Metabolic and ionic coupling factors in amino acid-stimulated insulin release in pancreatic β-HC9 cells

Nicolai M. Doliba,1,2 Suzanne L. Wehrli,3 Marko Z. Vatamaniuk,1,2 Wei Qin,1,2 Carol W. Buettger,1,2 Heather W. Collins,1,2 and Franz M. Matschinsky1,2

1Department of Biochemistry and Biophysics and 2Institute for Diabetes, Obesity, and Metabolism, University of Pennsylvania School of Medicine and 3Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania

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Doliba NM, Wehrli SL, Vatamaniuk MZ, Qin W, Buettger CW, Collins HW, Matschinsky FM. Metabolic and ionic coupling factors in amino acid-stimulated insulin release in pancreatic β-HC9 cells. Am J Physiol Endocrinol Metab 292: E1507–E1519, 2007. First published January 30, 2007; doi:10.1152/ajpendo.00282.2006.—Fuel stimulation of insulin secretion from pancreatic β-cells is thought to be mediated by metabolic coupling factors that are generated by energized mitochondria, including protons, adenine nucleotides, and perhaps certain amino acids (AA), as for instance aspartate, glutamate, or glutamine (Q). The goal of the present study was to evaluate the role of such factors when insulin release (IR) is stimulated by glucose or AA, alone or combined, using 31P, 23Na and 1H NMR technology, respirometry, and biochemical analysis to study the metabolic events that occur in continuously superfused mouse β-HC9 cells contained in agarose beads and enhanced by the phosphodiesterase inhibitor IBMX. Exposing β-HC9 cells to high glucose or 3.5 mM of a physiological mixture of 18 AA (AAM) plus 2 mM glutamine caused a marked stimulation of insulin secretion associated with increased oxygen consumption, cAMP release, and phosphorylation potential as evidenced by higher phosphocreatine and lower Pi peak areas of 31P NMR spectra. Diazoxide blocked stimulation of IR completely, suggesting involvement of ATP-dependent potassium (KATP) channels in this process. However, levels of MgATP and MgADP concentrations, which regulate channel activity, changed only slowly and little, whereas the rate of insulin release increased fast and very markedly. The involvement of other candidate coupling factors was therefore considered. High glucose or AAM + Q increased pH. The availability of temporal pH profiles allowed the precise computation of the phosphate potential (ATP/Pi × ADP) in fuel-stimulated IR. Intracellular Na+ levels were greatly elevated by AAM + Q. However, glutamine alone or together with 2-aminooxobornanocarboxylic acid (which activates glutamate dehydrogenase) decreased β-cell Na levels. Stimulation of β-cells by glucose in the presence of AAM + Q (0.5 mM) was associated with rising cellular concentrations of glutamate and glutamine and strikingly lower aspartate levels. Methionine sulfoximine, an inhibitor of glutamine synthetase, blocked the glucose enhancement of AAM + Q-induced IR and associated changes in glutamine and aspartate but did not prevent the accumulation of glutamate. The results of this study demonstrate again that an increased phosphate potential and a functional KATP channel are essential for metabolic coupling during fuel-stimulated insulin release but illustrate that determining the identity and relative importance of all participating coupling factors and second messengers remains a challenge largely unmet.

amino acids; energy metabolism; oxygen consumption; sodium; mitochondria

THE BIOCHEMICAL BASIS OF FUEL SENSING by the insulin-secreting β-cells of the pancreatic islets of Langerhans is an issue of fundamental importance. Mechanisms have evolved over time that depend on the metabolism of the fuels rather than direct receptor-mediated recognition of the fuel molecules themselves, which contrast strikingly with the molecular processes mediating smell and taste (5, 10, 24, 39, 42, 45, 46). The study of metabolic events and of the processes that couple metabolism to hormone secretion is therefore central to pancreatic islet cell function. However, limited availability and, equally important, the cellular diversity of the microscopical islet organs make this task very difficult. The use of fuel-sensitive pancreatic β-cell lines provides an alternative that allows the application of analytically powerful technologies as for instance NMR and respirometry, to mention just two possibilities. This research tends to focus on the following three aspects of fuel-stimulated insulin release: 1) metabolic coupling factors (MCFs); 2) second messengers (SMs); and 3) metabolic indicators (MIs). We here define MCFs narrowly as those intermediates and cofactors of intermediary metabolism that change their cellular concentration as a function of fuel supply and have proven interactions with macromolecular targets that play an essential role in fuel-stimulated insulin release. We identify only the following three MCFs in this strict sense of the term: ATP, ADP, and S′-AMP. ATP and ADP serve as inhibitor and activator, respectively, of the ATP-dependent potassium (KATP) channel complex (2), whereas S′-AMP activates AMP kinase (33, 52), which has been identified as an important fuel sensor and deserves to be seriously considered in this context. In the group of SMs, we follow Sutherland and Robison’s (61) seminal definition and list as important examples free intracellular Ca2+, cAMP, diacylglycerol, and inositol trisphosphate, all with well-established target proteins. MIs are defined as processes, metabolites, and cofactors of intermediary metabolism that exhibit significant rate or level changes when extracellular fuel supply is altered and have a proven track record as indicators of altered metabolic potentials and flux. Of the potentially very large list of candidates, we name only the most prominent: the oxygen consumption or respiratory rate (OCR), the phosphorylation potential (P-potential; ATP/P × ADP), the pH, the redox potential as indicated by lactate/pyruvate, malate/oxalacetate, α-glycerophosphate/dihydroxyacetone phosphate, NAD(P)H/NAD(P) or glutathione/reduced glutathione,

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Address for reprint requests and other correspondence: N. Doliba or F. Matschinsky, Univ. of Pennsylvania, Biochemistry/Biophysics, 501 Stemmler Hall, 36th & Hamilton Walk, Philadelphia, PA 19104-6015 (e-mail: nicolai@mail.med.upenn.edu or matsch@mail.med.upenn.edu).
the levels of malonyl-CoA, succinyl-CoA, long-chain acyl-CoA, citrate, alanine, aspartate, glutamate, and glutamine.

