Methyl pyruvate stimulates pancreatic β -cells by a direct effect on K_{ATP} channels, and not as a mitochondrial substrate

Martina DÜFER*, Peter KRIPPEIT-DREWS*, Linas BUNTINAS†, Detlef SIEMEN† and Gisela DREWS*¹

*Institute of Pharmacy, Department of Pharmacology, University of Tübingen, Auf der Morgenstelle 8, D-72076 Tübingen, Germany, and †Department for Neurology, University of Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany

In pancreatic β -cells, methyl pyruvate is a potent secretagogue and is widely used to study stimulus–secretion coupling. In contrast with pyruvate, which barely stimulates insulin secretion, methyl pyruvate was suggested to act as an effective mitochondrial substrate. We show that methyl pyruvate elicited electrical activity in the presence of 0.5 mM glucose, in contrast with pyruvate. Accordingly, methyl pyruvate increased the cytosolic free Ca^{2+} concentration after an initial decrease, similar to glucose. The initial decrease was inhibited by thapsigargin, suggesting that methyl pyruvate stimulates ATP production. This assumption is supported by the observation that methyl pyruvate hyperpolarized the mitochondrial membrane potential, similar to glucose. However, in contrast with glucose, methyl pyruvate even slightly decreased NAD(P)H autofluorescence and did not influence ATP production or the ATP/ADP ratio. This observation questions the suggestion that methyl pyruvate acts as a powerful mitochondrial substrate. The finding that methyl

pyruvate directly inhibited a cation current across the inner membrane of Jurkat T-lymphocyte mitochondria suggests that this metabolite may increase ATP production in β -cells by activating the respiratory chains without providing reduction equivalents. We conclude that this mechanism may account for a slight and transient increase in ATP production. We further show that methyl pyruvate inhibited the K_{ATP} current measured in the standard whole-cell configuration, an effect that was at least partly antagonized by diazoxide. Accordingly, single-channel currents in inside-out patches were blocked by methyl pyruvate. We conclude that inhibition of K_{ATP} channels, and not activation of metabolism, mediates the induction of electrical activity in pancreatic β -cells by methyl pyruvate.

Key words: cytosolic calcium, Jurkat T-lymphocytes, mitochondrial membrane potential ($\Delta\Psi$), mitoplast channels, NAD(P)H autofluorescence.

INTRODUCTION

Mitochondrial ATP production is a key step in glucose-induced stimulus–secretion coupling in pancreatic β -cells [1,2]. The increase in the ATP/ADP ratio leads to closure of K_{ATP} channels, depolarization and opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx and eventually insulin secretion [3,4]. It is commonly believed that the reduction equivalents for mitochondrial ATP production are derived from the metabolism of pyruvate in the citrate cycle [5]. However, two elegant studies have shown that β -cells exclusively use NADH produced in the cytosol for the production of that portion of ATP that closes K_{ATP} channels [6,7]. These reports explain the puzzling fact which has been known for more than 20 years [8,9] that pyruvate, the main glycolytic intermediate of glucose metabolism, does not act as a primary stimulus for insulin secretion in β -cells. However, it has been shown that methyl pyruvate (MPyr) is a powerful stimulator of insulin secretion [7,10–14]. This effect has been ascribed to supranormal ATP production due to the lipophilic nature of MPyr, which leads to accumulation in the mitochondria [11,12]. However, the experimental proof for this suggestion is still lacking. In contrast, a recent report demonstrated that MPyr induces electrical activity and insulin secretion in the absence of an increase in the ATP/ADP ratio in mouse islets [14]. Thus, although MPyr has often been used to study stimulus–secretion

coupling, the exact mechanism by which this agent activates pancreatic β -cells is still a matter of debate.

In the present paper we provide evidence that the insulinotropic action of MPyr is mainly a consequence of a direct inhibition of K_{ATP} channels, rather than an indirect effect via augmented ATP production due to its metabolism.

MATERIALS AND METHODS

Cell, islet and mitoplast preparation

Experiments were performed using single pancreatic β -cells from fed female NMRI mice (25–30 g) which had been killed by cervical dislocation. Islets were isolated by collagenase digestion of the pancreas. The cells were dispersed in Ca^{2+} -free medium and cultured for up to 4 days in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin [15].

Mitoplasts for patch-clamp experiments were prepared from Jurkat T-lymphocytes grown in culture using Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 μ M non-essential amino acids, 100 units/ml penicillin, 100 μ g/ml streptomycin and 12 μ g/ml tylosin. Cells were harvested by centrifugation (280 g). Homo-

Abbreviations used: a.u., arbitrary units; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; α -CHC, α -cyano-4-hydroxycinnamate; LDH, lactate dehydrogenase; MPyr, methyl pyruvate; P_o , open probability of a channel; NP_o , number of active channels in the patch $\times P_o$; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; $\Delta\Psi$, mitochondrial membrane potential.

¹ To whom correspondence should be addressed (e-mail Gisela.DreWS@uni-tuebingen.de).

genization, preparation of the mitochondria, and the swelling procedure in hypotonic solution for obtaining mitoplasts were carried out as described elsewhere for glia mitochondria [16].

Solutions and chemicals

Recordings of cell membrane potential were performed at 32 °C with amphotericin B in the pipette solution (250 µg/ml). The pipette solution also contained (in mM): 10 KCl, 10 NaCl, 70 K₂SO₄, 4 MgCl₂, 2 CaCl₂, 10 EGTA, 20 Hepes, pH 7.15, adjusted with KOH. The bath solution was composed of (in mM): 140 NaCl, 5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 0.5 glucose, 10 Hepes, pH 7.4, adjusted with NaOH. This bath solution was also used for the determination of K_{ATP} currents, the cytosolic free Ca²⁺ concentration ([Ca²⁺]_c), mitochondrial membrane potential (ΔΨ) and NAD(P)H fluorescence at 37 °C (glucose concentrations as indicated).

