Suppression of Adenylate Kinase Catalyzed Phosphotransfer Precedes and Is Associated with Glucose-induced Insulin Secretion in Intact HIT-T15 Cells*

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L. Karl Olson §*, William Schroeder ††, R. Paul Robertson †, Nelson D. Goldberg ‡‡, and Timothy F. Walseth ‡‡

From the *Department of Pharmacology, †Division of Diabetes, Endocrinology, and Metabolism, Department of Medicine, and ‡‡Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Adenine nucleotide metabolism was characterized in intact insulin secreting HIT-T15 cells during the transition from non-stimulated (i.e. 0.2 mM glucose) to the glucose-stimulated secretory state. Metabolic dynamics were monitored by assessing rates of appearance of [18O]labeled phosphoryls of endogenous nucleotides in cells incubated in medium enriched in [18O]water. Most prominent of the metabolic alterations associated with stimulated insulin secretion was the suppression in the rate of adenylate kinase (AK)-catalyzed phosphorylation of ADP by ATP. This was manifest as a graded decrease of up to 50% in the rate of appearance of β-[18O]-labeled species of ADP and ATP and corresponded to the magnitude of the secretory response elicited over a range of stimulatory glucose concentrations. The only nucleotide exhibiting a significant concentration change associated with suppression of AK activity was AMP, which decreased by about 50% irrespective of the glucose concentration. Leucine-stimulated secretion also decreased the rate of AK-catalyzed phosphotransfer. This secretory stimulus-related suppression of AK-catalyzed phosphotransfer occurs within 45 s of glucose addition, precedes insulin secretion, depends on the internalization and metabolism of glucose, and is independent of membrane depolarization and the influx of extracellular calcium. The secretory stimulus-induced decrease in AK-catalyzed phosphotransfer, therefore, occurs prior to or at the time of K$_{ATP}$ channel closure but it is not associated with or a consequence of events occurring subsequent to K$_{ATP}$ channel closure. These results indicate that AK-catalyzed phosphotransfer may be a determinant of ATP to ADP conversion rates in the K$_{ATP}$ channel microenvironment; secretory stimulus-linked decreased rates of AK-catalyzed ADP generation from ATP (and AMP) would translate into an increased probability of ATP-ligated and, therefore, closed state of the channel.

Although there has been considerable progress in developing an understanding of how a glucose signal is transduced to elicit an insulin secretory response in pancreatic islets (for review, see Ref. 1), some major aspects of the overall mechanism have not been elucidated. Well established is an absolute requirement for glucose to be metabolized, this leads to membrane depolarization due to a decreased conductance of K$^+$ by ATP-sensitive K$^+$ (K$_{ATP}$) channels in the β-cell plasma membrane. This membrane potential change results in the influx of Ca$^{2+}$ via L-type voltage-dependent Ca$^{2+}$ channels. How the metabolism of glucose is coupled to bringing about an increased frequency of K$_{ATP}$ channel closures is not known nor is the mechanism by which K$_{ATP}$ channel behavior is controlled. From in vitro studies it has been established that the K$^+$ conductance by this channel is suppressed when it is liganded with ATP which increases the probability of its “closed” status (2–6); when liganded with ADP the “open” state predominates and the K$^+$ conductance increases (7, 8). How the transition from ATP- to ADP-ligated status of the channel is achieved is not understood.

One currently held view of how glucose effects a more closed (ATP-ligated) state of this channel is through changing the intracellular concentration of ATP or the ATP/ADP ratio (for reviews, see Refs. 1 and 6). The basic premise is that by enhancing glycolytic flux, cytosolic ATP concentration increases and this promotes ATP liganding to the K$_{ATP}$ channels. Opposition to this concept is several fold. Ghosh et al. (9) found no significant changes in β-cell ATP concentration or that of any other adenine nucleotide when they examined nucleotide levels during glucose-induced insulin secretion in a perfused rat pancreas system. The concept can also be challenged on theoretical grounds. For example, the intracellular ATP concentration (e.g. 3–5 mM) is over 100-fold greater than the K$_i$ ATP value for K$_{ATP}$ channels (e.g. 15 μM (2)) and whether any additional increase of an apparently saturating ATP concentration would alter the liganded status of the channel can be seriously questioned. Additionally, the rate of ATP generation is generally conceded to be governed by its rate of utilization rather than driven by the availability of a metabolizable substrate.