It was the strategy of the present study to select a technically manageable panel of these MCFs, SMs, and MI s and study them dynamically, whenever possible on line, during glucose- and amino acid (AA)-stimulated insulin release in perfused β-HC9 cells to identify those processes and molecules that might be essential participants in stimulus secretion coupling. The results demonstrate the feasibility and potential of the approach and show that the OCR, the pH, the P-potential, cAMP release, and the levels of aspartate are most clearly associated with fuel stimulation of insulin release consistent with the classical view that the KATP channel and the cAMP system are essential in fuel-stimulated insulin release in this cell model.

RESEARCH DESIGN AND METHODS

General Procedures

The β-HC9 cell line was obtained from Dr. D. Hanahan. Cells were maintained in DMEM with 15% FCS and 24 mM glucose. β-HC9 cells of passages 22–28 were used in this study. Cells were supplemented with 25 mM creatine 48 h before the experiment to better visualize the phosphocreatine (PCr) peak by \(^{31}\)P NMR spectroscopy. Creatine loading did not have an effect on insulin release or oxygen consumption of superfused β-HC9 cells. The cellular protein content was measured by the method of Lowry et al. (36).

Formation of Beads

Cells were placed in agarose beads (bead size: 800–1,000 μm; 2,000–2,500 beads/cm\(^3\)) to maintain them in a stable environment and prevent their escaping from the perfusion system (13). Briefly, cells were suspended in 1 ml Krebs-Ringer-bicarbonate buffer containing (in mM): 114 NaCl, 5 KCl, 24 NaHCO\(_3\), 1 NaHPO\(_4\), 1 MgCl\(_2\)-6H\(_2\)O, 25 creatine, 2.2 CaCl\(_2\), 10 HEPES (pH 7.4), and 3 glucose. Cells suspended in this buffer were then added to 1 ml of 6% agarose (Sigma type VII, low gelling temperature) and mixed at 37°C. This suspension was then decanted into 50 ml of paraffin oil (37°C) and stirred continuously. The interaction (difference in surface tension) of the oil and agarose caused bead formation. Beads were cooled to 10°C by adding ice to the water bath under continuous stirring for 5 min to allow them to become firm and to maintain their shape. The beads were irregularly shaped with an average size of 1 mm diameter, as was determined by phase-contrast microscopy (Nikon TMS). Cell-containing beads were suspended in buffer and washed several times.

Perfusion Apparatus for Oxygen Consumption Experiments

Cell-containing beads were placed between two ~100-μm filters in a flow through column (Bio-Rad). Oxygen partial pressure was recorded polarographically with Clark-type oxygen electrodes placed in inflow and outflow. The perfusion medium was maintained at 37°C using a water bath. Note that all perfusion media contained 0.1 mM IBMX because the increase of insulin release of β-HC9 cells stimulated by glucose or AA in its absence is only small. The OCR was determined by phase-contrast microscopy (Nikon TMS). Cell-containing beads were suspended in buffer and washed several times.

Perfusion Apparatus for NMR Experiments

Cell-containing beads were placed in a 10-mm-diameter glass NMR tube and maintained in place by a filter (100 μm pore size) (12). Each NMR sample contained ~1.5 ml of the beads suspended in 1 ml buffer (20 mg of cell protein). The cells and the capillary tube containing methylene diphosphonate and NaCl with disopropyl standards were placed within the sensitive volume of the NMR coil. The perfusion medium contained (in mM): 114 NaCl, 5 KCl, 24 NaHCO\(_3\), 1 NaHPO\(_4\), 1 MgCl\(_2\)-6H\(_2\)O, 25 creatine, 2.2 CaCl\(_2\), and 10 HEPES (pH 7.4), was equilibrated with 95% O\(_2\)-5% CO\(_2\), and was maintained at 37°C using a water bath. The temperature in the probe was also maintained by the internal variable temperature controller. The transfer lines were insulated. The buffer was introduced through an in-flow line (at 2.7 ml/min). A suction line, placed above the beads, removed the superfusate, which was not recirculated.

\(^{31}\)P NMR Methods

The \(^{31}\)P NMR measurements were performed with an Avance DMX-400 spectrometer at 162 MHz. \(^{31}\)P NMR spectra were acquired consecutively in 5-min periods (500 transients) for up to 8 h without proton decoupling [because the resonances are broad and the \(^{31}\)P-\(^{1}\)H couplings are relatively small (3–12 Hz)]. The following conditions were used: pulse width 36°, sweep width 13 kHz, 16 K data points, repetition time 0.6 s. Free induction decays were processed with a Lorentz-Gauss window function for resolution enhancement [Bruker parameters: line broadening (LB) = −8, maximum of the Gaussian function = 0.003].

