Anaplerosis via pyruvate carboxylase is required for the fuel-induced rise in the ATP:ADP ratio in rat pancreatic islets

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ABSTRACT

Aims/hypothesis: The molecular mechanisms of insulin release are only partially known. Among putative factors for coupling glucose metabolism to insulin secretion, anaplerosis has lately received strong support. The anaplerotic enzyme pyruvate carboxylase is highly expressed in beta-cells, and anaplerosis influences insulin secretion in beta-cells. By inhibiting pyruvate carboxylase in rat islets, we aimed to clarify the hitherto unknown metabolic events underlying anaplerotic regulation of insulin secretion. *Methods*: Phenylacetic acid (5 mmol/l) was used to inhibit pyruvate carboxylase in isolated rat islets, which were then assessed for insulin secretion, fuel oxidation, ATP:ADP ratio, respiration, mitochondrial membrane potential, exocytosis and K_{ATP}-channel conductance. *Results*: We found that the glucose-provoked rise in ATP:ADP ratio was suppressed by inhibition of pyruvate carboxylase. In contrast, fuel oxidation, respiration, mitochondrial membrane potential, as well as Ca^{2+}-induced exocytosis and K_{ATP}-channel conductance in single cells were unaffected. α-ketoisocaproic acid -induced insulin secretion was suppressed., whereas methyl succinate-stimulated secretion remained unchanged. Perifusion of rat islets revealed that inhibition of anaplerosis decreased the second phase of insulin secretion, during which K_{ATP}-independent actions of fuel secretagogues are operational, as well as the first and K_{ATP}-dependent phase. *Conclusion/interpretation*: Our results are consistent with a model in which anaplerosis via pyruvate carboxylase determines pyruvate cycling, which previously has been shown to correlate with glucose responsiveness in clonal beta-cells. These processes, controlled by pyruvate carboxylase, seem crucial for generation of an appropriate ATP:ADP ratio, which may regulate both phases of fuel-induced insulin secretion.

Keywords: Insulin secretion, mitochondria, type 2 diabetes

Abbreviations: α-KIC: α-ketoisocaproic acid, BCH: 2-amino-2-norbornane-carboxylic acid HBSS: HEPES balanced salt solution, GDH: glutamate dehydrogenase, GSIS: glucose stimulated insulin secretion, K_{ATP}-channels: ATP-sensitive K^+ channels, MTS: 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, PAA: phenylacetic acid, PC: pyruvate carboxylase, PDH: pyruvate dehydrogenase, TCA: tricarboxylic acid

INTRODUCTION

Type 2 diabetes is characterized by dysregulated insulin secretion [1], which is manifested as an inadequate secretory response to glucose. The stimulus-secretion coupling of glucose in beta-cells involves at least two different pathways. In the well defined triggering or K_{ATP}-dependent pathway, glucose metabolism increases the ATP:ADP ratio, closing the ATP-sensitive K^+ (K_{ATP}) channels. This depolarizes the plasma membrane, voltage-dependent Ca^{2+}-channels open, and cytosolic Ca^{2+}-concentrations $[Ca^{2+}]_i$ increase [2], triggering exocytosis. The less well-characterized amplifying or K_{ATP}-independent pathway is also dependent on the rise in cytosolic Ca^{2+}, but does not necessarily involve the K_{ATP}-channels, since glucose stimulates insulin secretion in a dose-dependent manner also when these channels are bypassed [3, 4]. The K_{ATP}-dependent pathway has been suggested to account for the rapid first phase, in which a readily releasable pool of insulin granules is exocytosed. The slower, sustained, second phase requires metabolism of glucose and, presumably, the K_{ATP}-independent pathway acts on another pool of granules, converting them to a state in which they become readily releasable [5, 6].

The mechanisms underlying the K_{ATP}-independent pathway are poorly defined, and there are several hypotheses regarding the identity of the factors coupling glucose metabolism to insulin secretion. Glutamate [7], malonyl-CoA [8], ATP [9], and NADPH [10] are among the proposed signals, all of which, however, are subject to considerable controversy. Nonetheless, a unifying aspect of these candidates for metabolic coupling in beta-cells is anaplerosis, i.e. the filling-up of carbon intermediates into the TCA cycle [10, 11]. The anaplerotic enzyme pyruvate carboxylase (PC) is highly expressed in beta-cells as compared to islet non-beta-cells [11], and ~ 40% of pyruvate entering the TCA cycle during glucose stimulation is carboxylated by PC [12], indicating a critical role of the enzyme. indeed, inhibition of PC with phenylacetic acid (PAA) [13] decreases insulin secretion in INS-1 cells,

832/13 cells, and rat islets [14-16]. PAA is metabolized to phenylacetyl-CoA intracellularly, and inhibits PC by competing with acetyl-CoA, a key regulator of the enzyme [17].

The aim of the present study was to investigate the metabolic events behind these observations, addressing the role of PC-mediated anaplerosis for insulin secretion. We used PAA as a tool to inhibit PC and anaplerosis in rat islets, and examined the consequences for insulin secretion and islet metabolism. We found that PC, and thereby anaplerosis, is crucial for an appropriate rise in the ATP:ADP ratio, in response to fuel metabolism, a rise thought to trigger and sustain insulin secretion.

EXPERIMENTAL PROCEDURES

Reagents and animals. All chemicals were from Sigma (St. Louis, MO, USA) if not stated otherwise. Female Sprague-Dawley rats (180-250g; B&K, Stockholm, Sweden) were used for the experiments. The studies were approved by the Regional Animal Ethics Committee in Lund, Sweden.