K_{ATP} currents in the standard whole-cell configuration were measured with a pipette solution composed of (in mM): 130 KCl, 4 MgCl₂, 2 CaCl₂, 0.65 Na₂ATP, 10 EGTA, 20 Hepes, pH 7.15, adjusted with KOH. For recording of single K_{ATP}-channel currents, the bath solution was replaced before patch excision by a Mg²⁺-free solution containing (in mM): 130 KCl, 4.6 CaCl₂, 10 EDTA, 20 Hepes, pH 7.2, adjusted with KOH. In this case the pipette solution was composed of (in mM): 130 KCl, 2 CaCl₂, 1 MgCl₂, 10 EGTA, 20 Hepes, pH 7.4, adjusted with KOH.

Single-channel currents from mitoplasts were recorded with a pipette solution containing (in mM): 150 KCl, 10 Hepes, 0.5 CaCl₂, pH 7.2, adjusted with KOH. Mitoplasts were formed in a hypotonic solution containing (in mM): 5 Hepes, 0.5 CaCl₂, pH 7.2, adjusted with KOH. Isotonicity was then restored by adding 1 part of a hypertonic solution containing (in mM) 750 KCl, 30 Hepes and 0.5 CaCl₂, pH 7.2, adjusted with KOH to 4 parts of the hypotonic solution. MPyr was added to an isotonic mixture of the latter two solutions, and the pH was adjusted to 7.2 with KOH. Aliquots of this solution were stored frozen and thawed just before the experiment.

For all other experiments, MPyr solutions were freshly prepared immediately before use. As MPyr undergoes slow hydrolysis, the medium pH was checked and if necessary corrected throughout the experiments to avoid marked acidification. Fura 2 acetoxymethyl ester and rhodamine 123 were obtained from Molecular Probes (Eugene, OR, U.S.A.). Dulbecco's modified Eagle's medium, serum and amino acids were from PAA (Linz, Austria), RPMI 1640 medium, penicillin/streptomycin and glutamine were from GIBCO/BRL (Karlsruhe, Germany), and luciferase was from Calbiochem (Darmstadt, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) in the purest form available.

Recording methods

Patch-clamp recordings

Patch pipettes were pulled from borosilicate glass capillaries (Clark, Pangbourne, Berks., U.K.). They had resistances of between 3 and 5 MΩ for whole-cell measurements, and between 6 and 10 MΩ for single-channel recordings when filled with pipette solution. Membrane currents and potential were recorded with an EPC-9 patch-clamp amplifier and 'Pulse' software (HEKA, Lambrecht, Germany) in the voltage-clamp or current-clamp mode respectively. The potential difference across the cell membrane was determined in the current-clamp mode of the patch-clamp technique. The β-cells were identified by the charac-

teristic spike activity that occurred in the presence of a stimulatory glucose concentration. Whole-cell K_{ATP} currents were measured at a holding potential of -70 mV and during 300 ms pulses to -80 mV and -60 mV at 15 s intervals. Under these experimental conditions the current is almost entirely K_{ATP} current, blockable by tolbutamide [17]. Single-K_{ATP}-channel currents were recorded at a holding potential of -50 mV. NP_o [the number of active channels in the patch × the open probability of these channels (P_o)] was determined in segments of 30 s.

For single-channel measurements in mitoplasts, the pipettes had a resistance of 17 ± 2 MΩ. An EPC-7 amplifier was used in combination with an LM-1 AD/DA-converter and pClamp software (Axon-Instruments, Foster City, CA, U.S.A.). More details are given in Siemen et al. [16]. P_o was determined by all-points analysis. Records of 1 min duration were taken and the amplitude was determined point by point. Histograms of this analysis were then fitted by Gauss curves. The area below the Gaussians, representing the open channels (weighted accordingly if more than one channel was present in the patch), was divided by the total time of the record. Potentials are always given as at the inner side of the membrane, and outward currents always deflect upwards. Measurements were performed at room temperature (24 °C).

Determination of ATP and ADP

Between 18 and 25 islets were incubated in bath solution (see above) containing 0.1 % BSA and 0.5 or 15 mM glucose or 0.5 mM glucose/20 mM MPyr for 30 min at 37 °C. The incubation medium was removed and cells were lysed in a solution of 200 mM NaOH and 0.5 mM EDTA. Aliquots were stored at -20 °C for up to 7 days. ATP and ADP were measured luminometrically with a Luminometer 1253 (Bioorbit, Turku, Finland). For determination of ADP, intrinsic ATP was completely converted into AMP in a solution consisting of (in mM): 5 MgCl₂, 10 NaMoO₄, 2.5 GMP and 50 Tris/HCl, pH 8.0, plus 100–200 µg/ml ATP sulphurylase (specific activity 6 units/mg). An aliquot of 10 µl of the islet lysate was added to 50 µl of reaction buffer, incubated for 20 min at 30 °C, boiled for 2 min and centrifuged for 2 min at 5000 g. To convert ADP into ATP, 30 µl of the resultant supernatant was added to 30 µl of buffer solution composed of (in mM): 4.7 MgCl₂, 0.5 phosphoenolpyruvate, 38 KCl and 47 Tris/HCl, pH 8.0, plus 22 µl/ml pyruvate kinase (5.4 mg of protein/ml). After incubation at 37 °C for 20 min, the mixture was boiled for 2 min and centrifuged for 2 min at 5000 g. Bioluminescence assays were performed in triplicate by adding 10 µl of test solution to 490 µl of assay buffer containing (in mM): 5 MgCl₂, 0.1 EDTA, 0.1 Na₂S₂O₃, 1 dithioerythritol and 25 Tricin/HCl, pH 7.8, plus 0.1 % BSA, 25 µg/ml luciferin and 5 µg/ml luciferase.

Measurement of [Ca²⁺]_c

[Ca²⁺]_c was measured by the fura 2 method according to Grynkiewicz et al. [18]. Equipment and software were from TILL Photonics (Gräfelfing, Germany). The cells were loaded with fura 2 acetoxymethyl ester (5 µM) for 30 min at 37 °C. Intracellular fura 2 was excited with light of 340 nm or 380 nm wavelength adjusted by means of a diffractive grating. The excitation light was then directed through the objective (PlanNeofluar 40× objective; Zeiss, Stuttgart, Germany) by means of a glass fibre light guide and a dichroic mirror. The emitted light was filtered (long-pass 515 nm, i.e. wavelengths longer than 515 nm pass through this filter) and measured using a digital camera. The ratio of the emitted light intensity at

340/380 nm excitation wavelength was used to calculate $[Ca^{2+}]_c$ by means of an *in vitro* calibration with fura 2 K^+ salt.