**\(^{23}\)Na NMR Methods**

Intracellular Na\(^+\) was determined using thulium-DOTP (3.5 mM) as shift reagent. The following conditions were used: pulse width 90°, sweep width 6.3 kHz, 4 K data points, repetition time 0.325 s. LB was applied at 3 Hz.

**Calculation of pH**

\[ \text{pH}_i = \text{pH}_o + \log(\Delta - 3.27/5.69 - \Delta), \]

where Δ is the chemical shift difference between signal of inorganic phosphate (P\(_i\)) and PCr.

**Calculation of Intracellular ADP**

The parameters of the creatine kinase equilibrium reaction were used to calculate the concentration of free intracellular ADP (21):

\[ \text{Cr-P}^2^- + \text{MgADP}^- + \text{H}^+ \rightarrow \text{Cr} + \text{MgATP}^2^- \]

\[ [\text{MgADP}]/[\text{MgATP}] = ([\text{Cr}]/[\text{PCr}] - 1) \times 10^{40}/K \]

where \( K = 1.66 \times 10^9 \text{ mol}^{-1}. \)

**Insulin and cAMP Measurements**

Insulin content in efflux samples was measured by RIA with charcoal separation (25). Rat insulin from Linco Research served as standard, and Miles anti-insulin antibody from ICN was the primary antibody. The cAMP assay was performed according to the RIA method of Albano et al. (1) using the Biotrak \([125\)I]cAMP assay system from Amersham Pharmacia Biotech.

**Statistical Analysis**

In appropriate cases, significant differences between groups were determined by one-way ANOVA with post hoc analysis using Dunnett’s multiple-comparison test. Values of \( P \leq 0.05 \) were accepted as significant. In some instances, typical results are presented for clarity of the temporal profiles (n = 3–5), and the statistical data with comparisons between the different groups are presented in Table 1.
Glutamine and $P_i$ are Two Critical Factors That Affect AA-stimulated Insulin Release in β-HC9 Cells

Figure 1, A and B, presents the results of continuous measurements of insulin and cAMP release and oxygen consumption by perfused β-HC9 cells in response to stimulation with low (3 mM) and high (30 mM) glucose followed by addition of a physiological 3.5 mM mixture of 18 AA (in mM: 0.44 alanine, 0.19 arginine, 0.038 aspartate, 0.094 citrulline, 0.12 glutamate, 0.30 glycine, 0.077 histidine, 0.094 isoleucine, 0.16 leucine, 0.37 lysine, 0.05 methionine, 0.70 ornithine, 0.08 phenylalanine, 0.35 proline, 0.57 serine, 0.27 threonine, 0.073 tryptophan, and 0.20 valine) plus 2 mM glutamine (AAM + Q). Comparable to isolated pancreatic islets (11, 23, 26), β-HC9 cells responded to increased levels of oxygen consumption by enhancing insulin and cAMP release and oxygen consumption. However, in contrast to islets, β-HC9 cells respond to AAM + Q by a burst in insulin release. AAM + Q also elevated cAMP release and oxygen consumption. Glucose or 2-amino-norbornanecarboxylic acid (BCH), a nonmetabolized leucine analog and allosteric activator of glutamate dehydrogenase (GDH; see Ref. 57) did not further increase hormone releases in β-HC9 cells in the presence of AAM + Q, suggesting that a maximum rate of hormone release had been reached. It is noteworthy, that glucose in the presence of AAM + Q influenced oxygen consumption and cAMP only slightly. The uncoupling of respiration and oxidative phosphorylation by FCCP (5 μM) blocked insulin and cAMP release and markedly increased oxygen consumption, indicating that respiration and oxidative phosphorylation are well coupled in this preparation and that both cAMP and insulin release require enhanced ATP production.

The omission of glucose from the perfusate reduced AAM-stimulated insulin and cAMP release (Fig. 1C) and oxygen consumption (Fig. 1D). Low and high glucose in the presence of AAM without glutamine (AAM – Q) greatly increased insulin release and oxygen consumption. The cAMP response was significantly blunted when glutamine was absent from the medium. The omission of $P_i$ from the AAM + Q mixture reduced AA-stimulated insulin release (Fig. 1E) similarly to leaving out glutamine but did not affect cAMP release or AAM + Q-stimulated oxygen consumption (Fig. 1F). In fact, the respiratory profiles of Fig. 1, B and F, are virtually the same. Low glucose (3 mM) further increased insulin release, and high glucose (30 mM) produced a rapid increase of insulin release that was not sustained. BCH was ineffective under these conditions. However, glucose alone was not sufficient to support sustained insulin release in this β-cell line (Fig. 2). In contrast, glutamine with BCH (Fig. 2A) or glucose (Fig. 2C) greatly increased insulin release, indicating the effects of these substances are interdependent. Glutamine alone stimulated oxygen consumption and BCH (Fig. 2B), low and high glucose (Fig. 2D) further increased oxidative metabolism.

These data suggest that glutamine is the major contributing stimulator in an AAM but that its action requires the presence of $P_i$ to stimulate glutaminase (43) and BCH to activate GDH (57) when it is used in the absence of the physiological AAM. This implies that the involvement of augmented AA catabolism and energy production in stimulation of hormone release is essential.