Islet isolations, batch incubations and islet perifusions. Pancreatic islets from fed rats were isolated by standard collagenase digestion [18] , and subsequently handpicked under a stereo microscope. All experiments were conducted on freshly isolated islets if not stated otherwise. For culture overnight, the isolated islets were kept in RPMI-1640 medium, containing 11.1 mmol/l glucose, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in 95% air and 5% CO_2. For secretion studies, batches (n=8) of 3 islets for each condition were kept in HEPES balanced salt solution (HBSS; in mmol/l: 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 20 HEPES, 1.3 $CaCl_2$, 0.1% BSA; pH 7.35) containing 2.8 mmol/l glucose for 60 min at 37°C. The islets were then incubated in 200 μl buffer combined with the respective secretagogue and inhibitor at 37°C for an additional hour. Afterwards, a sample from the buffer was measured for insulin by ELISA (Mercodia, Uppsala, Sweden). Perifusion of islets was performed as previously described [19]. In brief, islets were pre-incubated in HBSS at 2.8 mmol/l glucose for 60 min. Then, batches of 20 islets were sandwiched between two layers of gel (Bio-Gel P4, Bio-Rad, Richmond, CA, USA). The HBSS was warmed to 37°C. Islets were perifused at a flow rate of 0.5 ml/min, fractions were collected at 1 min intervals, and insulin in the perifusate was measured by ELISA.

ATP and ADP determinations. Isolated islets were cultured overnight followed by 2 h incubation in HBSS containing 16.7 mmol/l glucose, 2.5 µmol/l forskolin, and 35 mmol/l KCl. These measures were taken to degranulate beta-cells, depleting them of ATP contained in insulin granules, which obscures changes in ATP generated by metabolism [20]. Islets were washed in HBSS, followed by preincubation for 1 h in HBSS at 2.8 mmol/l glucose; the buffer was then replaced by HBSS containing 2.8 or 16.7 mmol/l glucose, or 2.8 mmol/l glucose and 10 mmol/l methyl-succinate. After an additional 5 or 30 minutes, trichloroacetic acid was added at a final concentration of 2% to the islets, which were kept on ice for 20 min. After centrifugation (13000 g), the supernatant was assayed for ATP content using bioluminescence (BioThema, Haninge, Sweden). ADP was measured after ATP had been depleted by ATP sulfurylase followed by conversion of ADP to ATP by pyruvate kinase, as described in detail [21].

Fuel oxidation. Isolated islets were kept in HBSS containing 2.8 mmol/l glucose for 60 min at 37°C. Batches of islets (n=20) were transferred to a cup (Kimble-Kontes, Vineland, NJ, USA) suspended from a rubber sleeve stopper (Fisher, Pittsburgh, PA, USA), which was inserted into a glass scintillation vial. For glucose oxidation, a 50 µl reaction mixture containing 13 kBq D-[14C(U)]glucose (NEN Life Science Products, Boston, MA, USA) and glucose at a final concentration of 2.8 or 16.7 mmol/l was added to the cups and the vials were sealed. For palmitate oxidation, a 100 µl reaction mixture consisting of 0.5 mmol/l palmitic acid complexed to 1% BSA (essentially fatty acid-free), with approximately 9.2 kBq [1-14C]palmitic acid (NEN) as tracer, 0.8 mmol/l Lcarnitine, and glucose, at a final concentration of 2.8 or 16.7 mmol/l, was added. For glutamine oxidation, a 50 µl reaction mixture containing 3.7 kBq L-[14C(U)]glutamine (PerkinElmer, Life Sciences, Boston, MA,

USA), 10 mmol/l glutamine and 10 mmol/l 2-amino-2-norbornane- carboxylic acid was added. The reaction was terminated after 1 h by injection of 100 µl 10% trichloroacetic acid into the suspended cup. The oxidation rate was measured as 14CO2 trapped in 300 µl benzethonium hydroxide added to the bottom of the sealed vials, followed by an additional incubation overnight at room temperature. 14CO2 was determined by scintillation counting.

MTS assay. Isolated islets were kept in HBSS containing 2.8 mmol/l glucose for 60 min at 37°C. Batches of islets (n=10) were transferred to a 96-well plate containing 100 µl HBSS and 20 µl [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-(MTS) tetrazolium compound (Promega, WI, USA) and 2.8 or 16.7 mmol/l glucose. After incubation for 2 h at 37°C, the absorbance was measured at 490 nm.

Oxygen consumption. Oxygen consumption rates were monitored with an oxygen electrode (Hansatech, Cambridge, UK). Islets were kept in HBSS containing 2.8 mmol/l glucose for 30 min at 37°C. Thereafter 100 islets were added to a magnetically stirred oxygen electrode chamber thermostated to 37°C containing 300 µl HBSS. Oxygen consumption was measured during 4 minutes at 2.8mmol/l glucose, 16.7 mmol/l glucose and 16.7 mmol/l glucose with 5mmol/l PAA.

Mitochondrial membrane potential.

Mitochondrial membrane potential was measured by Rh123 fluorescence [22]. Isolated islets were incubated with 13 µmol/l Rh123 (Sigma-Aldrich, Sweden) for 30-60 min in Hanks' balanced salt solution (Sigma-Aldrich, Sweden), supplemented with 4.1 mmol/l $NaHCO_3$. Upon investigation, a single islet was transferred to a separate glass-bottomed dish containing 1000 µl Hanks' balanced salt solution and placed on the heated and gas-equilibrated (37°C,

95% air-5% CO_2) microscope stage (Carl Zeiss, Göttingen, Germany). Time-lapse images were recorded every 20 s. Rh123 fluorescence was excited using the 488 nm line of a krypton-argon laser of a Zeiss LSM 510 META confocal microscope, and emitted light was collected via a Zeiss Plan-APOCHROMAT x10 objective using a band-pass 526 to 537 nm filter. The pinhole was maximized, resulting in a confocal section thickness of 140 μm. Rh123 fluorescence intensity was measured from the entire islet for each image frame. The osmolarity of the solutions were kept constant throughout the experiment.