Measurement of $\Delta\Psi$ and NAD(P)H fluorescence

$\Delta\Psi$ and NAD(P)H fluorescence were measured with the same equipment as for $[Ca^{2+}]_c$. Rhodamine 123 fluorescence was excited at 480 nm and the intensity of the emitted light (long-pass 515 nm) was measured. Cells were loaded with rhodamine 123 (10 μ g/ml) for 10 min at 37 °C. An increase in rhodamine 123 fluorescence corresponds to a decrease in $\Delta\Psi$ [1,2]. NAD(P)H autofluorescence was measured every 1 s at 360 nm excitation wavelength. For clarity in the graph, a double-exponential curve was fitted to the baseline and subtracted from the curve. Thus only changes in fluorescence are given.

Presentation of results

Electrophysiological experiments and $[Ca^{2+}]_c$, $\Delta\Psi$ and NAD(P)H fluorescence measurements are illustrated by recordings that are representative of the indicated numbers of experiments performed with different cells. Cells from at least three different cell preparations were used for each series of experiments. If possible, means $\pm$ S.E.M. are given in the text for the indicated numbers of experiments. The statistical significance of differences between means was assessed by a one-sample *t* test, or Student's *t* test for paired values when two samples were compared. Multiple comparisons were made by ANOVA followed by the Student–Newman–Keuls test. $P \leq 0.05$ was considered to indicate a significant difference.

RESULTS

Comparison of the effects of MPyr, pyruvate and glucose on the membrane potential of mouse pancreatic β -cells

Figure 1(A) shows the well known effect of glucose on the membrane potential of pancreatic β -cells. At 0.5 mM glucose the membrane potential was stable and close to the K^+ reversal potential. After switching to a stimulatory glucose concentration (10 mM), the cell membrane potential depolarized and the characteristic spike activity appeared. A concentration of 20 mM MPyr (equivalent to 10 mM glucose with respect to carbon atoms) induced electrical spike activity similar to that with glucose (Figure 1B), while 20 mM pyruvate had no effect (Figure 1C). On average, MPyr depolarized the β -cell membrane potential from -75 ± 1 mV to a plateau potential (potential from which spikes start) of -44 ± 2 mV ($n = 3$); the values for glucose were depolarization from -73 ± 1 mV to -54 ± 3 mV ($n = 7$). In the experiments with pyruvate, the membrane potential was -76 ± 1 mV under control conditions with 0.5 mM glucose, and -78 ± 3 mV in the presence of 20 mM pyruvate ($n = 4$).

Effects of MPyr, pyruvate and glucose on $[Ca^{2+}]_c$

$[Ca^{2+}]_c$ was measured to reveal whether MPyr could mimic the effects of glucose on this important signal for insulin secretion. An increase in the glucose concentration from 0.5 to 10 mM evoked the characteristic response in $[Ca^{2+}]_c$ (Figure 2A). The initial fall in $[Ca^{2+}]_c$ indicates the activation of sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPases (SERCAs) [19]. Thereafter, the opening of L-type Ca^{2+} channels led to a drastic increase in $[Ca^{2+}]_c$ and finally to oscillations. Comparable with the effect of glucose, addition of 20 mM MPyr also resulted in an initial decrease in $[Ca^{2+}]_c$, suggesting an activation of Ca^{2+} uptake prior to opening of L-type Ca^{2+} channels. On average,

Figure 1 MPyr activates electrical activity in mouse pancreatic β -cells

The membrane potential of single β -cells was recorded in the current-clamp mode of the patch-clamp technique. Solution changes are indicated by the horizontal bars (the same applies to subsequent Figures). (A) Increasing the glucose (G) concentration from 0.5 to 10 mM led to a depolarization of the membrane potential to the plateau potential from which spikes started ($n = 7$). (B) Similar to glucose, 20 mM MPyr induced electrical spike activity in β -cells ($n = 3$). (C) Pyruvate (Pyr) was without effect on the membrane potential of β -cells ($n = 4$).

10 mM glucose and 20 mM MPyr decreased $[Ca^{2+}]_c$ from 67 ± 12 nM to 58 ± 15 nM ($n = 4$) and from 64 ± 7 nM to 46 ± 7 nM ($n = 7$) respectively. The subsequent elevation in $[Ca^{2+}]_c$ was 251 ± 37 nM for glucose and 263 ± 17 nM in the presence of MPyr. The inset of Figure 2(A) shows that 20 mM pyruvate did not induce any detectable change in $[Ca^{2+}]_c$ ($n = 5$). Figure 2(B) confirms that the initial decrease in $[Ca^{2+}]_c$ observed with glucose and MPyr was a result of ATP-induced SERCA activation, because it was completely prevented by preincubation of the β -cells with 1 μ M thapsigargin, a specific SERCA inhibitor (for comparison of Ca^{2+} decreases, note arrowheads in Figures 2A and 2B). Figure 2(B) also demonstrates that the rise in $[Ca^{2+}]_c$ evoked by 20 mM MPyr was suppressed by 200 μ M diazoxide, a K_{ATP} -channel opener ($n = 4$).