AA-induced Increase of $P_i$-potential In β-HC9 Cells

To test if the effect of AAM + Q on insulin release is indeed the result of an increase of energy production and inhibition of K$_{ATP}$ channels, we used two different approaches. In the first, diazoxide, a K$_{ATP}$ channel opener, was added during (Fig. 3A) or before (Fig. 3B) stimulation of insulin secretion by the AAM. In both cases, diazoxide blocked AAM-stimulated insulin release, demonstrating that the K$_{ATP}$ is essential for coupling. A remarkable observation of these experiments is the lack of effect of diazoxide on the OCR, which implies that cytosolic Ca$^{2+}$ levels and the work load imposed on the system by the process of exocytosis have little influence on respiration. To further explore the relationship between insulin release and energy metabolism, a second approach, noninvasive NMR technology, was used in studies of phosphorus metabolism. Figure 4A presents a typical $^{31}$P NMR spectrum of superfused β-HC9 cells with low glucose (3 mM). The spectrum clearly displays the resonances of P$_i$, PCr, and ATP ($\gamma$, $\alpha$, and $\beta$, respectively) peaks. Frequent monitoring of phosphorus metabolites (every 5 min) (Fig. 4, B and C) demonstrated that increasing the glucose concentration from 3 to 30 mM elevates

Table 1. Effect of glucose and amino acids on insulin release, metabolic and ionic parameters in perfused β-HC9 cells

<table>
<thead>
<tr>
<th>Glucose (3 mM)</th>
<th>Glucose (30 mM)</th>
<th>AAM + Q</th>
<th>AAM + Q + Glucose (3 mM)</th>
<th>AAM + Q + Glucose (30 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin Release, ng mg$^{-1}$ Protein$^{-1}$</td>
<td>OCR, nmol min$^{-1}$ Protein$^{-1}$</td>
<td>pH</td>
<td>Na$^+$, %change</td>
<td>P$_i$ Peak Area</td>
</tr>
<tr>
<td>1.05 ± 0.18</td>
<td>7.39 ± 0.13</td>
<td>7.15 ± 0.03</td>
<td>100.00 ± 8.05</td>
<td>1.65 ± 0.08</td>
</tr>
<tr>
<td>1.91 ± 0.17*</td>
<td>8.72 ± 0.04*</td>
<td>7.18 ± 0.01</td>
<td>79.07 ± 1.35*</td>
<td>1.41 ± 0.10*</td>
</tr>
<tr>
<td>7.80 ± 1.05*</td>
<td>12.33 ± 0.20*</td>
<td>7.38 ± 0.02*</td>
<td>62.79 ± 4.03*</td>
<td>0.78 ± 0.10*</td>
</tr>
<tr>
<td>7.30 ± 1.97*†</td>
<td>13.07 ± 0.33*†</td>
<td>7.24 ± 0.01*†</td>
<td>114.66 ± 1.75*†</td>
<td>1.22 ± 0.06*†</td>
</tr>
</tbody>
</table>

For each experiment, the average value of steady-state measurements for each intervention was calculated and then mean value and SD were determined from 18528.
E1510 ENERGY METABOLISM AND INSULIN RELEASE IN β-HC9 CELLS

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the P-potential markedly as evidenced by increased PCr and 
decreased Pi peak areas. However, it is striking that the 
changes are relatively slow compared with the brisk, 15- to 
20-fold hormone and cAMP secretory responses (compare with 
Figs. 1 and 2). It is also remarkable that the ATP level changed 
only little because of high glucose (30 mM). After removal of 
secretagogues, the PCr decreased and Pi increased markedly, 
but the ATP levels were much less affected. Addition of 
AAM/H11001 Qt o 
H9252 
HC9 cells increased the P-potential, as evi-
denced by rising PCr and declining Pi without significant 
changes in 
H9252 
ATP peak areas of 31P NMR spectra of super-
fused 
H9252 
HC9 cells. Low (3 mM) and high (30 mM) glucose in 
the presence of AAM 
H11001 Q further augmented the P-potential of 
H9252 
HC9 cells by increasing PCr and 
H9252 
ATP (P = 0.048) and 
decreasing Pi. Figure 4C demonstrates the importance of 
glutamine for the P-potential, as influenced by the AAM. 
AA without glutamine were unable to influence the P-
potential of 
HC9 cells in a major way. However, 3 and 30 
mM glucose in the presence of AAM were more effective and 
raised the P-potential.

Cytosolic pH and MgADP Levels During Glucose- and 
AA-Stimulated Insulin Release

It was suggested from previous work (49) that ADP rather 
than ATP could serve as the crucial MCF. However, the 
pertinent calculations of free ADP were based on the untenable 
asumption that cytosolic pH remained constant during fuel
stimulation (19, 49). With NMR technology, cytosolic pH can be monitored continuously by the chemical shift between Pi and PCr of the 31P spectrum. Figure 5A shows the marked changes in pH that result from the exposure to glucose and the AAM, both in the presence and absence of glutamine. Perfusion of β-HC9 cells without glucose lowered the pH as the endogenous fuel stores were depleted. Low (3 mM) glucose arrested the decline and elevated pH slightly. High (30 mM) glucose led to a marked and rapid rise of pH by 0.22 units. A large fall of 0.32 units occurred when glucose was omitted from the medium. The AAM increased pH, and a switch from 3 to 30 mM glucose in the presence of the AAM further increased pH. Omitting glutamine from the medium significantly reduced the effect of AA on pH, illustrating the important role of glutamine. These pH data were taken into account in calculations of the MgADP levels and derivative parameters. MgADP decreased when 3 mM glucose was added to the medium containing the AAM but was not altered when glucose was raised further to 30 mM. The corresponding temporal profiles of the ATP-to-MgADP ratio and the P-potential provide a more comprehensive picture of β-cell energy metabolism in the >5 h course of fuel stimulation. The impact of glutamine is perhaps the most striking, as apparent in Fig. 5, B and C. The ATP-to-MgADP ratio and the energy potential correlate most closely with fuel-stimulated insulin release. It must, however, be reemphasized that metabolic changes trailed behind the insulin and cAMP release responses.