Electrophysiology. Measurements in dispersed islet cells were conducted using an EPC-10 patch-clamp amplifier in conjunction with the Pulse software suite (version 8.53; HEKA Elektronik, Lambrecht/Pfalz, Germany). Exocytosis was monitored as increases in cell capacitance using the sine+DC mode of the lock-in amplifier included in the Pulse software suite. The experiments were made in the standard whole-cell configuration patch in which the pipette solution dialyses the cell and replaces the cytosol. The pipette solution consisted of (in mmol/l) 125 K-glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 0.1 cAMP, 10 EGTA and 9 $CaCl_2$ (pH 7.2 with KOH). The resulting free intracellular Ca^{2+} concentration of this Ca^{2+} /EGTA buffer was estimated to 1.5 μM using the binding constants of Martell and Smith [23]. The extracellular solution contained (in mmol/l) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose, and 5 HEPES with 5 PAA added as indicated (pH 7.4 with NaOH). The bath (~1.5 ml) was continuously perfused (6 ml/min) and temperature maintained at ~34^{o}C. Effects of PAA on K_{ATP} channel conductance were measured using the standard and perforated-patch whole-cell configuration of the patch clamp technique. The same extracellular buffer as above was used. In standard whole-cell experiments the pipette solution (=intracellular) consisted of (in mmol/l) 125 K^+-glutamate, 10 KCl, 10 NaCl,

1 $MgCl_2$, 5 HEPES, 1 Mg-ATP, 3 Mg-ADP with pH 7.2 (KOH). In the perforated-patch mode the pipette solution contained 76 mmol/l K_2SO_4.

Statistical analysis. Student's *t* test was used for comparision of two groups. When multiple groups were compared, analysis of variance (ANOVA) was performed followed by Bonferroni's test *post hoc*.

RESULTS

Static incubation of isolated islets

It has previously been shown that PAA decreases insulin secretion in INS-1 cells and rat islets [14]. To confirm this model for inhibition of PC and insulin secretion, we performed secretion studies in static incubations with or without PAA (fig. 1). Upon an increase in glucose from 2.8 to 16.7 mmol/l, insulin secretion rose nine-fold. In the presence of 5 mmol/l PAA insulin secretion at 16.7 mmol/l glucose was decreased by 50%.

To further define the role of PC in glucose-stimulated insulin secretion (GSIS), we studied secretion under conditions where the K_{ATP}-dependent pathway was bypassed using 35 mmol/l KCl and 250 μM diazoxide. Here, insulin secretion was stimulated 11-fold by the rise in glucose; again, insulin secretion at 16.7 mmol/l glucose was decreased by 50% when PAA was added. These results show that the actions of PC and anaplerosis involve K_{ATP} - independent processes.

According to a recent study, activation of glutamate dehydrogenase (GDH), is sufficient to fully activate the K_{ATP}-independent pathway of insulin secretion [24]. Therefore we also investigated the effect of PAA on insulin secretion stimulated by non-glucidic fuels. Stimulation of islets with 10 mmol/l α-ketoisocaproic acid (α-KIC), which is transaminated to leucine activating GDH, increased insulin secretion 14-fold at 2.8 mmol/l glucose compared to 2.8 mmol/l glucose alone (Fig. 2A). Addition of PAA decreased α-KIC-induced insulin secretion by 47% ($P < 0.01$). Similar results were obtained when leucine was used (data not shown). Succinate is another widely used anaplerotic insulin secretagogue. 10 mmol/l mono-methyl succinate, which is converted into succinate intracellularly, provoked a six-fold increase in insulin secretion (Fig. 2B). Notably, this increase remained unaffected by PAA.

Perifusion of islets

To obtain information about the dynamics of insulin secretion regarding the two phases, we analyzed insulin secretion of islets in a perifusion system. Islets were perifused for 10 min with 2.8 mmol/l, followed by 30 min with 16.7 mmol/l glucose, in the presence or absence of PAA. Then the glucose concentration was lowered to 2.8 mmol/l. As shown in fig. 3A, this stimulated a clear biphasic secretion pattern in the control islets with a rapid increase in insulin secretion followed by a nadir and then a gradually increasing rate of secretion reaching a plateau after 20 min.When PAA was added, insulin secretion rose to a level that was markedly lower, and no clear first or second phase was distinguished. PAA reduced insulin secretion significantly during both the first phase (1-10 min after elevated glucose) ($P<0.01$), and the second phase (11-30 min) ($P<0.01$).

ATP:ADP measurement

Since the increase in ATP:ADP ratio is the generally accepted trigger of GSIS and responsible for initiation of the first phase of insulin secretion, we next examined the effects of PAA on adenine nucleotide levels. Measurements of ATP and ADP were performed after a 5 or 30 minute incubation in 16.7 mmol/l glucose (table 1-3). After 5 min, the ATP:ADP ratio increased significantly ($P< 0.01$) when the concentration of glucose was elevated from 2.8 to 16.7 mmol/l glucose; this increase was substantially decreased by PAA ($P<0.05$). ATP-levels increased significantly when glucose concentrations were elevated ($P<0.05$), whereas ADP-levels decreased ($P<0.01$). In PAA-treated islets, ATP-levels were not significantly increased in response to glucose, and ADP-levels were consistently higher than in the control islets ($P < 0.05$). Similar changes were observed after 30 minutes (Table 2).Thus, anaplerosis

appears to be essential for a sustained glucose-induced rise in the ATP:ADP ratio that may both trigger and amplify insulin secretion.