Effects of MPyr, pyruvate and glucose on $\Delta\Psi$

Since the measurements of $[Ca^{2+}]_c$ suggested an effect of MPyr on cell metabolism, we tested the action of the compound on $\Delta\Psi$, which under certain conditions can be taken as a measure of ATP production [1,2,20]. Figure 3(A) illustrates that 20 mM MPyr hyperpolarized $\Delta\Psi$ to the same extent as 10 mM glucose. On average, addition of MPyr or glucose decreased rhodamine 123 fluorescence by 704 ± 128 arbitrary units (a.u.) ($n = 4$) and 599 ± 94 a.u. ($n = 8$) respectively. Partial depolarizations of $\Delta\Psi$ after the initial hyperpolarization are a consequence

Figure 2 Effects of MPyr on $[Ca^{2+}]_c$ in the absence and presence of thapsigargin

(A) An increase in the glucose (G) concentration from 0.5 to 10 mM evoked the characteristic triphasic response in $[Ca^{2+}]_c$. MPyr (20 mM) evoked a very similar response to glucose. Note the initial decrease in $[Ca^{2+}]_c$ (arrowheads). The record is representative of four (glucose) or seven (MPyr) experiments with similar results for the individual manoeuvres. The inset shows that 20 mM pyruvate (Pyr) did not induce any changes in $[Ca^{2+}]_c$ ($n = 5$). (B) Preincubation of the β -cells with 1 μ M thapsigargin (Thapsi) completely prevented the initial decreases in $[Ca^{2+}]_c$ (arrowheads) induced by glucose and MPyr. Diazoxide (Diaz; 200 μ M) completely counteracted the $[Ca^{2+}]_c$ -elevating effect of MPyr. One record is shown that is representative of three (glucose) or four (MPyr) with similar results for the individual manoeuvres.

of oscillations in $[Ca^{2+}]_c$, as described previously [2]. Figure 3(B) shows that, in contrast with 10 mM glucose, 20 mM pyruvate only slightly hyperpolarized $\Delta\Psi$ by 120 ± 43 a.u. ($n = 5$).

Changes in NAD(P)H autofluorescence caused by glucose, pyruvate and MPyr

Substances which serve as mitochondrial substrates increase the concentration of reduction equivalents that can be measured as NAD(P)H autofluorescence, and thus the effects of glucose, MPyr and pyruvate on this parameter were tested. Figure 4 shows that NAD(P)H fluorescence was increased by 10 mM glucose and was even slightly elevated by 20 mM pyruvate, but was decreased by 20 mM MPyr. In the presence of glucose and pyruvate, increases of 5.8 ± 0.6 a.u. ($n = 10$) and 0.6 ± 0.2 a.u. ($n = 5$) respectively were observed. In contrast, MPyr lowered NAD(P)H fluorescence by -2.3 ± 0.5 a.u. ($n = 9$). To rule out the possibility that NAD(P)H autofluorescence was decreased due to a rise in cytosolic NADH consumption, MPyr was also tested in the presence of 40 mM oxamate, an inhibitor of lactate dehydrogenase (LDH) [21]. Under these conditions the effects of glucose and MPyr were identical with those in the experiments without LDH inhibition. On average, 10 mM glucose elevated NAD(P)H fluorescence by 6.3 ± 1.0 a.u. and 20 mM MPyr decreased autofluorescence by -3.1 ± 0.9 a.u. ($n = 8$; results not shown). To exclude the possibility that the MPyr-induced decrease in NAD(P)H autofluorescence was due to an unspecific interaction, the influence of 20 mM MPyr on the fluorescence of

a 100 μ M NADH solution was tested. The fluorescence remained unchanged ($n = 8$; results not shown).

Effect of MPyr on ATP production by pancreatic islets

To investigate whether 20 mM MPyr influenced mitochondrial ATP production, changes in ATP and ADP content were measured in freshly prepared isolated islets. MPyr increased neither ATP production nor the ATP/ADP ratio (Figure 5). On average, the concentration of ATP was 4.90 ± 0.86 pmol/islet with 0.5 mM glucose and 4.75 ± 0.44 pmol/islet in the presence of 20 mM MPyr after a 30 min incubation period. The ATP/ADP ratios were 1.73 ± 0.07 and 1.89 ± 0.21 respectively ($n = 4$). To ensure that cellular metabolism was intact, islets of each preparation were stimulated with 15 mM glucose. In these control experiments the ATP concentration rose to 8.18 ± 0.99 pmol/islet and the ATP/ADP ratio was 2.77 ± 0.14 ($n = 4$).

Effects of MPyr, pyruvate and glucose on K_{ATP} currents

This series of experiments was intended to reveal whether MPyr has a direct effect on K_{ATP} currents, which could explain the depolarization of the plasma membrane potential evoked by this agent. Figure 6(A) shows that 20 mM MPyr diminished the K_{ATP} current (measured by a 10 mV depolarizing step) from 176 ± 23 to 24 ± 3 pA ($n = 8$) in the standard whole-cell configuration, where the cell interior was dialysed. After wash-out of MPyr, the

Figure 3 Effects of glucose, MPyr and pyruvate on $\Delta\Psi$

(A) Addition of 20 mM MPyr or 10 mM glucose (G) led to a decrease in rhodamine 123 fluorescence, indicating a hyperpolarization of $\Delta\Psi$ due to increased ATP production. Partial depolarizations of $\Delta\Psi$ after the initial hyperpolarization are a consequence of the oscillations in $[Ca^{2+}]_i$ as described previously [2]. (B) In contrast with 10 mM glucose, 20 mM pyruvate (Pyr) induced only a slight hyperpolarization of $\Delta\Psi$. The results are representative of four (MPyr), eight (glucose) and five (pyruvate) experiments with similar results.

Figure 4 Changes in NAD(P)H autofluorescence induced by glucose, pyruvate and MPyr

Whereas glucose (G; 10 mM) and even pyruvate (Pyr; 20 mM) increased NAD(P)H autofluorescence, MPyr (20 mM) diminished it. This points to a stimulation of production of reduction equivalents by the first two substances and to increased use induced by the latter. The experiment is representative of ten (glucose), five (pyruvate) and nine (MPyr) with similar results.

K_{ATP} current first dropped to 9 ± 2 pA ($n = 6$) before it increased again to 147 ± 16 pA ($n = 8$). Figure 6(B) shows that pyruvate slightly diminished the K_{ATP} current from 142 ± 26 to 92 ± 14 pA ($n = 3$). This effect was reversible. As expected, 10 mM glucose had no effect on the K_{ATP} current registered in the standard whole-cell configuration (Figure 6C; $n = 3$).

