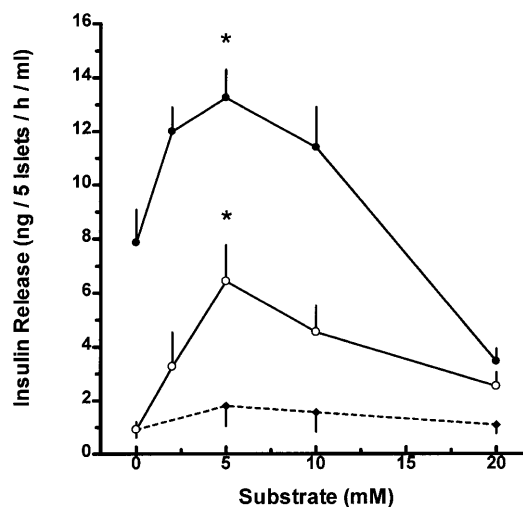
Figure 1   Insulin release induced by glucose, pyruvate or MP

Batches of five mouse islets were incubated for 1 h in 1 ml of Krebs–Ringer–Hepes buffer at 37 °C. (○) Insulin secretion induced by MP as sole substrate; (●) insulin secretion induced by MP in the presence of 20 mM glucose; (■) insulin secretion induced by pyruvate as the sole substrate. Values are means ± S.E.M. observed in at least five experiments. Statistics were performed by Student's t test. *$P < 0.05$.
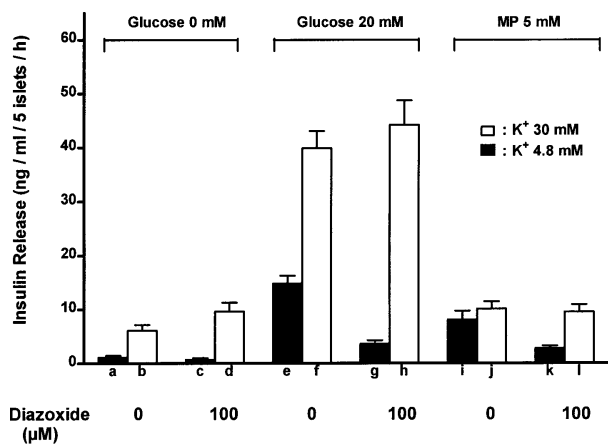
Figure 2   Effect of diazoxide and K$^+$ on insulin release induced by glucose or MP

Batches of five mouse islets were incubated in 1 ml of Krebs–Ringer–Hepes buffer for 1 h at 37 °C. The buffer was supplemented with K$^+$ and diazoxide as indicated. Substrates are present as indicated. Values are means ± S.E.M. of 10–14 batches of islets. Statistics were performed after ANOVA using Newman–Keuls post test for comparison of multiple mean values at a probability level of $P < 0.05$. Statistical analysis of various groups resulted in $P > 0.05$ for b versus d, i, j and l; $P < 0.05$ for d versus e, f and h; $P < 0.05$ for e versus g; $P < 0.05$ for i versus k.

concentration dependent manner. A significant stimulation of glucose-induced insulin was observed at 5 mM MP. Addition of 20 mM MP inhibited glucose-induced insulin secretion.

The effects of diazoxide and K$^+$ on insulin secretion induced by glucose or MP are summarized in Figure 2. K$^+$ (30 mM) enhanced insulin secretion in the absence of metabolic substrates. Addition of diazoxide (100 μM) to a medium containing 30 mM K$^+$ did not further enhance insulin secretion. Glucose-induced insulin release was inhibited by diazoxide and maximally potentiated upon addition of K$^+$. An equally high insulin secretion was observed in a medium containing glucose, K$^+$ (30 mM) and diazoxide. MP-induced insulin secretion was inhibited by diazoxide. No further increase of MP-induced insulin release was observed by addition of K$^+$. In addition, the combined presence of diazoxide and high K$^+$ concentrations failed to enhance MP-induced insulin release. Maximal release induced by MP was comparable to K$^+$-enhanced secretion of the substrate-free control.