Since PAA was without effect on succinate-stimulated insulin secretion we also investigated the impact of PAA on nucleotide levels in succinate-stimulated islets. Like glucose, methyl succinate induced an increase in the ATP:ADP ratio (table 3). Again, this increase was abolished by PAA.

Fuel oxidation studies

A low ATP:ADP ratio could reflect an overall reduction of beta-cell metabolism and a disturbance of the TCA-cycle. Thus, to investigate the influence of PC inhibition on beta-cell metabolism, we performed oxidation measurements in the presence and absence of PAA. Glucose oxidation increased six-fold when the glucose concentration was increased from 2.8 to 16.7 mmol/l (0.68 ±0.14 vs. 4.5 ± 0.76 pmol . islet-1. h-1). Addition of PAA did not affect the oxidation of glucose at either 2.8 or 16.7 mmol/l (1.22 ± 0.55 pmol . islet-1. h-1 vs. 3.83 ± 0.83 pmol . islet-1. h-1). This indicates that the replenishment of metabolites via PC into the TCA-cycle and the oxidation of acetyl-CoA to CO_2 are separated events. Also, the oxidation of glutamine, was unaffected by PAA (6.32 ± 0.96 in control islets vs. 6.76 ± 0.87 pmol . islet-1. h-1 in PAA-treated islets). In both control and PAA-treated islets, palmitate oxidation was reduced by an increase in glucose from 2.8 to 16.7 mmol/l. However, at neither low nor high glucose was there any significant difference in the rate of palmitate oxidation between the two groups (3.42 ± 0.57 vs. 2.55 ± 0.58 pmol . islet-1 . h-1 at 2.8 mmol/l glucose and 1.85 ± 0.37 vs. 1.52 ± 0.36 pmol . islet-1. h-1 at 16.7 mmol/l glucose). Together, these data indicate that fuel oxidation in beta-cell mitochondria is undisturbed when anaplerosis is inhibited. Furthermore, PAA has not depleted the cell of CoA, since oxidation of the lipid which requires this co-factor was unaffected.

MTS assay

To further verify that the treatment with PAA has no adverse effects on cell metabolism, we incubated islets in presence of MTS. This agent is reduced to coloured formazan, requiring NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. It is thus an indicator of overall mitochondrial metabolism. Again, no difference was seen between PAA-treated and control islets, neither at low nor high glucose concentrations (101% vs. 90% of control), ensuring that the cells were viable with metabolism intact.

Oxygen consumption measurements

To ascertain whether PAA has any influence on the electron transport chain *per se*, we performed oxygen consumption measurements (fig. 4). Oxygen consumption increased by 43 % ($P<0.01$) when the glucose concentration was changed from 2.8 to 16.7 mmol/l. When PAA was added to the buffer, there was no change in respiration. Hence, we can conclude that while PAA specifically inhibits PC and consequently anaplerosis, there appears to be no general effect on metabolism in beta-cells.

Mitochondrial membrane potential

If PAA acts as an uncoupler, then hyperpolarisation of the mitochondrial membrane will be reduced. To address this possibility, the mitochondrial membrane potential was assessed using the potentiometric dye Rh123 (13 μmol/l). Raising the glucose concentration from 1 to 20 mM resulted in mitochondrial hyperpolarisation, as evidenced by the average 5.8 +/- 0.4 % decrease in Rh123 fluorescence measured after 1-2 min at steady-state. When 5 mmol/l PAA was added to the islets either 5 min before, or concomitantly with, the sugar, Rh123 fluorescence decreased by 5.9 +/- 1.1 % (ns; n=10 islets for both conditions).

Electrophysiology

To verify that PAA has no effect on the exocytotic machinery *per se*, we performed single cell capacitance measurements where ATP was added in the pipette solution. Ca^{2+}-elicited exocytosis in beta-cells was monitored as increases in whole-cell membrane capacitance in the whole-cell configuration. Exocytosis commenced immediately after establishment of the whole-cell configuration at t=0, and remained unaffected in the presence of PAA (5 mmol/l) in the extracellular solution (25±7 and 27±7 fF/s, n=10 and 11, with or without PAA, respectively; Fig. 5A-B). Thus, PAA appears to exert no direct effects on exocytosis.

Next, measurements of K_{ATP}-channel conductance assessed the effect of PAA on the K_{ATP}-channel activity. In intact cells (perforated-patch configuration) the addition of 5 mmol/l PAA increased the K_{ATP} channel conductance (G) from 5.3±0.3 to 7.8±0.3 nS (5 min after addition; $P<0.01$, n=4). The effect was reversible as (G) returned to 5.1±0.2 nS upon wash-out ($P<0.01$). In the standard whole-cell experiments, where the intracellular ATP:ADP ratio was clamped at 1:3, (G) remained unaffected and averaged 12.7±2.0, 12.2±1.2 and 12.6±1.8, before, 3 min after addition, as well as after wash-out of the acid, respectively.. Taken together, the data indicate that PAA exerts no direct effect on K_{ATP} channel activity, and that the observed effect of PAA requires a process in intact cell.

DISCUSSION

It has been recognized that anaplerosis plays an important role in the regulation of insulin secretion. Despite this realization, little is known about the precise mechanisms of this control. We show here that anaplerosis via PC is required for an appropriate rise in the ATP:ADP ratio induced by glucose, a rise thought to trigger insulin secretion.