Figure 7 demonstrates that the effect of MPyr on the K_{ATP} current could, at least in part, be counteracted by diazoxide. On average, the K_{ATP} current in the presence of MPyr was 62 ± 20 pA directly before the addition of $100 \mu\text{M}$ diazoxide, and was increased by diazoxide to 112 ± 21 pA ($n = 5$). This effect was fully reversible. To confirm that MPyr influences K_{ATP} channels directly, inside-out patches of single β -cells were used. Figure 8 shows that K_{ATP} -channel activity was almost completely inhibited

Figure 5 MPyr does not change the ATP/ADP ratio in intact islets

In each experiment, 18–24 islets were incubated for 30 min with 0.5 mM glucose (control) or control solution supplemented with 20 mM MPyr or 15 mM glucose (15 G). Thereafter, concentrations of ATP and ADP were determined luminometrically. Values are means $\pm$ S.E.M. for four experiments with separate preparations.

Figure 6 Effects of MPyr, pyruvate and glucose on K_{ATP} current

K_{ATP} current (I) was monitored in β -cells in the conventional whole-cell mode in the presence of 0.5 mM glucose (0.5 G). The holding potential was -70 mV (solid line), and 300 ms voltage steps to -80 mV and -60 mV (lower and upper broken traces respectively) were applied every 15 s. (A) MPyr (20 mM) strongly inhibited the K_{ATP} current, thus explaining the depolarizing effect of this substance on the cell membrane potential. One representative experiment of eight with similar results is shown. (B, C) Whereas pyruvate (Pyr; 20 mM) decreased the K_{ATP} current slightly, 10 mM glucose (10 G) had no effect. Each experiment is representative of three with similar results.

in the presence of 20 mM MPyr. On average, NP_o was 0.73 ± 0.17 under control conditions and 0.03 ± 0.03 in the presence of 20 mM MPyr ($n = 6$). After wash-out of MPyr, NP_o rose to 0.43 ± 0.21 ($n = 5$). The effect of MPyr was of slow onset, with maximal inhibition after $3:07 \pm 0:46$ min ($n = 6$) (where 3:07 min denotes 3 min 7 s etc.). For comparison, addition of $500 \mu\text{M}$ ATP decreased NP_o from 0.60 ± 0.26 to zero within $0:26 \pm 0:06$ min, which represents the dead-time of the perfusion system ($n = 3$).

Effects of MPyr on single-channel cation currents from mitoplasts of Jurkat T-lymphocytes

MPyr hyperpolarized $\Delta\Psi$, suggesting an increase in ATP production. However, MPyr cannot serve as a mitochondrial sub-

Figure 7 Diazoxide antagonizes the effect of MPyr on the K_{ATP} current

MPyr (20 mM) inhibited the K_{ATP} current (same protocol as in Figure 6). Diazoxide (Diaz; 100 μ M) counteracted this effect. One representative experiment out of five with similar results is shown. 0.5 G, 0.5 mM glucose.

strate, because NAD(P)H autofluorescence was in fact decreased by MPyr. This apparent discrepancy could be explained if MPyr hyperpolarizes mitochondria directly, thereby activating respiratory chains. Inhibition of a cation current across the mitochondrial inner membrane would explain such a hyperpolarization.

In order to test for the presence of ion channels in the mitochondrial inner membrane that might be blocked by MPyr, we recorded single-channel currents from mitoplasts of Jurkat T-lymphocytes. In addition to a small channel with single-channel conductances lower than 30 pS, a larger channel could be

resolved. The current/voltage relationship at positive potentials of this larger channel is shown in Figure 9(A). The slope of the fitted straight line reveals a conductance of 58 pS. In four experiments, exchanging 84 % of the KCl in the bath solution for NaCl decreased the single-channel current to 62 ± 12 %, pointing to a preference of the channel for K^+ ions (Figure 9B). This channel was closed or showed only rare brief openings immediately after patch formation. It became very active after about 5 min, reaching a P_o of 0.94 ± 0.03 , which was decreased to 0.26 ± 0.13 by 20 mM MPyr ($n = 4$; Figures 9C and 9D). The single-channel current amplitude did not change: it was 1.55 ± 0.07 pA under control conditions and 1.60 ± 0.06 pA with MPyr ($n = 4$). In contrast, similar experiments performed with pyruvate only slightly decreased single-channel activity (Figure 9C). On average, in the presence of 20 mM pyruvate, P_o was decreased from 0.86 ± 0.06 to 0.59 ± 0.18 ($n = 3$). This observation is in good agreement with the small effect of 20 mM pyruvate on $\Delta\Psi$ and the whole-cell K_{ATP} current in pancreatic β -cells (see Figures 3B and 6B).

DISCUSSION

Detailed knowledge about the pathways that deliver ATP used for stimulus–secretion coupling in pancreatic β -cells is of tremendous interest, especially since steps preceding the closure of K_{ATP} channels may be disturbed in Type II diabetes mellitus. Agents such as MPyr have been proposed to have therapeutic benefit in the treatment of diabetes associated with a deficiency of hepatocyte nuclear factor-1 α and other forms of diabetes [13]. In contrast with pyruvate, MPyr stimulates insulin secretion

Figure 8 MPyr inhibits K_{ATP} currents in inside-out patches

Experiments were performed with inside-out patches of mouse β -cells at a holding potential of -50 mV. The number of open channels is indicated by the arrowheads on the left; c represents the closed state. The activity of K_{ATP} single-channel currents was reversibly decreased by 0.5 mM ATP and 20 mM MPyr. Representative parts of the recording (indicated by the black horizontal bars) are shown with an extended time scale in the lower part of the figure. One representative experiment out of five (MPyr) and three (ATP) with similar results is shown.

