Concentration Dependence and Time Course of the Effects of Glucose on Adenine and Guanine Nucleotides in Mouse Pancreatic Islets*

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Changes in the ATP:ADP ratio in pancreatic B cells may participate in the regulation of insulin secretion by glucose. Here, we have investigated the possible role of guanine nucleotides. Mouse islets were incubated in a control medium (when K+–ATP channels are the major site of regulation) or in a high K+ medium (when glucose modulates the effectiveness of cytosolic Ca2+ on electro-tosis). Glucose induced a concentration-dependent (0–20 mM) increase in GTP and a decrease in GDP in both types of medium, thus causing a progressive rise of the ATP:GTP ratio. ATP and ADP levels were 4–5-fold higher but varied in a similar way as those of guanine nucleotides. Insulin secretion was inversely correlated with ADP and GDP levels and positively correlated with the ATP:ADP ratio between 6 and 20 mM glucose in control medium and between 0 and 20 mM glucose in high K+ medium. The increases in the GTP:GDP and ATP:ADP ratios induced by a rise of glucose were faster than the decreases induced by a fall in glucose, but the changes of both ratios were again parallel. In conclusion, glucose causes large, concentration-dependent changes in guanine as well as in adenine nucleotides in islet cells. This raises the possibility that both participate in the regulation of nutrient-induced insulin secretion.

The regulation of pancreatic B cell function differs from that of other secretory cells in that the control of insulin secretion does not depend on the binding of the major physiological stimulator, glucose, to a receptor, but on its metabolism within B cells (reviewed in Refs. 1–5). It is now widely accepted that this metabolism generates several signals that close ATP-sensitive K+ channels (K+–ATP channels)† in the plasma membrane. This closure (hereafter referred to as the “primary mechanism” of control) leads to membrane depolarization with subsequent opening of voltage-dependent Ca2+ channels, Ca2+ influx, rise in cytoplasmic free Ca2+ concentration ([Ca2+]i), and eventual triggering of insulin secretion (4–9). A "second mechanism" of control by glucose exists, which also depends on changes in metabolism (10–12). It does not involve a further change in [Ca2+]i, but an increase in the effectiveness of Ca2+ on its intracellular targets (12). Despite numerous studies, the nature of the signals that link the acceleration of metabolism to the closure of K+–ATP channels and to the increase in Ca2+ action has only partially been elucidated. Purine nucleotides clearly stand out as potential candidates.

The popular hypothesis that variations in the cytosolic concentrations or ratio of adenine nucleotides are involved in the primary mechanism of control by glucose rests primarily on the fact that K+–ATP channels, which control the B cell membrane potential, are regulated by intracellular ATP and ADP (6, 8, 9, 13). Although this hypothesis has long been disputed (reviewed in Ref. 14), our demonstration of a correlation between insulin release and the ATP:ADP ratio in islets incubated in the presence of 6–20 mM glucose at least makes it plausible (15). We have also provided evidence supporting the role of adenine nucleotides in the second mechanism of control by glucose (12).

It has been reported that GTP levels and/or the GTP:GDP ratio in islet cells are increased by glucose (16–19). However, the reported changes were smaller (17–19) and slower (18) than those of adenine nucleotides. Moreover, they were generally observed at one single glucose concentration and, when evaluated further, no concentration dependence could be found (17, 18). This paucity of information on the influence of glucose on guanine nucleotides can largely be explained by the difficulty of measuring GTP and GDP in the small amounts of islet tissue that can be collected. It contrasts with the growing indirect support for a role of GTP in stimulus-secretion coupling in B cells (19–26).