Since altered ATP and/or ADP concentrations are not readily detectable nor correlated with secretory stimulus-induced changes in K$_{ATP}$ channel operation, we reasoned that the dynamic transitions of the open/closed states of the channel may also be related to a dynamic rather than a static characteristic of adenine nucleotide metabolism. This was examined by assessing the kinetic behavior of adenine nucleotide metabolism in intact HIT T-15 cells stimulated to secrete insulin by glucose or other secretagogues. Enzyme-catalyzed phosphotransfer velocities were monitored by measuring [18O]phosphoryl exchange rates (10). HIT-T15 cells, an SV-40 transformed Syrian hamster pancreatic β-cell line (11) were chosen because they: 1)
secretes insulin in response to glucose, sulfonlureas, and other metabolic fuel secretagogues (11–13), 2) possess KATP channels with characteristics similar to these channels in isolated pancreatic β cells (14, 15), and 3) provide a sufficient cell mass to permit analysis by the [18O]phosphoryl oxygen exchange procedure.

The results show that stimulus-induced insulin secretion is associated with a marked and glucose concentration-dependent suppression of AK1-catalyzed phosphotransfer process manifest as a reduced rate of AMP phosphorylation by ATP which translates into a decreased rate of ATP conversion to ADP. This occurs when KATP channel conductance is diminished and could account for extending the duration of the ATP-ganged state of the KATP channel or a closely related regulatory component.

EXPERIMENTAL PROCEDURES

HIT Cell Cultures—HIT cells were grown and maintained in RPMI 1640 culture media supplemented with 10% fetal bovine serum, under 5% CO2, 95% O2 at 37°C, as described previously (16). All studies were performed on HIT cell passages between 70 and 75 which have been previously shown to secrete insulin in response to glucose (17). HIT cells were subcultured at a density of 15–20 × 10^6 cells in 100-mm Corning culture dishes 2-3 days before each study. Sixteen hours before each experiment the RPMI 1640 culture media was exchanged with fresh culture media.

Labeling of Endogenous Nucleotide Phosphoryls with 32P in Intact HIT Cells—The standard preincubation procedure protocol for 32P labeling experiments was as follows. HIT cells were preincubated for 70 min at 37°C in 15 ml of Krebs-Ringer bicarbonate (KRB) medium consisting of 118.5 mM NaCl, 2.54 mM CaCl₂·H₂O, 1.19 mM K₂HPO₄, 4.74 mM KCl, 25 mM NaHCO₃, 1.19 mM MgSO₄·7H₂O, 10 mM HEPES pH 7.4, 0.1% bovine albumin, and 0.2 mM glucose to lower insulin secretion to basal levels. During the 70-min preincubation the KRB medium was exchanged once. The cells were then rinsed twice, within a 1-min period, with 10 ml of KRB medium containing 0.2 mM glucose. After the 71-min preincubation the KRB medium was removed and 5 ml of KRB, enriched with a 10–40% atom excess of [18O]water, was added to the cells. The [18O]water-enriched KRB medium also contained a predetermined glucose concentration ranging from 0.2 to 2.8 mM, along with other agents described in the figure legends. 32P labeling experiments were performed as described above except that KRB medium containing 0.25 μCi of 32P, was substituted for the [18O] water-enriched KRB medium. Incubations were terminated by rapid removal of [18O]water-enriched KRB and immediate addition of ice-cold 0.5 mM perchloric acid. While on ice cells were scraped from the surface, transferred along with the perchloric acid to a test tube, and then sonicated. The acidified sonicated cell suspension was centrifuged at 14,500 × g for 10 min to remove precipitated protein. These acid extracts were then neutralized with 2 M KHC₂O₄, the precipitated KClO₄ was removed by centrifugation, and the supernatant was evaporated to dryness in a SpeedVac (Savant). The protein pellet was dissolved in 1 M NaOH and the protein concentration was determined by the BCA method (Pierce). Cellular concentrations of AMP, ADP, ATP, and creatine phosphate were determined by enzymatic fluorometric analysis (18). The cellular levels of ATP, GTP, UTP, and ADP were also determined by UV-absorption upon their elution from Mono Q high performance liquid chromatograph.

Purification and Isotopic Analysis of 5'-Nucleotide Phosphoryls—The purification and analytical procedure for determining the 18O in the phosphoryls of the 5'-nucleotides, orthophosphate, and creatine phosphate has been previously described (10). The only modification of this procedure was the use of a Mono Q HR 5/5 FPLC column equilibrated with triethylammonium bicarbonate, pH 8.8, instead of AG MP-1 chromatography for the purification of the 5'-nucleotides. All the 5'-nucleotides bind to Mono Q resin in 10 mM triethylammonium bicarbonate and are sequentially eluted by increasing the triethylammonium bicarbonate concentration to 1 M. Purification of Experimental Results—The appearance of 18O in the phosphoryls of the 5'-nucleotides is presented as the percentage of phosphoryl oxygen that has been replaced with 18O during the indicated time of incubation. The percentage of nucleotide phosphoryl oxygens replaced by 18O is calculated by the formula, ([%18O]O₂ + 2[18O]O₂ + 3[18O]O₂) / (2[18O]O₂ + [18O]O₂ + [18O]O₂) × 100%. This data was not modeled to obtain estimated velocities (19) except in the case of the results shown in Figs. 2 and 3.