**Opposing Effects of AA and Glutamine on Internal Na⁺ Levels in β-HC9 Cells**

The rise of pH in response to glucose is probably the result of activation of Na⁺/H⁺ exchange (28). The latter effect should be associated with changes in intracellular Na⁺ concentration ([Na⁺]i) levels. To test this hypothesis, we used 23Na NMR to monitor the free Na⁺ concentration in β-HC9 cells. Previously, we reported that glucose induces a dose-dependent decrease in [Na⁺]i (12). Figure 6 shows the changes in intracellular Na (A) and insulin release (B) during perfusion with AA and glutamine alone. When cells were perfused with glutamine alone, [Na⁺]i levels decreased slightly compared with baseline. BCH caused a marked reduction of internal [Na⁺]i. Exposure of β-HC9 cells to the AAM without glutamine increased [Na⁺]i. The subsequent addition of BCH to the glutamine-free AAM increased [Na⁺]i further. Glucose at

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**Fig. 3.** Effect of diazoxide on amino acid-stimulated insulin release in β-HC9 cells. A: diazoxide was added during stimulation of hormone secretion by amino acids. B: diazoxide was added before stimulation of insulin release by amino acids. Each curve represents a typical experiment (n = 3).
when superimposed on this stimulus mixture, decreased 

\[ \text{Na}^+ \] slightly, yet glutamine addition to the perfusate resulted in a 40\% decrease of cytosolic \[ \text{Na}^+ \]. BCH markedly potentiated insulin release in the presence of glutamine but was unable to enhance insulin release in the presence of AAM without glutamine (Fig. 6B). This refractory state was partially reversed by the addition of 3 mM glucose. It is not clear why the combination of AAM + Q + Glucose (3 mM) + BCH was

Fig. 4. Effect of glucose and amino acids on Pi, phosphocreatine (PCr), and \( \beta \)-ATP of perifused \( \beta \)-HC9 cells. A: \( ^{31} \)P NMR spectra of perifused \( \beta \)-HC9 cells at baseline glucose (3 mM). Peak assignments are indicated as follows: methylene diphosphonate (MDP) standard, PCr, and ATP (\( \gamma \), \( \alpha \), and \( \beta \), respectively). B: continuous monitoring (every 5 min) of phosphorus metabolites during stimulation of insulin release by glucose and amino acids. C: experiments were done in the same order as in B except that glutamine was removed from the amino acid mixture. \( ^{31} \)P NMR spectra were acquired consecutively in 5-min periods. For clarity, typical experiments are presented (\( n = 5 \)). See Table 1 for statistical information.

Fig. 5. Effect of glucose and amino acids on pH, and MgADP, the ATP-to-ADP ratio, and the P-potential (ATP/ADP) in perifused \( \beta \)-HC9 cells. Changes in pH were used to calculate free MgADP. Note: data are presented as moving average of 3 adjacent points (Microcal Origin 5). The results of all experiments (\( n = 6 \)) were comparable up to the 190-min time point and were combined. After that, one set of experiments contained 2 mM glutamine in the amino acid mixture (\( G_0 \), pH; \( \omega \), MgADP, and ATP/ADP), whereas in the second set glutamine was omitted (\( G_0 \), pH; \( + \), MgADP, ATP/ADP, and ATP/ADP, ADP). A: changes in pH, and free MgADP (in mM). B: relative changes in the ATP-to-MgADP ratio (relative units). C: relative changes in the phosphorylation potential (P-potential; ATP/Pi, ADP). Note that the pH data are shown in A–C to facilitate comparison of kinetics. Also note that the insulin release dynamics were comparable to those in Fig. 1, A and B.
Proton and phosphorus NMR analysis was applied to measure intracellular metabolites, including lactate, alanine, aspartate, glutamate, glutamine, PCr, and ATP in extracts prepared from perfused β-HC9 cells. The concentration of glutamine in the medium was reduced to 0.5 mM to magnify the effect of high glucose and to diminish analytical difficulties resulting from contamination of the cell extracts by extracellular glutamine. The focus is here on the portion of the experiment that deals with the glucose effect. High glucose doubled AA-induced insulin release transiently (Fig. 7A) and increased oxygen consumption slightly but persistently (Fig. 7B). These effects were associated with increased cellular concentrations of lactate, alanine, glutamate, and glutamine but markedly decreased aspartate levels (Table 2 and Fig. 8, note that the lactate and alanine data are not shown because the focus is on the tricarboxylic acid cycle derivatives). At the same time, the total concentration of PCr and ATP increased by 65 and 44%, respectively. Methionine sulfoximine (MSO), an inhibitor of glutamine synthetase, decreased the delayed phase of insulin secretion resulting from AAM + Q and blocked glucose-induced insulin release (Fig. 7C) but did not prevent the increase in oxygen consumption, nor did it impair the energy potential as indicated by normal and higher levels of ATP and PCr, respectively (Fig. 7D and Table 1). The baseline level of aspartate was somewhat reduced after addition of MSO but did increase barely under the influence of glucose, consistent with the inhibition of glutamine synthase by MSO.