The aim of the present study was to compare the effects of various glucose concentrations on adenine and guanine nucleotide levels in normal mouse islets and to determine whether the observed changes correlate with insulin secretion from the same islets. The time course of the changes was also investigated. The experiments were carried out with overnight cultured islets because the partial degradation that these islets undergo decreases the pool of nucleotides present in insulin granules (15) and, hence, lowers the background above which variations in metabolically active pools are sought. The islets were incubated in a control medium under conditions where the primary mechanism of glucose control (at the K+–ATP channel level) plays the predominant role. They were also incubated in a medium containing diazoxide to keep K+–ATP channels open and high K+ to depolarize the membrane (27), i.e. under conditions where the second mechanism of glucose control can be studied (12, 27).

**EXPERIMENTAL PROCEDURES**

Solutions—The medium used was a bicarbonate-buffered solution that contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2,

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The abbreviations used are: K+–ATP channels, ATP-sensitive K+ channels; [Ca2+]i, cytoplasmic free Ca2+ concentration; NDPK, nucleoside diphosphate kinase.
1 mM Na$_2$HPO$_4$, and 24 mM NaHCO$_3$. It was gassed with O$_2$:CO$_2$ (94:6).

Preparation—Islets were isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), followed by hand-picking. They were cultured for 18–20 h at 37°C in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The concentration of glucose was 10 mM.

Measurements of Insulin Release from Incubated Islets—After culture, the islets were preincubated for 60 min at 37°C in a control medium containing 15 mM glucose, a concentration that causes half-maximal stimulation of insulin release in mouse islets (15). They were then distributed in batches of 10 and incubated for 60 min in 1 ml of medium containing various concentrations of glucose. At the end of the incubation, an aliquot (0.625 ml) of the medium was taken and appropriately diluted for measurement of insulin by a double-antibody radioimmunoassay using rat insulin as a standard (Novo Research Institute, Bagsvaerd, Denmark).

Measurements of Nucleotides in Incubated Islets—After the aliquot for insulin assay was taken (while the tubes remained at 37°C), the islets were incubated for another 5 min. The incubation was stopped by the addition of 0.125 ml of trichloroacetic acid to a final concentration of 5%. The tubes were then mixed by vortex, left on ice for 5 min, and centrifuged (1000 g for 5 min in a Microfuge). A fraction of the supernatant was mixed with 1.5 ml of diethylether, and the ether phase containing trichloroacetic acid was discarded. This step was repeated three times to ensure complete elimination of trichloroacetic acid. The extracts were then diluted with 0.4 ml of a buffer containing 20 mM HEPES, 3 mM MgCl$_2$, and KOH as required to adjust pH to 7.75, followed, except that the first incubation step was performed in the absence of UDP-glucose pyrophosphorylase. UTP levels were estimated with 1 mM NAD, 2 mM glucose-1-phosphate, 1 unit/ml hexokinase, and 5 units/ml UDP glucose pyrophosphorylase. This was followed by boiling for 1 min to destroy the enzyme. Samples were then calculated by the difference between the sum of GTP + UTP and GDP.

GTP levels were then calculated by the difference between the sum of GTP + UTP and GDP. Blank and GTP standards were run through the entire procedure, including the incubation steps. The light emission increased linearly with the GTP concentration within the range of 0.33–2.67 pmol/tube. GTP standards were devoid of UTP but were contaminated by 7% GDP, as verified by high-performance liquid chromatography (30). The standard curve was corrected for this contamination when the sum of GTP + GDP was measured. We also verified that the samples did not cause any quenching of the signal by adding known amounts of GTP to buffer or extracts that had been processed similarly.

One potential pitfall of the procedure is linked to the transformation of GDP into ATP by NDPK. Because the enzyme operates close to equilibrium, ADP must be present in excess to ensure complete transformation. However, commercially available NDPK is contaminated by adenylate kinase that converts ADP into AMP + ATP, and the latter increases the background over which ATP derived from GTP has to be measured. We overcame this difficulty in two ways. NDPK was purified by anion exchange chromatography (DEAE-Sephacel), and the fractions containing the lowest adenylate kinase activity were pooled and concentrated 5–10-fold on Amicon YM 10 filters. Because of this purification, the amount of NDPK used in the reaction is only approximately known. We also fortuitously noticed that phosphoenolpyruvate inhibited the low residual adenylate kinase activity without preventing complete transformation of GTP into ATP by NDPK. Phosphoenolpyruvate (1.5 mM) was, therefore, empirically added to the reaction mixture to lower the background maximally.