Figure 9 MPyr causes extended closed times of a cation channel in Jurkat T-lymphocyte mitoplasts

(A) Single-channel current/voltage relationship of the larger of two different channels seen in the T-lymphocyte mitoplast attached mode. Values are means $\pm$ S.E.M. for the indicated numbers of experiments (n); error bars are shown only where they exceed symbol size. (B) K^+ -selectivity of the 58 pS channel at +40 mV. The single-channel current in symmetrical KCl solutions on both sides of the membrane was set as 100% (open bar). Substituting 84% of the KCl in the bath solution with NaCl lowered the current to $62 \pm 12\%$ (hatched bar) ($n = 4$). (C) P_o of the 58 pS channel at +40 mV under control conditions (cont; open bars) and with 20 mM MPyr or 20 mM pyruvate (Pyr) (hatched bars; $n = 4$ and $n = 3$ respectively). (D) Patch-clamp current traces at a holding potential of +60 mV showing single-channel events in the mitoplast-attached mode. The closed and open levels of the 58 pS channel are denoted by c and 1 respectively. The channel is open almost continuously under control conditions (top trace). For the middle trace, 20 mM MPyr was added. The bottom trace shows the recovery of the channel after wash-out of MPyr.

[11,12,14]. Interestingly, in this context it has been shown that the ATP production necessary to inhibit the K_{ATP} channels derives solely from reduction equivalents produced in the cytosol during glycolysis, and not in the citrate cycle [6,7]. This might explain why pyruvate is not a primary stimulus (Figure 1; [11,14]). However, the literature about the actions of pyruvate and MPyr on β -cells is very confusing, and many contradictory effects have been reported. For instance, it was found that MPyr and pyruvate are both metabolized to the same extent in intact islets [22], but only MPyr is an effective primary stimulus [11,12,14,23,24]. Other groups have reported that MPyr is only a secondary stimulus, needing at least a subthreshold concentration of glucose for its action [25]. In contrast, the results of Lombert and co-workers [14] and our own data clearly demonstrate that MPyr is able to depolarize the plasma membrane potential in the presence of a very low glucose concentration or even in the absence of the sugar. One of the most important unresolved questions is how MPyr activates β -cells. Some observations support the view that the insulinotropic effect of MPyr can be attributed to mitochondrial ATP production [11–13], assuming that MPyr serves as a substrate for the citrate cycle [8,9]. However, our data (Figure 5) and the results of Lombert and co-workers [14] clearly speak against ATP production induced by MPyr in intact cells. In the present paper we show that MPyr influences mitochondrial function presumably by inhibiting the activity of a mitochondrial ion channel, and not by increasing mitochondrial metabolism. These new data may on the one hand reconcile contradictory results in the literature and on the other

hand encourage the re-interpretation of certain results obtained with MPyr.

One argument in support of the hypothesis that MPyr induces ATP production comes from experiments with α -cyano-4-hydroxycinnamate (α -CHC), an inhibitor of the mitochondrial pyruvate transporter [11]. Mertz et al. [11] showed that the insulinotropic effect of MPyr could not be inhibited by α -CHC, and deduced from this observation that MPyr, in contrast with pyruvate, accumulates in the mitochondria due to its lipophilic nature. Another possibility to explain the lack of effect of α -CHC and pyruvate in intact cells might be the low activity of the membrane monocarboxylate transporter that has been demonstrated for isolated rat islets and insulinoma cells [26]. However, our data clearly show that, in mouse pancreatic β -cells, pyruvate is transported across the plasma membrane, as it affects NAD(P)H autofluorescence as well as $\Delta\Psi$. Furthermore, in isolated mitochondria from *ob/ob* mouse islets, in which pyruvate and MPyr induce ATP synthesis to a similar extent in the presence of malate, the effect of both agents could be prevented by α -CHC [14].

Reports on the effects of MPyr on K_{ATP} channels are also inconsistent. MPyr has been shown to increase an outward current and to abrogate electrical activity [25] in the perforated-patch mode, but the opposite effect, i.e. a complete inhibition of the K_{ATP} current, has also been reported [11,25]. Mertz et al. [11] additionally found that MPyr had no effect on single-channel K_{ATP} currents in inside-out patches of β -cell membranes, suggesting the involvement of metabolism in the action of MPyr.

Moreover, they found that a blocker of respiratory chains, sodium azide, which inhibits cytochrome a_3 , diminished MPyr-induced elevations in $[Ca^{2+}]_c$. Mertz and co-workers [11] deduced from these observations that MPyr acts via 'supranormal' ATP production which inhibits the K_{ATP} current. However, we have recently shown [27] that NaN_3 directly blocks L-type Ca^{2+} channel currents, and therefore the effect of NaN_3 in the experiments of Mertz et al. [11] does not necessarily mean that decreased ATP production counteracts the effect of MPyr on $[Ca^{2+}]_c$. Moreover, we have shown in the present study that MPyr inhibited the K_{ATP} current in the standard whole-cell mode (Figure 6A), where the cytosol is dialysed by the pipette solution and cell metabolism is absent (see Figure 6C), as well as in inside-out patches (Figure 8). These findings are in contrast with the data reported by Mertz et al. [11], who observed no effect of MPyr on single K_{ATP} channels in excised patches. However, in our experiments the effect of MPyr is a clear but slow one that appears after several minutes. As Mertz et al. [11] did not state how long MPyr was administered for prior to the analysed part of their inside-out experiments, the discrepancy may simply be due to different perfusion times. Possibly, MPyr inhibits the channels from the extracellular side and therefore needs some time to accumulate to a sufficient concentration at the outside of an inside-out patch by diffusion across the patch membrane.