Previous studies have shown that glucose-induced insulin secretion is decreased when the anaplerotic key enzyme PC is inhibited by PAA [14]. This was believed mainly to affect the second phase of insulin secretion, which requires activation of the K_{ATP}-independent pathway in addition to K_{ATP} channel closure. Here, we have extended these studies and found that inhibition of PC decreased both the K_{ATP}-dependent and K_{ATP}-independent components of insulin secretion. In agreement with these observations, when examining insulin secretion in a dynamic perifusion system, we found that both phases of insulin secretion were inhibited by PAA. This indicates that anaplerosis is essential not only for sustained insulin secretion during the second phase, but also for initiating the K_{ATP}-dependent first phase of secretion. Our investigations of the effect of PAA on the K_{ATP} channels agree with this suggestion. In the perforated-patch configuration, where metabolism presumably is unperturbed, PAA increased K_{ATP} channel activity. In contrast, K_{ATP} channel conductance in disrupted cells (standard whole-cell configuration), with a clamped ATP:ADP ratio, was unaffected by PAA. Collectively, this indicates that inhibition of PC influences metabolism, where the most apparent candidate is the ATP:ADP ratio. An increased K_{ATP} channel activity could be a cause of the lacking first phase, because the closure of the K_{ATP} channels is the generally accepted trigger of first phase insulin secretion. Increased K_{ATP} channel activity is not a likely explanation of the abrogated second phase, because PAA decreased insulin secretion also under K_{ATP}-independent conditions. However, adenine

nucleotides may play a critical role in the second and K_{ATP}-independent phase via other mechanisms. This is supported by our findings that the ATP:ADP ratio was decreased also 30 minutes after a rise in glucose concentration, when the K_{ATP}-independent pathway is thought to be operating. The hypothesis was originally based on the ability of glucose to increase the ATP:ADP ratio in a concentration-dependent manner [9, 25]. Moreover, the priming of insulin granules, which modifies them into a readily releasable state, is ATP-dependent, whereas exocytosis of the immediately releasable pool is not [5]. While priming mechanisms are still to be elucidated, they may be attributed to the K_{ATP}-independent pathway.

An increased ATP:ADP ratio depends on glucose metabolism [26], and anaplerosis may be the pivotal regulator. We found that the glucose-induced rise in ATP was absent when anaplerosis was inhibited; hence our results show that influx of carbon intermediates into the TCA-cycle is critical for appropriate ATP production in beta-cells. Although the underlying mechanisms have yet to be resolved, pyruvate cycling may explain the regulatory role of anaplerosis via PC [16]. The model is based on the existence of two pools of pyruvate in beta-cells, one feeding acetyl-CoA into the TCA-cycle through pyruvate dehydrogenase (PDH), and one exchanging with TCA-cycle intermediates via PC [16]. In clonal beta-cells, only the latter correlates with glucose responsiveness and insulin secretion [16]. Pyruvate cycling will generate flux through either the pyruvate-malate [10] or pyruvate-citrate shuttles [14, 27], both of which have been proposed to generate coupling signals. Supportive evidence for this hypothesis is that inhibition of PC by PAA decreases pyruvate cycling and GSIS in parallel [16]. This agrees with the fact that we observed no effect of PC-inhibition on glucose oxidation, while insulin secretion was markedly decreased. This model and our current results indicate that PC and PDH are acting on different pools of pyruvate, and that these processes are separate from each other. The decrease of α-KIC-stimulated insulin secretion when PC was inhibited suggests that α-KIC or leucine exerted some of its effects on insulin secretion

via PC. In line with this, leucine provokes an increased PC-mediated anaplerotic flux in beta-cells, whereas the flux through GDH is unchanged [28]. Collectively, these observations further support the view that pyruvate cycling with flux via PC is critical and the most relevant anaplerotic pathway for regulation of insulin secretion. The stimulatory effect on insulin secretion by succinate, another anaplerotic fuel, was not affected by PAA, indicating that succinate mediates its effect through other pathways, independent of PC and pyruvate cycling. The ATP:ADP measurements, where succinate was used as secretagogue and which showed a similar decrease in ATP:ADP ratio in presence of PAA as with glucose suggest that succinate stimulates insulin secretion in an ATP-independent way.

Throughout the study, we have used phenylacetic acid (PAA), the most widely used inhibitor of PC; so far, effects of PAA other than on metabolism and insulin secretion have not been reported in beta-cells (13; 14). However, we acknowledge that pharmacological inhibitors must be used with great caution, since unspecific effects can never be entirely excluded. To address this concern, as well as to investigate the mechanisms of anaplerosis-regulated insulin secretion, we have examined relevant metabolic and functional parameters, such as fuel oxidation, respiration, mitochondrial membrane potential, exocytosis and K_{ATP}-channel conductance, and found that none was changed, with the exception of the ATP:ADP ratio. Many of the effects attributed to PAA when used in other systems involve more long-term alterations in function, presumably via changes in gene expression. [29]. We believe that they may be of less relevance in our experiments, where acute effects were assessed.

Whether the regulation of adenine nucleotides is the sole effect of flux through PC and pyruvate cycling, or if other molecules or signals involved in insulin secretion also are generated, remains to be elucidated. NADPH is a candidate for such a messenger [10], and its role has recently received additional support [30]. Nevertheless, we show that anaplerosis via PC is a key regulator of fuel-induced insulin secretion, essential for both phases of insulin

secretion. We suggest that this regulation is exerted via generation of an increase in the ATP:ADP ratio, for which pyruvate cycling plays an important role.

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FIGURE LEGENDS

Fig. 1: PAA inhibits insulin secretion under both K_{ATP}-dependent and -independent conditions.

Static incubations of islets at 2.8 and 16.7 mmol/l glucose in the presence (grey bars) or absence (black bars) of 5 mmol/l PAA, and in combination with 35 mmol/l K^+ and 250 mmol/l diazoxide (dz). Values are means ± S.E.M. for 3 independent experiments. * $P<0.05$.

Fig. 2: Effects of PAA on non-glucidic fuel-induced insulin secretion.