Evaluation of the Technique—One drawback inherent to luminometric techniques, like that we have used, is that ADP levels are calculated as a difference between ATP and the sum of ADP + ATP. The accurate measurement of ADP critically depends on the precision of the assay, especially when the absolute levels are low and the ATP:ADP ratio is high. The same holds true for guanine nucleotides, and the greatest difficulty is encountered for islets incubated in the presence of 6 mM glucose or more, when GDP levels are low. Under these conditions, in addition to the great care consistently exercised in the whole procedure, duplicate aliquots of each extracted sample were processed through the different steps of the assay. For these duplicate determinations, the average coefficient of variation was 0.95% for both GDP and the sum of GTP + GDP, with a range of 0.5–1.3%.

Materials—Diazoxide was from Schering-Plough Avondale (Rathburn, Ireland). ATP, ADP, AMP, GTP, GDP, UTP, phosphoenolpyruvate, glucose-1-phosphate, NAD, pyruvate kinase, adenylate kinase, glucose-6-phosphate dehydrogenase, NDPK, UDP-glucose pyrophosphorylase, and hexokinase were from Boehringer-Mannheim. Other reagents were of analytical grade and usually obtained from UCB (Brussels, Belgium).

Presentation of Results and Statistical Analysis—Results are presented as mean ± S.E. for the indicated number of batches of islets that were obtained from 3–5 separate experiments. The statistical significance of differences between means was assessed by an analysis of variance, followed by a Newman-Keuls’ test for multiple comparisons (31).

RESULTS

Concentration Dependence of Glucose-induced Changes in Nucleotide Levels in Islets Incubated under Control Conditions—The nucleotide content of the islets was measured at the end of 1 h of incubation in the presence of various glucose concentrations. As shown in Fig. 1, ATP levels increased 2-fold between 0 and 10 mM glucose (EC$_{50}$ ~3.2 mM) and then plateaued. Simultaneously, ADP levels progressively decreased from 3.8 ± 0.2 pmol/islet in 0 mM glucose to 1.7 ± 0.1 pmol/islet in 20 mM glucose. The first significant effect was observed at 6 mM glucose, and the difference between ADP levels in 10 and 20 mM was still significant (p < 0.01). As a result, the ATP:ADP ratio increased over the whole range of glucose concentrations.

Importantly, the increase was significant (p < 0.01) between 10 and 20 mM glucose (9.0 ± 0.9 versus 12.4 ± 1.4, i.e. when insulin secretion is stimulated.)

GTP levels similarly doubled between 0 and 10 mM glucose (EC$_{50}$ ~5.4 mM) and then remained constant up to 20 mM glucose (Fig. 1), whereas GDP levels progressively decreased from 1.48 ± 0.06 pmol/islet in the absence of glucose to 0.39 ±...
0.04 pmol/islet in 20 mM glucose. The difference between GDP levels in 10 and 20 mM glucose was still significant ($p < 0.05$). These reciprocal changes led to a marked increase in the GTP:GDP ratio over the whole range of glucose concentrations, in particular between 10 and 20 mM glucose (7.6 ± 0.5 versus 13.8 ± 1.4; $p < 0.01$) when insulin secretion is stimulated. UTP levels increased about 5-fold between 0 and 10 mM glucose (EC$_{50}$; 4.8 mM) and then remained constant at higher glucose levels (Fig. 1).