The electrical activity of pancreatic β-cells after stimulation with glucose or MP is given in Figure 3. Glucose stimulation (16.7 mM) provoked the occurrence of slow waves in pancreatic β-cells (Figure 3A). Stimulation with pyruvate (20 mM) in the absence of glucose failed to depolarize the membrane (Figure 3B). MP depolarized the β-cell membrane potential in a dose-dependent manner. MP (5 mM) provoked a transient depolarization with initial spike activity and a depolarization period of about 5 min (Figure 3C). MP (10 mM) provoked a sustained depolarization with initial spike activity (Figure 3D). MP (20 mM) induced a sustained depolarization with constant spike activity (Figure 3E). MP never provoked the appearance of slow waves at concentrations ranging from 5 to 20 mM.

Addition of diazoxide (100 μM) completely suppressed glucose-induced membrane depolarization (Figure 4A). However, diazoxide (100 μM) had no effect on MP-induced depolarization (Figure 4B). Even at a concentration of 500 μM, diazoxide failed to prevent MP-induced membrane depolarization, but shortened the period of spike activity (Figure 4C).
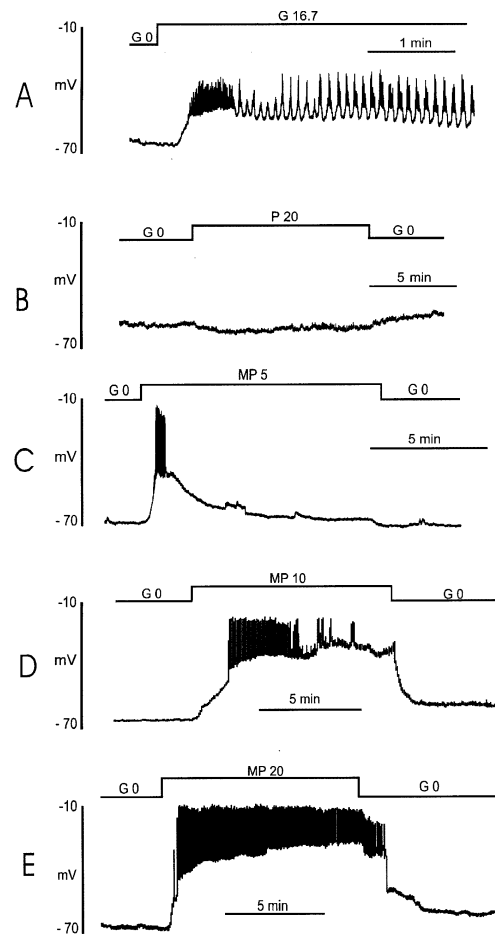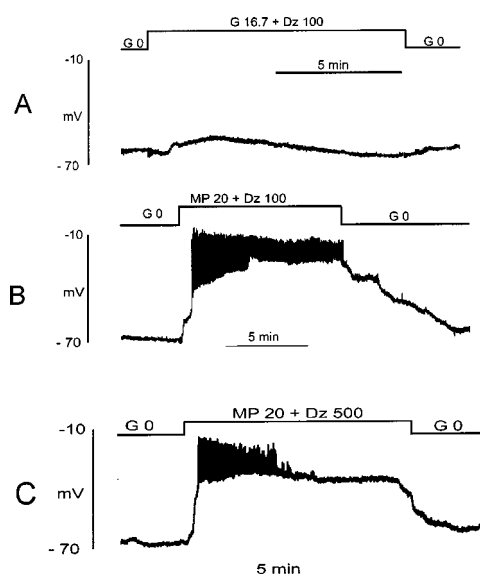
Figure 3   Effect of glucose, pyruvate or MP on membrane potential of mouse pancreatic β-cells