Islets were incubated for 1 h in 2.8 mmol/l glucose combined with 10 mmol/l α-ketoisocaproic acid (KIC) (a) or 10 mmol/l methyl succinate (b) in the presence (striped bars) or absence (white bars) of 5 mmol/l PAA. 16.7 mmol/l glucose (grey bars) was used as a stimulatory control. Values are means ± S.E.M. for 6 (a) or 3 (b) independent experiments; ** $P<0.01$

Fig. 3: PAA inhibits insulin secretion during both first and second phase of insulin secretion.

Islets were perifused for 10 min with 2.8 mmol/l glucose, followed by 30 min with 16.7 mmol/l glucose, in the presence (open squares) or absence (closed squares) of PAA. Then the glucose concentration was lowered to 2.8 mmol/l. Fig b shows total amount of insulin released, where the first and second phase is defined as 1-10 min and 11-30 min, respectively, after the rise in glucose . Grey bars PAA; black bars no PAA. Values are means ± S.E.M. for 5 independent experiments. ** $P<0.01$.

Fig. 4: Oxygen consumption in rat islets is not affected by PAA.

Oxygen consumption was measured in islets by an oxygraph, where consumption was calculated as pmol O_2 consumed /islet/min (a). O_2 consumption increased significantly when the glucose concentration was increased from 2.8 (white bar) to16.7 mmol/l (black bar), whereas no effect was seen when PAA was added (grey bar). Figure b shows a representative experiment where arrows indicate addition of glucose and PAA. Values are means ± S.E.M. for 6 independent experiments. *** P<0.001.

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Fig. 5: Effects of PAA on single beta-cell electrophysiology

Effects of PAA on single beta cell electrophysiology. **a** Increases in cell capacitance (ΔC) evoked by intracellular dialysis of a Ca2+-containing patch electrode solution (free intracellular Ca2+ ~1.5 μmol/l; Mg-ATP 3 mmol/l) in rat beta cells. Data represent average values±SEM every 10 s after establishing the standard whole-cell configuration (at t=0), and are from 11 cells when 5 mmol/l PAA was present in the extracellular medium (open circles), or from ten control cells (closed circles). **b** In intact cells using the perforated-patch configuration, the addition of 5 mmol/l PAA, increased the KATP-channel conductance. ctrl control. This effect was reversible upon wash-out. Data represent responses in seven different beta cells, summarised in (c). GM, membrane conductance; nS, nanoseconds. Left column control, middle column PAA, right column control. **p<0.01