Relationship between Insulin Release and Nucleotide Levels under Control Conditions—We also measured insulin secretion from the same islets in which nucleotide levels were determined. As shown in Fig. 1, the relationship between the rate of release and glucose concentration was sigmoidal (1, 3, 4), with a threshold for stimulation of release at 6 mM glucose. Therefore, correlations between insulin release and nucleotide levels were sought only for glucose concentrations between 6 and 20 mM. No correlation was found between insulin release and either ATP or GTP levels, but a negative correlation was found with ADP or GDP levels (Fig. 2). Good positive correlations were also found with the ATP:ADP or GTP:GDP ratios (Fig. 2). There was no correlation between glucose-induced insulin release and UTP changes in islets (data not shown).

Time Course of Glucose-induced Changes in Nucleotide Levels in Islets Stimulated under Control Conditions—The islets were first incubated for 30 min in a medium containing 1 mM glucose. The concentration of glucose was then increased to 10 mM or kept at 1 mM for an additional period of 5 or 60 min. Raising the glucose concentration led to increases in ATP and GTP levels and to decreases in ADP and GDP levels, which were already maximal (or almost) within 5 min (Fig. 3). The increase in the GTP:GDP ratio paralleled that in the ATP:ADP ratio. Since the increases in ATP and GTP were larger than the decreases in ADP and GDP, the sums ATP + ADP and GTP + GDP were significantly increased ($p < 0.05$ or less) already after 5 min of stimulation with 10 mM glucose. The changes in UTP were much more progressive than those of ATP or GTP: an increase from -0.7 to -1.0 and -2.0 pmol/islet occurred after 5 and 60 min, respectively. To measure the effects of a decrease in glucose concentration, the islets were first incu-
bated for 30 min in a medium containing 10 mM glucose. The concentration of glucose was then decreased to 1 mM (open symbols) or maintained at 10 mM (closed symbols). Values are means (bars, S.E.) for 16 batches of islets from four separate experiments.

Concentration Dependence of Glucose-induced Changes in Nucleotide Levels in Islets Incubated under Depolarizing Conditions—In these experiments, the medium contained 30 mM K⁺ and 100 μM diazoxide to depolarize the B-cell membrane and raise [Ca²⁺], independently of the glucose concentration. As shown in Fig. 5, ATP levels increased between 0 and 10 mM glucose (EC₅₀ = 5.3 mM) and then plateaued, whereas ADP levels decreased mainly between 6 and 20 mM glucose. Within these ranges, there was a significant (p < 0.01) difference between each successive concentration of glucose. This resulted in a linear increase in the ATP:ADP ratio, which reached 5-fold at 20 mM glucose.

GTP levels increased (p < 0.01) up to 15 mM glucose (EC₅₀ = 6.5 mM), whereas GDP levels significantly (p < 0.01) decreased throughout the range of glucose concentrations tested. This resulted in a marked increase in the GTP:GDP ratio between 0 and 20 mM glucose (Fig. 5). UTP levels increased in a similar manner to GTP levels (Fig. 5).

Relationship between Insulin Release and Nucleotide Levels under Depolarizing Conditions—When the islets were incubated in a medium containing 30 mM K⁺ and 100 μM diazoxide, insulin release was already stimulated in the absence of glucose and increased as the glucose concentration was raised (Fig. 5). In a previous study using freshly isolated islets, 3 and 6 mM glucose were found to increase insulin release by ~30% under similar conditions (10). In the present study using cul-
tured islets, 3 mM glucose was ineffective, and the ~20% increase observed in 6 mM glucose did not reach statistical significance. Correlations between insulin release and nucleotide levels were, however, sought over the whole range of glucose concentrations tested (Fig. 6).

Positive correlations were found between insulin release and ATP or GTP levels, whereas negative correlations were found with ADP or GDP levels. This explains the good correlations with both the ATP:ADP and GTP:GDP ratios (Fig. 6).

**DISCUSSION**

This study shows that variations in the ambient glucose concentration induce rapid, parallel changes in guanine and adenine nucleotides in normal mouse islets, and that most of these changes correlate well with insulin secretion from the same islets.