Glucose was completely omitted for a period of 10 min before the addition of substrates. Changes in the composition of the medium were made as indicated. Traces show the effect of (**A**) glucose (G; 16.7 mM), (**B**) pyruvate (P; 20 mM), (**C**) MP, 5 mM, (**D**) MP, 10 mM and (**E**) MP, 20 mM. A,B,C; A,B,D and A,B,E were performed in different cells. Similar effects for each substrate were observed in at least four independent experiments.
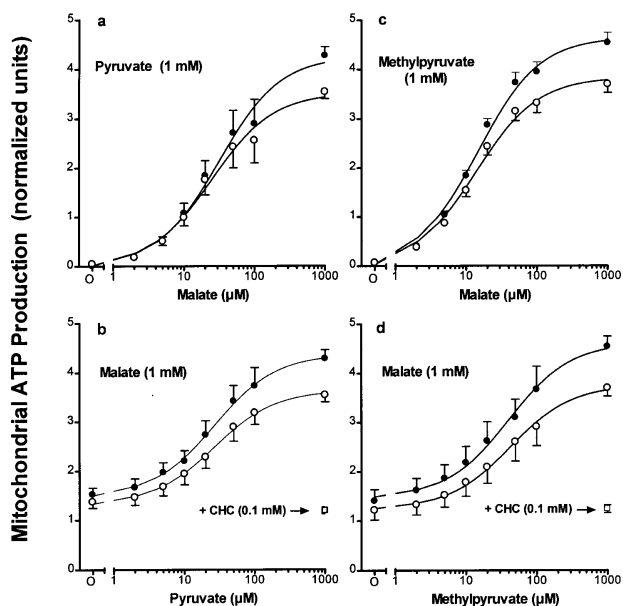
Mitochondrial ATP production induced by pyruvate, malate and MP is summarized in Figure 5. Pyruvate alone (1 mM) did not induce ATP production in β-cell mitochondria (Figure 5a). Addition of malate stimulated ATP production in a dose-dependent manner, with half-maximal effect (EC$_{50}$) at 24.6 ± 3.4 μM or 31.2 ± 4.3 μM in the absence or presence of 300 nM Ca^{2+}. Maximal stimulation was observed in the presence of 1 mM malate. Mitochondria produced ATP in the presence of malate alone (Figure 5b). Addition of pyruvate increased ATP production dose-dependently, with a pyruvate EC$_{50}$ of 26.7 ± 2.2 μM in the absence or 27.0 ± 1.9 μM in the presence of Ca^{2+}. Maximal activity was observed in the presence of 1 mM pyruvate. Ca^{2+} (300 nM) significantly stimulated maximal ATP production (Figures 5a and 5b). Higher concentrations of pyruvate (10 or 20 mM) did not further enhance mitochondrial ATP production either in the absence or presence of Ca^{2+} (results not shown).

ATP production induced by MP/malate was very similar to that obtained by pyruvate/malate (Figures 5c and 5d). The EC$_{50}$ of malate in the presence of 1 mM MP was 13.8 ± 1.5 μM or 14.6 ± 1.3 μM in the absence or presence of 300 nM Ca^{2+}. The

Figure 4 Effect of diazoxide on electrical activity induced by glucose or MP

Glucose was completely omitted for a period of 10 min before the addition of substrates. Changes in the composition of the medium were made as indicated. In (**A**) glucose (G; 16.7 mM) and diazoxide (Dz; 100 μM) were added simultaneously, in (**B**) and (**C**) MP 20 mM and Dz (100 μM or 500 μM) were added together. Typical traces observed in three independent pancreas preparations are shown.

Figure 5 ATP production in isolated mitochondria from pancreatic β-cells in the presence of malate and pyruvate or MP

Isolated mitochondria from pancreatic β-cells were incubated for 10 min at 37 °C with pyruvate/malate or MP/malate at the indicated substrate concentrations. Normalized mitochondrial ATP production is the ratio of ATP production induced by oxidative phosphorylation and ATP production induced by mitochondrial adenylate kinase. ($\bigcirc$) ATP measurements in the absence of Ca^{2+}; ($\bullet$) ATP production in the presence of 300 nM Ca^{2+}. Curves are calculated by fitting a hyperbolic function to the mean values with the allowance of zero activity in (**b**) and (**d**). Values are means $\pm$ S.E.M. of at least five different observations.

Table 1 ATP production in isolated mitochondria from pancreatic β-cells in the presence of glutamate and pyruvate, MP or KIC

Isolated mitochondria from pancreatic β-cells were incubated for 10 min at 37 °C with glutamate (10 mM) in combination with pyruvate (1 mM), MP (1 mM) or KIC (0.1 mM). Mitochondrial ATP production is the ratio of ATP production by oxidative phosphorylation and ATP production induced by mitochondrial adenylate kinase. Values are means $\pm$ S.E.M. of at least five experiments. *Significant stimulation by Ca^{2+} at a probability level of $P < 0.05$ determined by independent Student's t test.