Figure 1

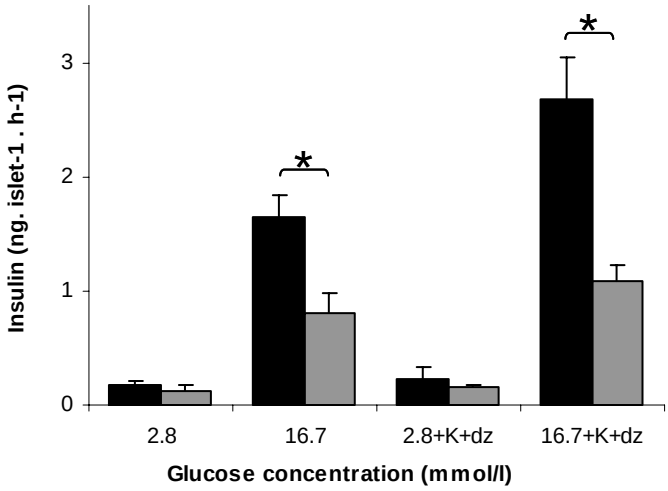

Figure 2a

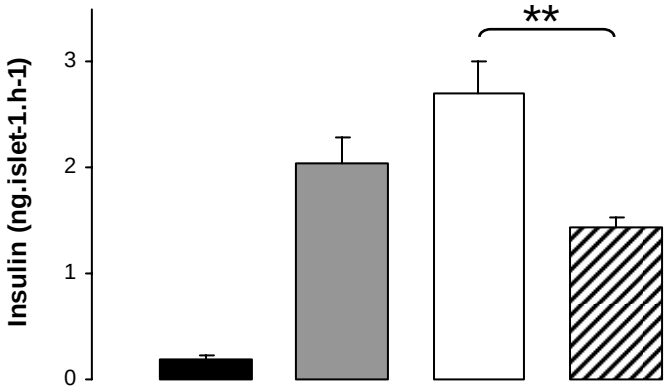

Figure 2b

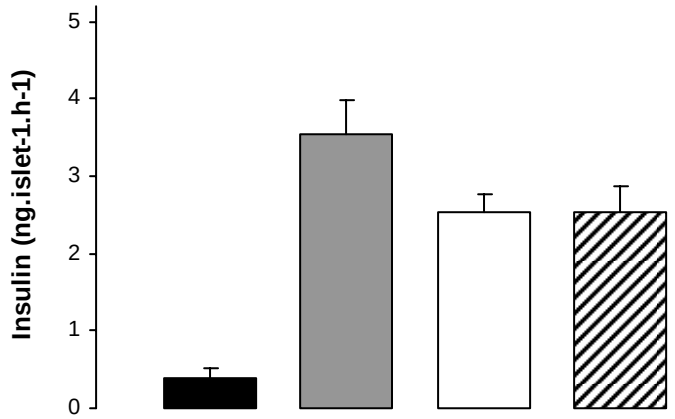

Figure 3a

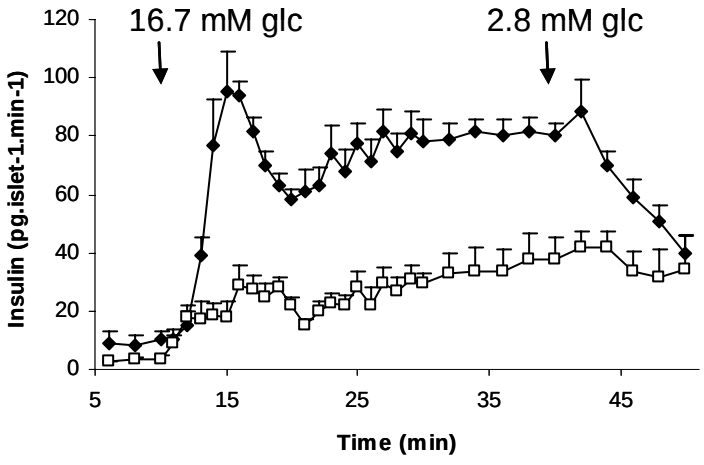

Figure 3b

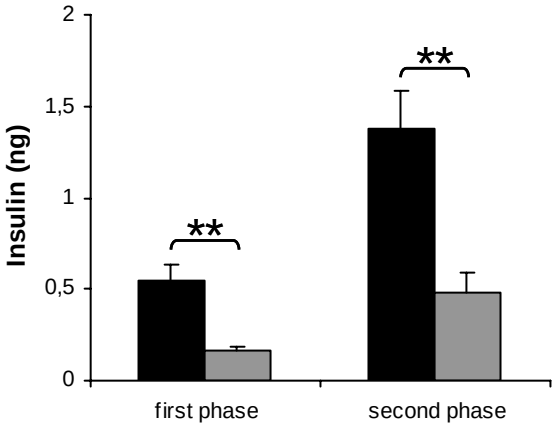

Figure 4a

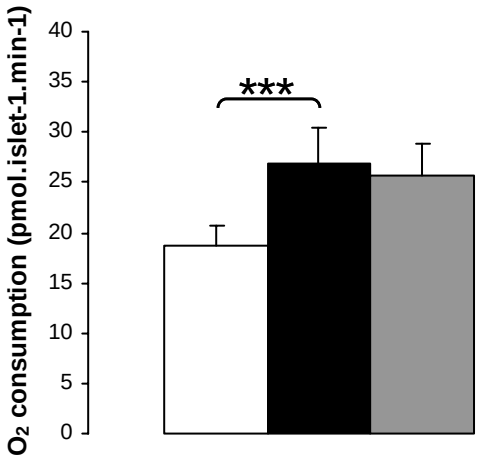

Figure 4b

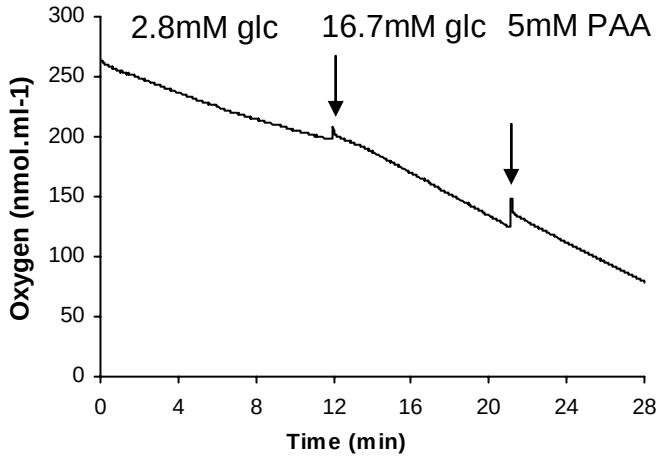

Figure 5a

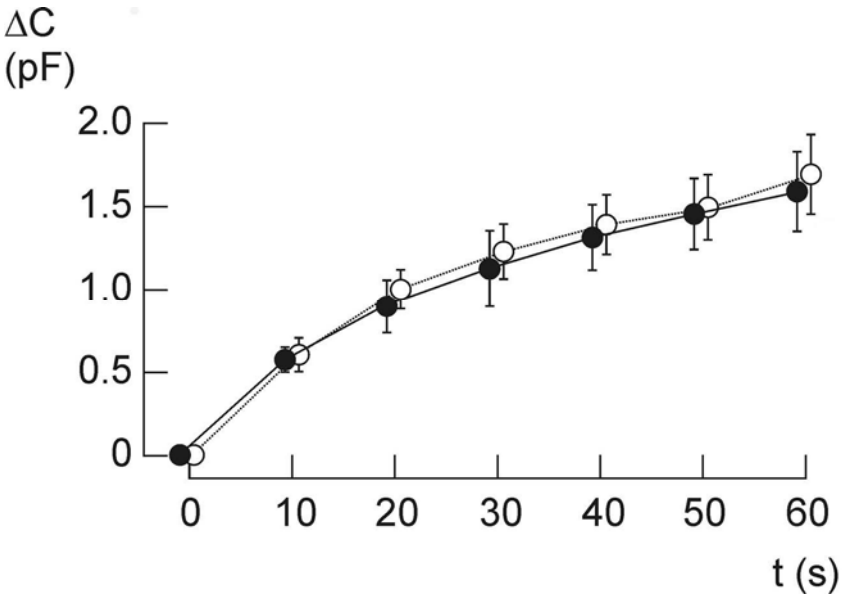

Figure 5b

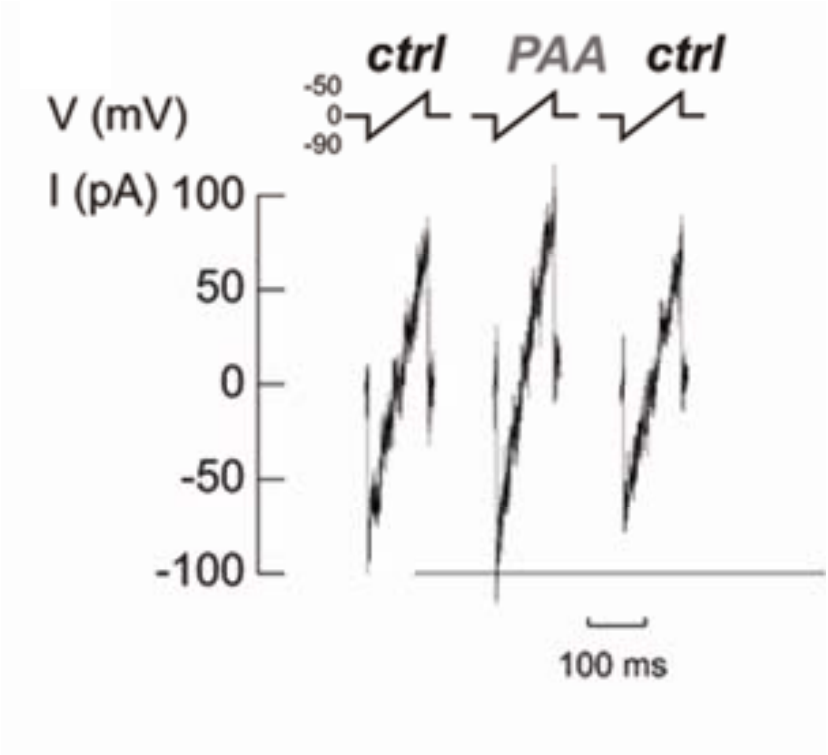

Figure 5c

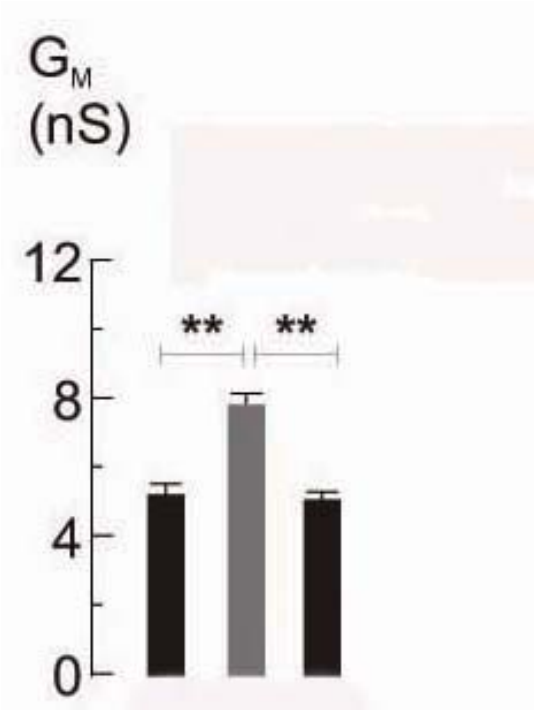

Table 1. Islet nucleotide content 5 minutes after glucose stimulation.

	ATP (pmol.islet-1)	ADP (pmol.islet-1)	ATP/ADP
2.8 mmol/l glucose	3.22 ± 0.24	0.66 ± 0.04	5.03 ± 0.33
2.8 mmol/l glucose + PAA	3.69 ± 0.32	0.84 ± 0.10	4.60 ± 1.03
16.7 mmol/l glucose	3.93 ± 0.32[a]	0.44 ± 0.007[b]	9.36 ± 0.88[d]
16.7 mmol/l glucose + PAA	3.75 ± 0.29	0.54 ± 0.013[c]	7.06 ± 1.27[e]

[a] $p < 0.05$ for difference between 2.8 and 16.7
[b] $p < 0.01$ for difference between 2.8 and 16.7
[c] $p < 0.05$ for difference between 16.7 and 16.7 PAA
[d] $p < 0.01$ for difference between 2.8 and 16.7
[e] $p < 0.05$ for difference between 16.7 and 16.7 PAA

Values are means ± S.E.M. for 4 independent experiments.