Characteristics of the Changes in Adenine and Guanine Nucleotides—Several studies have investigated the effects of glucose on adenine nucleotides in islet cells. Although glucose was generally found to increase ATP levels and the ATP:ADP ratio, the changes were usually of small amplitude and restricted to glucose concentrations below those which induce insulin release (14, 32–36). It is only in the presence of high K⁺ and diazoxide that the ATP:ADP ratio was found to increase with glucose over a wide range (0–20 mM) of concentrations (12). Recently, we have reported that partial degranulation of the islets decreases a fairly stable (granular) pool of adenine nucleotides. This unmasked large changes in the ATP:ADP ratio and a glucose dependence of these changes, which persisted within the range of stimulatory concentrations also under control conditions (15). The present study entirely confirms our previous findings.

Here, we have also characterized the time course of these changes in adenine nucleotides. Raising the glucose concentration from 1 to 10 mM maximally increased the ATP:ADP ratio within 5 min (the shortest tested time), in agreement with previous studies (18, 34, 35) in which an almost maximal effect was sometimes observed after 1 min (34, 35). The effects of a
decrease in glucose concentration on islet nucleotides have not been reported previously. We show that the ATP:ADP ratio markedly decreased within 5 min and more slowly declined afterwards. These changes were due to a progressive and sustained decrease in ATP and a rise in ADP levels, which was maximal within 5–15 min. Interestingly, the changes in ATP were consistently larger than those in ADP, resulting in a stable –20% increase in the sum ATP + ADP upon elevation of glucose and in a progressive fall in this sum (by –30% after 60 min) upon diminution of glucose. In other, unpublished experiments, we observed only minor differences in AMP levels in islets incubated in low or high glucose. This suggests that variations in the ambient glucose concentration, at least between 0 and 10 mM, are associated with rapid changes in total adenine nucleotide concentrations. The mechanism involved in this synthesis of adenine nucleotides has not been identified, but the phenomenon must be borne in mind when interpreting the effects of the different glucose concentrations. On average, GTP levels corresponded to about 20% of ATP levels. This compares well with a proportion of 23% in two recent studies (19, 22) but is lower than the values of 33–38% of other reports (16, 18). The present study confirms that glucose increases GTP levels and the GTP:GDP ratio in islets (16, 18, 19) and, contrary to a previous study (18), shows that this effect is concentration-dependent and of rapid onset and reversibility. A central message emerging from our results is that guanine nucleotide levels in islet cells change in parallel with adenine nucleotide levels when the concentration of glucose varies, whether under control or depolarizing conditions. This rapid equilibration probably reflects the high NDPK activity present in the islets (37).

Functional Significance of the Changes in Adenine and Guanine Nucleotides—Cells, as a rule, maintain relatively stable levels of adenine and guanine nucleotides under physiological conditions. Pancreatic islet cells are thus peculiar in that their ATP:ADP and GTP:GDP ratios markedly and dose dependently change with the concentration of ambient glucose. This unusual characteristic is probably linked to the fact that glucose has to be metabolized by B cells to induce insulin release (1–5). It has long been known that ATP is necessary for insulin release (38), but whether its role is permissive or regulatory has proved much more difficult to establish. It is also not easy to define the respective roles of adenine and guanine nucleotides because they normally vary in parallel.

Correlations between the rate of insulin release and the ATP:ADP ratio in islets were first observed when the energy state of islet cells was progressively decreased by mitochondrial poisons or by lowering O₂ tension (27, 33, 35). However, it is only recently that correlations between both parameters could be found when the concentration of glucose was varied under normal conditions where the primary mechanism of control, involving K⁺-ATP channels, is studied (15). The present work extends our previous findings in an important manner. It shows that glucose-induced insulin release also correlates with ADP and GDP levels, which raises the possibility that nucleotide diphosphates might be the critical variable.