Substrate (mM)	Ca^{2+} (300 nM)	ATP production (normalized units)
Pyruvate	−	0.35 ± 0.03
Pyruvate	+	0.41 ± 0.03
MP	−	0.88 ± 0.08
MP	+	0.77 ± 0.07
KIC	−	1.48 ± 0.23
KIC	+	2.95 ± 0.47 *

Table 2 Effect of glucose, pyruvate or MP on whole islet ATP/ADP ratios

Four *ob/ob* mice islets were incubated for 1 h at 37 °C. Islet contents of ATP and ADP were assayed as described in the Material and Methods section. Values are means $\pm$ S.E.M. of eight separate experiments. Statistics were performed after ANOVA using Newman–Keuls post test for comparison of multiple mean values at a probability level of $P < 0.05$. $P < 0.05$ for D versus A,B,C. No significant difference between A,B and C.

Group	Substrate	ATP/ADP ratio
A	−	2.01 ± 0.09
B	Pyruvate (5 mM)	1.92 ± 0.03
C	MP (5 mM)	1.91 ± 0.05
D	Glucose (20 mM)	3.13 ± 0.22

EC$_{50}$ of MP in the presence of 1 mM malate was 43 ± 4.3 μM or 40.4 ± 4.8 μM in the absence or presence of Ca^{2+}. High concentrations of MP (10 or 20 mM) failed to further increase mitochondrial ATP production either in the absence or presence of Ca^{2+} (results not shown). In order to test the specificity of mitochondrial pyruvate or MP uptake, ATP production was measured in the presence of the pyruvate-transport inhibitor α-cyano-4-hydroxycinnamate (α-CHC). α-CHC (100 μM) reduced pyruvate/malate (1 mM/1 mM)-induced ATP production to the ATP production level observed in the presence of 1 mM malate alone (Figure 5b). Similar results were observed in the presence of MP/malate (1 mM/1 mM) (Figure 5d). These findings indicate that MP is transported specifically by the mitochondrial pyruvate transporter and that it is not unspecifically diffusing into the matrix.

The efficiency of mitochondrial glutamate metabolism in the presence of pyruvate or MP is given in Table 1. Mitochondrial ATP production in the presence of glutamate and MP significantly exceeded that induced by glutamate and pyruvate. However, pyruvate or its methyl ester was rather inefficient in stimulating mitochondrial ATP production when compared with α-ketoisocaproate and glutamate. Addition of Ca^{2+} (300 nM) clearly augmented KIC/glutamate-induced mitochondrial ATP production, but had no stimulatory effect on that induced by pyruvate/glutamate or MP/glutamate. There was no additive effect of MP when added to a substrate combination consisting of pyruvate, malate and glutamate, whether in the absence $(4.59 \pm 0.49$ versus $4.65 \pm 0.49)$ or presence of Ca^{2+} $(5.56 \pm 0.0.63$ versus $5.81 \pm 0.61)$.

The effects of pyruvate, MP or glucose on whole islet ATP, whole islet ADP and the corresponding ATP/ADP ratios in whole islets are shown in Table 2. Glucose (20 mM) significantly increased the ATP/ADP ratio compared to a substrate-free control. However, MP failed to elevate the ATP/ADP ratio when tested at the optimal concentration for insulin release. Similarly, no effect was observed on the ATP/ADP ratio after stimulation with pyruvate.

DISCUSSION

According to the substrate site hypothesis, all substrates that initiate insulin secretion stimulate β-cell mitochondria to increased ATP production [3,20]. The increased ATP/ADP ratio is considered the central metabolic signal which triggers depolarization, Ca^{2+} influx and insulin release. The insulinogenic effect of MP was attributed to a supra-normal mitochondrial ATP production caused by unspecific substrate uptake and accumulation in the matrix [7–10]. In this study, however, MP induces insulin release and β-cell membrane depolarization, but is no more effective than pyruvate as a stimulator of mitochondrial ATP production.