Table 2. Islet nucleotide content 30 minutes after glucose stimulation.

	ATP (pmol.islet-1)	ADP (pmol.islet-1)	ATP/ADP
2.8 mmol/l glucose	4.11 ± 0.26	0.33 ± 0.03	13.83 ± 1.59
2.8 mmol/l glucose + PAA	2.61 ± 0.25	0.38 ± 0.06	7.73 ± 0.82
16.7 mmol/l glucose	3.70 ±0.39	0.19 ± 0.01[a]	21.07 ± 1.11[b]
16.7 mmol/l glucose + PAA	4.07 ± 0.36	0.30 ± 0.04	14.47± 0.96[c]

[a] $p < 0.05$ for difference between 2.8 and 16.7
[b] $p < 0.01$ for difference between 2.8 and 16.7
[c] $p < 0.01$ for difference between 16.7 and 16.7 PAA

Values are means ± S.E.M. for 4 independent experiments.

Table 3. Islet nucleotide content 5 minutes after methyl-succinate stimulation.

5 min	ATP (pmol.islet-1)	ADP (pmol.islet-1)	ATP/ADP
2.8 mmol/l glucose	4.12 ± 0.24	1.01 ± 0.18	6.08 ± 0.88
2.8 mmol/l glucose + PAA	4.36 ± 0.16	1.26 ± 0.18	4.92 ± 0.53
10 mmol/l succinate	5.48 ± 0.23[a]	0.67 ± 0.08	10.40 ± 1.00[b]
10 mmol/l succinate + PAA	5.09 ± 0.31	0.85 ± 0.11	7.53 ± 0.37[c]

[a] $p < 0.01$ for difference between 2.8 and succinate
[b] $p < 0.01$ for difference between 2.8 and succinate
[c] $p < 0.05$ for difference between succinate and succinate PAA

Values are means ± S.E.M. for 5 independent experiments.