The effects of nucleotides on K⁺-ATP channels are complex. In the absence of Mg²⁺, ATP, ADP, GTP, and GDP inhibit the channels, albeit with markedly different potencies (ATP is by far the most potent) (6, 8, 39–41). In the presence of physiological concentrations of Mg²⁺, ATP remains inhibitory, but ADP is now stimulatory (6, 8, 9, 13, 40), which supports the proposal that the ATP:ADP ratio has a regulatory role on the channel. On the other hand, it may seem confusing that both GTP and GDP activate K⁺-ATP channels in the presence of Mg²⁺ (20, 40, 41). However, GTP is 12-fold less potent than GDP (41), and the activation could be explained by the contamination of GTP preparations with GDP that we (7% in this specific study) and others (42) have measured. It has also been suggested that GTP is partially degraded into GDP by membrane patches (41). Degradation of ATP into ADP by membrane patches has been demonstrated directly (43, 44). Finally, and most importantly, GDP, like ADP, opens K⁺-ATP channels in the presence of ATP, whereas GTP is ineffective under these conditions (40, 42, 45). Thus, the changes in the ATP:ADP ratio and those in the GTP:GDP ratio exert similar effects on K⁺-ATP channels. They might subserve the control of B-cell membrane potential not only by low, subthreshold glucose concentrations but also by concentrations that stimulate insulin release.

We have recently reported that a correlation exists between the ATP:ADP ratio in islet cells and insulin release under depolarizing conditions (12), when the second mechanism of control by glucose is investigated. The present study confirms our findings and further shows that the correlation holds for the GTP:GDP ratio. The mechanisms of this K⁺-ATP channel-independent regulation of insulin release are still elusive, but potential sites of action of nucleotides can be envisaged. Of particular interest is the ATP-stimulatable Ca²⁺-independent phospholipase A₂ identified in islet cells (46, 47). It is indeed activated by ATP in a concentration-dependent manner, and the effect of ATP is dose dependently inhibited by ADP. Moreover, pharmacologic inhibition of the enzyme decreases glucose-induced insulin release (46). GTP-binding proteins are another potential target. Thus, mastoparan, which is known to activate heterotrimeric (48) and low molecular weight (49) GTP-binding proteins, increases insulin release mainly by acting at a late stage of the secretory process (23, 24, 26). In experiments using permeabilized insulin-secreting cells, guanine nucleotides have been found to induce a Ca²⁺-independent release (21) and to potentiate the secretory response to a rise in [Ca²⁺] (25). The latter effect is particularly relevant to the present observation that the second mechanism of glucose control, which involves a sensitization at the action of Ca²⁺ (10, 12), correlated well with the GTP:GDP ratio.

In an interesting attempt to dissociate the effects of ATP and GTP in stimulus-secretion coupling, Metz et al. (19, 22) have used mycophenolic acid, an inhibitor of IMP dehydrogenase (50), to decrease the de novo synthesis of guanine nucleotides in islet cells. However, the interpretation of their experiments is not straightforward for two reasons. (a) The observed changes in GTP were attended by small changes in ATP (19). (b) The effects of mycophenolic acid on GTP levels and on insulin release were not quantitatively correlated (22). Nevertheless, their results are suggestive of a specific role of GTP in glucose-induced insulin release. The present study indicates that the GTP:GDP ratio and, more particularly, GDP levels might be the important factor.

In conclusion, variations in the ambient concentration of glucose produce rapid and concentration-dependent, parallel changes in adenine and guanine nucleotide levels in islet cells. These changes largely correlate with the changes in insulin release from the same islets, both under control conditions and when [Ca²⁺], in B cells is raised by high extracellular K⁺. This indicates that both types of nucleotides, not only adenine nucleotides as thought previously, may participate in stimulus-secretion coupling and exert their regulatory action at more than one site. Of particular interest are the inverse correlations between insulin release and ADP or GDP levels. They suggest that dinucleotides have a potentially more important function than heretofore suspected. The identification of large effects of glucose on guanine nucleotides in pancreatic B cells also calls...
for an investigation of their role in processes other than insulin secretion.

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