Our data demonstrate that MPyr elevated neither the ATP content nor the ATP/ADP ratio in intact islets. These results are in accordance with the observations of Lember et al. [14]. However, and puzzling at a first glance, the present results also demonstrate that MPyr evokes at least a slight or transient increase in ATP production. This is deduced from the fact that MPyr, like glucose [2,19], initially lowered $[Ca^{2+}]_c$ (Figure 2A; [11,24]). This initial decrease is due to increased ATP production, which activates Ca^{2+} pumps in the endoplasmic reticulum. Consequently this initial decline in $[Ca^{2+}]_c$ is inhibited by thapsigargin (Figure 2B). In addition, we found that MPyr hyperpolarized $\Delta\Psi$ (Figure 3), which can be taken as a measure of ATP production under certain circumstances [1,2]. When β -cells are stimulated by metabolic substrates (e.g. glucose), the hyperpolarization of $\Delta\Psi$ is a result of the increased production of reduction equivalents (NADH, FADH) (Figure 4), which in turn activates respiratory chains. The proton-pumping activity of the respiratory chains produces an electrochemical H^+ gradient across the mitochondrial inner membrane which is used for ATP synthesis by the F_0/F_1 -ATPase. However, in contrast with glucose, MPyr does not increase reduction equivalents, which is demonstrated by the observations that it did not increase the ATP/ADP ratio (Figure 5), ATP production or NAD(P)H autofluorescence. In fact, MPyr lowered NAD(P)H autofluorescence (Figure 4). In combination with the observed hyperpolarization of $\Delta\Psi$, this nevertheless points to an increase in the activity of respiratory chains [2]. Thus glucose leads to the production of reduction equivalents and an increase in NAD(P)H autofluorescence, resulting in the hyperpolarization of $\Delta\Psi$. In contrast, MPyr causes the hyperpolarization of $\Delta\Psi$, possibly by inhibition of a mitochondrial cation current (see below), and the increased consumption of reduction equivalents is the consequence of this hyperpolarization. Both sequences of events result in augmented ATP synthesis; however, in the case of MPyr this increase would be small and probably transient, because of the limited amount of reduction equivalents. This is the most likely reason why it is not detectable in a static incubation period of 30 min in intact islets (Figure 5 and [14]). Another possibility to explain the decrease in NAD(P)H fluorescence induced by MPyr could be that changes in the generation of mitochondrial reduction equivalents were compensated for by increased turn-

over of pyruvate into lactate induced by MPyr. However, this is rather unlikely, as it has been shown that LDH activity is extremely low in pancreatic β -cells [28,29]. In addition, our experiments with oxamate, which has been shown to inhibit lactate formation in intact cells [21], clearly demonstrate that the effect of MPyr is not prevented by inhibition of LDH.

The MPyr-induced hyperpolarization of $\Delta\Psi$ could be the result of inhibition of a depolarizing cation current across the mitochondrial inner membrane. Figure 9 shows that MPyr indeed partially inhibited such a current in mitoplasts of Jurkat T-lymphocytes. Since MPyr directly inhibited the K_{ATP} current in the plasma membrane of β -cells (Figures 6 and 8), the mitochondrial K_{ATP} current, which has been found in different cell types [30–32], may be a good candidate for a mitochondrial depolarizing current inhibitable by MPyr. Indeed the current shown here is sensitive to ATP (D. Siemen, unpublished work). However, the exact nature and properties of this current remain to be examined in further studies, and are beyond the scope of this investigation. The mitochondrial K_{ATP} channel of rat liver mitochondria has been reported to have a five times lower conductance [30] than the channel presented here; however, this may be due to different K^+ concentrations. Although the existence of a mitochondrial K_{ATP} channel in β -cell mitochondria has been proposed recently by Lember et al. [33], no direct proof for the existence of K_{ATP} channels in β -cell mitochondria has been published so far. An influence of diazoxide and tolbutamide on β -cell $\Delta\Psi$ has been reported [34,35]. However, these experiments do not really support the existence of mitochondrial K_{ATP} channels in β -cells, because both agents depolarized $\Delta\Psi$.

In conclusion, we have shown here that MPyr does not function as a mitochondrial substrate in pancreatic β -cells, since it did not increase the concentration of reduction equivalents. Nevertheless, MPyr seems to provoke a slight or transient increase in ATP production via hyperpolarization of the mitochondria. This hyperpolarization could be brought about by inhibition of a depolarizing current across the mitochondrial inner membrane. Thus ATP production induced by MPyr is an epiphenomenon caused by the effect of this agent on the mitochondria, but is not the reason for the stimulation of pancreatic β -cells. We conclude that MPyr exerts its insulinotropic effect via direct inhibition of K_{ATP} channels located in the plasma membrane.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Dr 225/6-1), the Deutsche Diabetesgesellschaft (to G.D.), the BMBF and the Deutsche Parkinsonvereinigung e.V. (to D.S.).

REFERENCES

- 1 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.
- 2 Krippeit-Drews, P., Düfer, M. and Drews, G. (2000) Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells. *Biochem. Biophys. Res. Commun.* **267**, 179–183.
- 3 Ashcroft, F. M. and Rorsman, P. (1989) Electrophysiology of the pancreatic β -cell. *Prog. Biophys. Mol. Biol.* **54**, 87–143.
- 4 Zhou, Z. and Misler, S. (1996) Amperometric detection of quantal secretion from patch-clamped rat pancreatic β -cells. *J. Biol. Chem.* **270**, 270–277.
- 5 Maechler, P. and Wollheim, C. B. (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature (London)* **402**, 685–689.
- 6 Dukes, I. D., McIntyre, M. S., Mertz, R. J., Philipson, L. H., Roe, M. W., Spencer, B. and Worley, III, J. F. (1994) Dependence on NADH produced during glycolysis for β -cell glucose signaling. *J. Biol. Chem.* **269**, 10979–10982.
- 7 Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T. et al. (1999) Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science* **283**, 981–985.