**DISCUSSION**

**Methodological Considerations**

At the outset, advantages and limitations of the present model system using perfused agarose-embedded β-HC9 cells need to be discussed. There are distinct benefits in using this model system for studying fuel-stimulated insulin secretion: 1) the availability of large batches of insulin-secreting cells allows the application of powerful techniques as, for instance, phosphorous, Na, and proton NMR, which are too insensitive for experimenting with isolated pancreatic islets; 2) the approach avoids the complication arising from the admixture of non-β-cells in the preparation, which compromises many studies with isolated islets of Langerhans; and 3) the glucose dose-response curve with a threshold of 3–4 mM is in the physiological range consistent with involvement of glucokinase as glucose sensor and normal operation of the KATP and Ca2+ channels. There are, however, certain features and drawbacks that have to be kept in mind when interpreting results obtained with this β-cell model system: 1) the phosphodiesterase inhibitor IBMX is required to permit optimal studies of fuel-stimulated insulin release; 2) cells are cultured and perfused in the presence of 25 mM creatine, a measure taken to facilitate studying the P-potential by NMR but creating perhaps an artificially high intracellular energy potential as indicated by normal and higher levels of ATP and PCr; and 3) the glucose metabolic effect. High glucose doubled AA-induced insulin release transiently (Fig. 7A) and increased oxygen consumption slightly but persistently (Fig. 7B). These effects were associated with increased cellular concentrations of lactate, alanine, glutamate, and glutamine but markedly decreased aspartate levels (Table 2 and Fig. 8, note that the lactate and alanine data are not shown because the focus is on the tricarboxylic acid cycle derivatives). At the same time, the total concentration of PCr and ATP increased by 65 and 44%, respectively. Methionine sulfoximine (MSO), an inhibitor of glutamine synthetase, decreased the delayed phase of insulin secretion resulting from AAM + Q and blocked glucose-induced insulin release (Fig. 7C) but did not prevent the increase in oxygen consumption, nor did it impair the energy potential as indicated by normal and higher levels of ATP and PCr, respectively (Fig. 7D and Table 1). The baseline level of aspartate was somewhat reduced after addition of MSO but did increase barely under the influence of glucose, consistent with the inhibition of glutamine synthase by MSO.

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backs, as illustrated by previous studies and to become apparent in the discussion of the present results.

Summary and General Discussion of Study

The present results, carefully considered as a whole, are best explained by the classical hypothesis that an increased P-potential is the critical driving force for insulin release stimulated by glucose and by AA, but they also illustrate the great difficulties inherent in mechanistic studies of stimulus-secretion coupling of this process. Using large batches of the pancreatic cell line β-HC9 in a continuous perifusion system, we studied seven primary parameters (the OCR, cytosolic pH, ...
free ATP, Pi, PCr, cAMP release, and insulin secretion) and three derivative parameters (free ADP, the ATP-to-ADP ratio and the P-potential ATP/P_{i} \times ADP) as influenced by glucose and AA stimulation, and we also determined the cellular levels of aspartate, glutamate, and glutamine at single time points following a glucose load superimposed on a physiological AAM. Among these parameters are MCF, SM, and MI, as defined in the Introduction. Furthermore, diazoxide, the specific activator of the K_{ATP} channel, MSO, an inhibitor of glutamine synthase, and omission of Pi in the perifusate were employed to facilitate the exploration of coupling mechanisms. The results of this study are summarized as follows: Insulin secretion, cAMP release, and the OCR respond rapidly and markedly to fuel stimulation, suggesting that they are causally related. However, the association is not absolute: 1) diazoxide blocked insulin release but had little or no effect on the OCR; 2) phosphate omission greatly reduced AA-induced insulin release but did not affect the OCR and the cAMP release; and 3) the level changes of MCFs and changes of derivative MIs are much slower than the rates of change observed in insulin and cAMP release and the OCR. This dissociation cannot be interpreted to indicate that increased oxidative phosphorylation and the participation of the protein kinase A (PKA) system are not essential for fuel-stimulated insulin release. The results merely show that it is possible to uncouple metabolism or SM production from insulin secretion by well-established (in the case of diazoxide) or less-well-understood (the omission of phosphate in AA stimulation) mechanisms. Levels of aspartate, glutamate, and glutamine change dramatically when β-HC9 cells are stimulated with glucose on a background of a physiological AAM. However, neither one of them qualifies as MCF according to our definition. In the paragraphs below, specific aspects of these results and conclusions are discussed in detail.