The present study demonstrates a qualitative difference in the action of pyruvate and MP that cannot be explained by a simple difference in cytosolic substrate concentrations due to facilitated membrane transport of the methylated form. None of the results obtained could be attributed to a stimulated mitochondrial ATP production as proposed by others [7–10]. The only difference in mitochondrial ATP production was observed in combination with glutamate, where MP was a more efficient substrate than pyruvate. However, maximal ATP production remained rather low when compared with that of the insulinogenic substrate KIC, which stimulates mitochondrial ATP production exclusively by glutamate transamination [18]. The differences between pyruvate and MP in mitochondrial ATP production by transamination are not paralleled by increased cytosolic ATP/ADP ratios, which indicates the minor contribution of pyruvate or MP transamination reactions to the cellular energy status.

Since α-CHC failed to inhibit insulin secretion by MP [7], an unspecific uptake of MP to the mitochondrial matrix was proposed [7]. However, an unspecific uptake of MP into the mitochondria can be excluded since α-CHC inhibited both pyruvate- and MP-induced mitochondrial ATP production.

An unspecific cytosolic effect of MP, which would increase the β-cell ATP/ADP ratio due to an activation of glycolytic ATP production, is also excluded. Similar ATP/ADP ratios, and similar mitochondrial ATP production in the presence of pyruvate or MP, are corroborated by previous measurements of pyruvate or MP decarboxylation in intact islets showing no difference in decarboxylation efficacy between the two substrates [6].

The participation of metabolic signals different from ATP which are produced by MP is evident from recordings of the membrane potential. Glucose (Figure 3 and [15]) or KIC [21] provoke slow waves which gradually change to constant depolarization at higher substrate concentrations. Depolarization without slow waves has not been described for insulinogenic substrates apart from MP.

The use of diazoxide and/or high K^+ concentrations demonstrate a clear difference in the insulin-releasing mechanism between glucose and MP. Glucose is able to augment insulin release under conditions in which its genuine effect on K^+_{ATP}-channel activity is eliminated by diazoxide. This phenomenon is usually referred to as the K^+_{ATP}-channel-independent pathway of glucose-induced insulin release and is also typical for other insulinogenic substrates such as KIC and some other keto acids [22–24]. However, no stimulation by MP is observed under these conditions. This indicates that MP lacks such a K^+_{ATP}-channel-independent mechanism. MP should be regarded primarily as an initiator of insulin secretion, acting by as yet undefined mechanisms.

Some of the results reported here may be influenced by the instability of MP during incubation. MP is an unstable compound: the slow hydrolysis is apparent from the concomitant fall in medium pH. This is not a new observation, and has been described earlier [6]. It should be noted also that Mertz et al. [7] faced MP instability which prevented direct MP oxidation studies. Thus the inhibitory effect of high MP concentrations on insulin secretion during static incubation may result from acidification of the medium over time, which has adverse effects on insulin release. Partial de-esterification may also explain the apparent difference between the MP concentrations optimal for insulin release and those maximally effective for membrane depolarization. All MP concentrations in the text are based on calculation only and may be lower since the de-esterification with time has not been corrected for. On the other hand, the results obtained in membrane potential measurements observed after 1 h of perifusion consistently showed a typical MP signal, which could not be mimicked by pyruvate. This constitutes a strong argument against explaining the different effects of pyruvate and MP by a mere difference in cytosolic concentration.

It is proposed here that MP or specific metabolites of MP produced in the cytoplasm initiate membrane depolarization by a mechanism different from ATP-dependent inhibition of K^+_{ATP}-channel activity. The participation of K^+_{ATP}-channel activity is indicated by the observed inhibition of insulin release in the presence of diazoxide. However, the classical assumption of an initial K^+_{ATP}-channel inhibition by mitochondrial ATP production [7] does not hold in the case of MP. Binding of MP or metabolites to the K^+_{ATP}-channel may result in a disturbed diazoxide binding site. Alternatively, MP-derived substances may induce membrane depolarization by binding to completely different sites. Both alternatives are compatible with the observation that diazoxide fails to prevent membrane depolarization in the presence of MP.

MP was proposed to be a prototype agent with possible therapeutic benefits in the treatment of some forms of Type II diabetes [9]. However, the narrow concentration range effective for insulin release, the instability at physiological pH and the yet unidentified mechanism of action may limit the usefulness of this substrate.

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