- 8 Sener, A., Kawazu, S., Hutton, J. C., Boschero, A. C., Devis, G., Somers, G., Herchuelz, A. and Malaisse, W. J. (1978) The stimulus-secretion coupling of glucose-induced insulin release. Effect of exogenous pyruvate on islet function. *Biochem. J.* **176**, 217–232
- 9 Lenzen, S. (1979) Effects of α -ketocarboxylic acids and 4-pentenoic acid on insulin secretion from the perfused rat pancreas. *Biochem. Pharmacol.* **27**, 1321–1324
- 10 Leclercq-Meyer, V., Garcia-Martinez, J. A., Villanueva-Penacarrillo, M. L., Valverde, I. and Malaisse, W. J. (1995) In vitro and in vivo insulinotropic action of methyl pyruvate. *Horm. Metab. Res.* **27**, 477–481
- 11 Mertz, R. J., Jennings, F. W., 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. *J. Biol. Chem.* **271**, 4838–4845
- 12 Zawalich, W. S. and Zawalich, K. C. (1997) Influence of pyruvic acid methyl ester on rat pancreatic islets. *J. Biol. Chem.* **272**, 3527–3531
- 13 Dukes, I. D., Sreenan, S., Roe, M. W., Levisetti, M., Zhou, Y. P., Ostrega, D., Bell, G. I., Pontoglio, M., Yaniv, M., Philipson, L. and Polonsky, K. S. (1998) Defective pancreatic β -cell glycolytic signaling in hepatocyte nuclear factor-1 α -deficient mice. *J. Biol. Chem.* **273**, 24457–24464
- 14 Lambert, N., Joos, H. C., Idahl, L. A., Ammon, H. P. T. and Wahl, M. A. (2001) Methyl pyruvate initiates membrane depolarization and insulin release by metabolic factors other than ATP. *Biochem. J.* **354**, 345–350
- 15 Plant, T. D. (1988) Properties and calcium-dependent inactivation of calcium currents in cultured mouse pancreatic B-cells. *J. Physiol. (Cambridge, U.K.)* **404**, 731–747
- 16 Siemen, D., Loupatatzis, C., Borecky, J., Gulbins, E. and Lang, F. (1999) Ca^{2+} -activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochem. Biophys. Res. Commun.* **257**, 549–554
- 17 Garrino, M. G., Plant, T. D. and Henquin, J. C. (1989) Effects of putative activators of K^{+} channel in mouse pancreatic β -cells. *Br. J. Pharmacol.* **98**, 957–965
- 18 Gryniewicz, 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
- 19 Grapengiesser, E., Gylfe, E. and Hellman, B. (1988) Dual effect of glucose on cytoplasmic Ca^{2+} in single pancreatic β -cells. *Biochem. Biophys. Res. Commun.* **150**, 419–425
- 20 Drews, G., Krämer, C., Düfer, M. and Krippeit-Drews, P. (2000) Contrasting effects of alloxan on islets and single mouse pancreatic β -cells. *Biochem. J.* **352**, 389–397
- 21 Coe, E. L. and Strunk, R. C. (1970) The effect of oxamate on glycolysis in intact ascites tumor cells. *Biochim. Biophys. Acta* **208**, 189–202
- 22 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
- 23 Schöffl, C., Börger, J., Lange, S., von zur Mühlen, A. and Brabant, G. (2000) Energetic requirement of carbachol-induced Ca^{2+} signalling in single mouse β -cells. *Endocrinology* **141**, 4065–4071
- 24 Lenzen, S., Lerch, M., Peckmann, T. and Tiedge, M. (2000) Differential regulation of $[Ca^{2+}]_i$ oscillations in mouse pancreatic islets by glucose, α -ketoisocaproic acid, glyceraldehyde and glycolytic intermediates. *Biochim. Biophys. Acta* **1523**, 65–72
- 25 Malaisse, W. J., Jijakli, H., Ulusoy, S., Cook, L., Best, L., Viñambres, C., Villanueva-Penacarrillo, M. L., Valverde, I. and Sener, A. (1996) Insulinotropic action of methyl pyruvate: Secretory, cationic, and biochemical aspects. *Arch. Biochem. Biophys.* **335**, 229–244
- 26 Ishihara, H., Wang, H., Drewes, L. R. and Wollheim, C. B. (1999) Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory response to pyruvate and lactate in β -cells. *J. Clin. Invest.* **104**, 1621–1629
- 27 Düfer, M., Krippeit-Drews, P. and Drews, G. (2002) Inhibition of mitochondrial function affects cellular Ca^{2+} handling in pancreatic B-cells. *Pflügers Arch. Eur. J. Physiol.* **444**, 236–243
- 28 Sekine, N., Cirulli, V., Regazzi, R., Brown, L. J., Gine, E., Tamarit-Rodriguez, J., Girotti, M., Marie, S., MacDonald, M. J., Wollheim, C. B. et al. (1994) Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic β -cells. Potential role in nutrient sensing. *J. Biol. Chem.* **269**, 4895–4902
- 29 MacDonald, M. J., Kelley, P. C. and Laclau, M. (2002) Histochemical evidence for pathways insulin secreting cells use to oxidize glycolysis-derived NADH. *Metab. Clin. Exp.* **51**, 318–321
- 30 Inoue, I., Nagase, H., Kishi, K. and Higuti, T. (1991) ATP-sensitive K^{+} channel in the mitochondrial inner membrane. *Nature (London)* **352**, 244–247
- 31 Bajgar, R., Seetharaman, S., Kowaltowski, A. J., Garlid, K. D. and Paucek, P. (2001) Identification and properties of a novel intracellular (mitochondrial) ATP-sensitive potassium channel in brain. *J. Biol. Chem.* **276**, 33369–33374
- 32 Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G. and Garlid, K. D. (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^{+} channel from rat liver and beef heart mitochondria. *J. Biol. Chem.* **265**, 26062–26069
- 33 Lambert, N., Idahl, L. A. and Ammon, H. P. T. (2001) Diazoxide inhibits ATP production in isolated β -cell mitochondria only in the presence of extramitochondrial Mg-nucleotides. *Diabetologia* **44** (Suppl. 1), A128 (Abstract)
- 34 Grimmsmann, T. and Rustenbeck, I. (1998) Direct effects of diazoxide on mitochondria in pancreatic β -cells and on isolated liver mitochondria. *Br. J. Pharmacol.* **123**, 781–788
- 35 Smith, P. A., Proks, P. and Moorhouse, A. (1999) Direct effects of tolbutamide on mitochondrial function, intracellular Ca^{2+} and exocytosis in pancreatic β -cells. *Pflügers Arch. Eur. J. Physiol.* **437**, 577–588

Received 24 April 2002/12 September 2002; accepted 27 September 2002

Published as BJ Immediate Publication 27 September 2002, DOI 10.1042/BJ20020657