Discussion of Specific Aspects of the Present Study

The role of ATP and free ADP in stimulus-secretion coupling in β-HC9 cells. The metabolism of AA and flux through GDH is dependent on the energy status of the cell (17, 35). In the fuel-deprived state, glutaminase and GDH are activated and glutamine synthesis is slow such that there is a net flux in the catabolic direction. The results of the present study suggest that, in glucose-free media, physiological AAM, including glutamine, stimulate insulin release of β-HC9 cells, and this effect is associated with enhanced flux through GDH in the direction of the tricarboxylic acid cycle as evidenced by an increase in oxygen consumption and the P-potential (ATP/P_{i} \times ADP). This AA-induced rise in the P-potential should lead to closure of the K_{ATP} channels and trigger insulin release as is seen when glucose is the stimulus. Indeed, hyperpolarization of the β-cell membrane by diazoxide completely abolished the AA- and glucose-induced insulin release, proving the involvement of K_{ATP} channels. However, it is still a matter of debate whether it is the change in the concentration of ATP, of MgADP, or both that influence K_{ATP} channel activity. A substantial fraction of the ATP (68%) and ADP (45%) within the β-cell is nondiffusible and contained in intracellular organelles (especially the insulin secretory granules; see Ref. 9). Measurements of intracellular ATP concentration ([ATP]_{i}) in purified rat β-cells suggest that [ATP]_{i} is ~2 mmol/l (2 pmol/10^{3} cells) in the absence of glucose and increases to >4 mmol/l (≥4 pmol/10^{3} cells) when glucose is raised to 10 mmol/l (8). However, a change in [ATP], on exposure to glucose is not universally observed (19). The present results demonstrate that free ATP of β-cells is not detectably altered by glucose. The β-ATP peak significantly increased (by 30%; P = 0.048) only when glucose was combined with AA. In support of these data, the authors of a recent paper reported only modestly increased ATP resulting from glucose metabolism and a relatively high ATP-to-ADP concentration ratio value even in low glucose (16). Therefore, one might question a role of ATP as a critical physiological regulator of K_{ATP} channel activity (51). On the other hand, intracellular free MgADP^{−} stimulates K_{ATP} channel activity, and it has been suggested that ADP, and therefore the ATP-to-ADP ratio, is responsible for channel regulation in vivo (2, 6, 31, 51). MgADP^{−} shifts the ATP concentration-inhibition curve to a higher intracellular ATP concentration (29). There have been few estimates of free MgADP in β-cells which show that increasing concentrations of glucose are associated with a decline in the concentration of free ADP in the range that can inhibit K_{ATP} channel activity (19, 49). However, all previous calculations of MgADP^{−} used the assumption that pH remained constant during fuel-stimulated insulin release (19, 49). If we assume that pH is constant then MgADP^{−} would decrease by ~50% when the perfusion is switched from low to high glucose in the present study. However, according to our data and calculations, marked alkalinization of β-cells resulting from high glucose results in unchanged MgADP^{−} when glucose was raised from 3 to 30 mM.

Results of the present study showed that AA and glucose also cause a marked increase in PCr and a fall in phosphate. These data are in good agreement with previous published...
results (30) showing that the PCr concentration in isolated mouse islets clearly increased in response to a rise of the glucose concentration from 0.5 to 15 mM. PCr reduces the ability of ADP to stimulate K$_{ATP}$ channel activity in β-cells (30).

Taranov et al. (63) suggested recently that, similarly to cardiac muscle (14), β-cells have an intracellular phosphotransfer network with creatine kinase linking ATP generation to K$_{ATP}$ channel closure and adenylate kinase regulating K$_{ATP}$ channel opening. Such a system might shuttle high energy-rich phosphates from the mitochondria to the plasma membrane without much change in ATP. In the microenvironment of the channel, adenylate kinase could convert AMP and ATP to ADP, promoting channel opening, whereas creatine kinase could catalyze the transfer of phosphate from PCr to ADP, producing creatine and ATP and, consequently, channel closure. This process could take place as an early response to fuel exposure but may manifest itself with a delay by the increased P-potential.

There are at least two other agents known to modulate the ATP sensitivity of the K$_{ATP}$ channel: phosphatidylinositol-4,5-bisphosphate and related phosphoinositides (3, 15), and also long-chain acyl-CoA esters (20, 32, 54). They both decrease the ability of ATP to close the K$_{ATP}$ channel.

**Activation of the cAMP system by fuel stimulation of β-HC9 cells.** The rate of cAMP release is markedly increased coincidentally with insulin secretion following glucose or AA stimulation of β-HC9 cells. Even though few replicates of the results are available, the data are internally consistent and derive great strength from their context and extended time course of their profiles. The increased production of the SM cAMP is most likely the result of enhanced fuel metabolism as apparent from the data set presented in Fig. 1. The most plausible explanation is that an elevated intracellular calcium level is the cause of adenylate cyclase activation (48, 64). It should be obvious that any discussion of metabolic coupling mechanisms involved in fuel-stimulated insulin release, including “triggering” and “amplification,” must consider the participation of the PKA signaling pathway as essential.

**Role of glutamine and Pi in insulin secretion.** The results also suggest a prominent role of glutamine in AA-stimulated insulin release in β-HC9 cells because omitting glutamine from the AAM markedly reduces insulin release and oxygen consumption and abolishes changes in the P-potential. However, glutamine alone is not sufficient to stimulate insulin release by β-HC9 cells. Stimulation of GDH by BCh or adding glucose enables the effect of glutamine on hormone release similarly to effects of l-leucine and BCh on pancreatic islets (56, 57).

The data also suggest a critical role for Pi in AA-stimulated insulin release. Pi regulates the activity of glutaminase and thus addition of Pi leads to an increase of the glutamate concentration and enhances insulin secretion evoked by the addition of leucine (43). It is remarkable that omitting Pi from the perfusate affects only insulin secretion but not oxygen consumption and cAMP release, suggesting that secretion and oxidation are not tightly coupled.

These studies with glutamine and Pi clearly implicate enhanced metabolism as essential for the stimulation of insulin secretion.

**Role of intracellular H$^+$ and Na$^+$ in hormone release.** The changes in intracellular H$^+$ and Na$^+$ are tightly coupled in pancreatic β-cells. High glucose induces an increase in pH, and this effect is dependent on the presence of extracellular Na$^+$ and is inhibited by 5-(N-ethyl-N-isopropyl)amiloride, a blocker of Na$^+$/H$^+$ exchange in HCO$_3^-$ free buffer (28). However, in the presence of a Na$^+$/H$^+$ exchange inhibitor, stimulation of β-cell with glucose leads to acidification, unmasking the acid load imposed by metabolism of the sugar (28). Activation of Na$^+$/H$^+$ exchange should lead to elevation of the intracellular Na$^+$ level. However, we (12) and others (65) have shown that glucose induces a dose-dependent decrease in intracellular Na$^+$. Shepherdd and Henquin (59) proposed that, in physiological HCO$_3^-$ buffer, the activity of the HCO$_3^-$/Cl$^-$ exchanger overcompensates for the acidification action of glucose metabolism and leads to the increase of pH. Recently, it has been shown that H$^+$ uptake by secretory granules may also contribute to the increase in pH during islet stimulation with glucose (60), consistent with the observation that cytosolic and granular pH change in opposite directions upon glucose stimulation. AA also cause an alkalinization of β-cells, and this effect depends on the presence of glutamine in the AAM. One should not ignore intracellular H$^+$ concentration ([H$^+$]) as a possible physiological regulator in stimulus-secretion coupling of β-cells, particularly because changes in pH may modulate ATP-regulated K$^+$ channel activity (28, 44, 50). Furthermore, pH affects glutaminase activity (43). The marked decrease in the rate of glutamine breakdown at a pH <7.4 indicates that, when [H$^+$] increases, glutamine catabolism decreases (43).

Glutamine alone or with BCh also lowers the Na$^+$ level, whereas an AAM with or without glutamine increases β-cell intracellular ion levels. Because glutamine metabolism influences the P-potential, it is logical to propose that this elevation of energy production may activate the Na$^+$/K$^+$ pump and cause an extrusion of Na$^+$ from the β-cells.

**Do intracellular aspartate, glutamate, or glutamine serve as a MCF in fuel-stimulated insulin secretion?** Glutaminolysis may be blocked in the fuel-energized β-cell (35). Glutaminase and GDH are inhibited while glutamine synthesis may be activated, resulting in net flux in the direction of glutamate and glutamine. Addition of glucose to β-HC9 cells oxidizing AA should therefore energize the cells and lead to inhibition of GDH and a redirection of the flux into anabolic pathways (e.g., a rise of glutamate and glutamine). Maechler and Wollheim (40) suggested that glutamate, released from the mitochondria, may act as an intracellular messenger that couples glucose metabolism to insulin secretion. Their hypothesis was based on the following observations and considerations: 1) high glucose generated glutamate from β-cell mitochondria, resulting in an increase of cellular glutamate concentration of about fivefold in rat insulinoma INS-1 cells or in isolated human pancreatic islets (27); 2) the mitochondrial poison FCCP inhibited the production of glutamate during glucose stimulation of INS-1 cells (40); 3) in permeabilized cells, under conditions of fixed intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), glutamate directly stimulated insulin exocytosis (18, 53); and 4) dimethyl glutamate had no effect on the mitochondrial membrane potential in INS-1 cells, suggesting that glutamate participates directly in the secretory process downstream of mitochondrial activation (40). It was also suggested that glutamate can be taken up by secretory granules because inhibitors of vesicular glutamate transport suppressed the glutamate-evoked exocytosis. However, the “glutamate hypothesis” was challenged by reports...
suggesting that glucose was unable to affect glutamate levels in islets from *ob/ob* mice (7, 22), rats (38), and insulinoma cells (37). Furthermore, the ability of glutamate dimethyl ester to increase insulin secretion was attributed to its use as a nutrient by β-cells (55). Altering the islet glutamate content by adding glutamine (38, 41, 43) did not induce insulin release unless GDH was activated by leucine or its nonmetabolized analog BCH (47, 57). Because of glutamate’s putative role in the amplification pathway, the glutamate hypothesis was tested in conditions of clamping of Ca\(^{2+}\) concentration at an elevated level by depolarizing β-cells either with sulfonfonylurea compounds or by high KCl in the presence of diazoxide (4). The amplification of secretion produced by glucose was accompanied by an increase in islet glutamate. However, glutamine (0.5–2 nM) augmented islet glutamate without affecting insulin secretion, whereas glucose augmented secretion without influencing glutamate levels. Activation of GDH by BCH lowered islet glutamate but increased insulin secretion (4). This dissociation between changes in islet glutamate and insulin secretion was used as argument against a role of β-cell glutamate in the amplification of insulin secretion by glucose (4).

In a recent paper (34), it was suggested that glutamine may serve as a cofactor of insulin release. The hypothesis was based on observations that glutamine elicited a secretory response under conditions of elevated [Ca\(^{2+}\)]; in sulfonfonylurea type 1 receptor (SUR1) knockout islets and because 6-diaz-o-5-oxo-L-norleucine (DON), a glutamate analog, reversed MSO inhibition of glucose-stimulated insulin release.

Because of the contradictory literature, we have revisited this topic. According to our \(^1\)H NMR analysis, the levels of glutamine and glutamate were elevated and aspartate levels lowered islet glutamate but increased insulin secretion (4). This dissociation between changes in islet glutamate and insulin secretion was used as argument against a role of β-cell glutamate in the amplification of insulin secretion by glucose (4).

**REFERENCES**