Insulin release, lactate production, and nucleotide levels are presented as the mean ± S.D. Lactate production is presented as the sum of nanomoles of cellular lactate plus the nanomoles of lactate determined in the extracellular media. Statistical significance was determined by Student's t test.

RESULTS

Glucose-induced Insulin Secretion and Lactate Production by HIT Cells—HIT T-15 cells, a clonal cell line of Syrian hamster pancreatic islet β cells (11), secrete insulin in response to glucose (and other secretagogues) in a concentration-dependent manner. These cells are about 10 times more sensitive to glucose than normal islets. Although the reason for this sensitivity difference has not been defined it has been suggested to result from an alteration in glucose transport (20, 21) and/or an expanded role for hexokinase-mediated glucose utilization (22). This transformed cell line behaves identically with islet β cells in almost all other respects. Fig. 1A shows the insulin secretory response in HIT cells at 5 min as a function of glucose concentration. Half-maximal and maximal secretions occur at approximately 1.6 and 2.8 mM glucose, respectively. The time course of insulin secretion and lactate production by a nearly maximal stimulatory (2.8 mM) compared to a minimally stimulatory (0.2 mM) concentration of glucose is shown in Fig. 1, B and C. With 0.2 mM glucose as the stimulus there is a small increase in the insulin as well as lactate generation but only during the first 1 to 2.5 min not thereafter. In the presence of 2.8 mM glucose, insulin secretion is increased fold relative to the 1-min time period by 2.5 min and this stimulated rate is sustained for at least the next 2.5 min. Enhanced secretion although at a submaximal rate occurs for the ensuing 35 min with 2.8 mM glucose but not with 0.2 mM glucose in the medium (not shown). An increase in lactate production is measurable at 45 s which precedes the detectable increase in insulin secretion occurring sometime after 1 min. These experiments were carried out under conditions identical to those in which glucose-induced changes in nucleotide metabolism were examined.

Cellular Nucleotide Levels during Glucose-induced Insulin Secretion—HIT cells were incubated with increasing glucose concentrations for 5 min, after an initial 71-min preincubation in KRB containing 0.2 mM glucose. After acid extraction 5'-nucleotides concentrations were determined by enzymatic fluorometric assay (18). In these experiments, increasing the glucose concentration from 0.2 to 2.8 mM led to increased insulin release from 131.3 ± 15.5 to 359.8 ± 19.6 micromolts of insulin/mg of protein (not shown). During this 5-min incubation with glucose concentrations from 0.2 to 2.8 mM there was no apparent change in cellular ATP concentration (Table I). The concentrations of the individual nucleotides GTP, UTP, CTP, and of creatine phosphate were also found to be unchanged (not shown). Although there appeared to be a decrease of 15–30% in ADP levels (Table I) which resulted in an apparent increase in ATP to ADP ratios (Table I) neither of these changes were determined to be statistically significant nor did they show any gradations that corresponded to the magnitude of the glucose stimulus. The cellular concentration of AMP exhibited a consistent and statistically significant decrease of approximately 50% when HIT cells were incubated with glucose concentrations greater than 0.2 mM (Table I). This decline in AMP concentration corresponds with the observation to be described below of a glucose-induced suppression of the rate of adenylyl kinase-catalyzed phosphorylation of AMP. However, this decrease in AMP concentration also did not exhibit any gradations related to the glucose concentration.

Dynamics of ATP and ADP Metabolism during Glucose-in-
rates of flux with glucose-induced insulin secretion. The calculated time course of nineteen nucleotide metabolism during glucose-induced insulin secretion—

...with glucose-induced insulin secretion. HIT cells were incubated with the indicated glucose concentrations for 5 min after the standard preincubation protocol. Each value represents the mean ± S.D. of triplicate samples from three independent experiments. B, time course of lactate production in glucose-treated cells. Cells were incubated in 0.2 mM (open squares) or 2.8 mM glucose (filled squares) for the subsequent times indicated. Insulin was assayed in the media, whereas lactate is the sum represented by both the media and cell acid extracts. Panels B and C show a representative experiment of three identical experiments that yielded very similar results. The results in panels B and C were obtained from the same cells used for the 18O labeling experiment shown in Figs. 2 and 3. Each value represents the mean ± S.D. of three determinations.

Reduced Insulin Secretion—The dynamics of intracellular adenine nucleotide metabolism during glucose-induced insulin secretion were monitored by preincubating HIT cells in KRB containing 0.2 mM glucose for 71 min, then replacing the medium with [18O]water-enriched KRB containing either 0.2 or 2.8 mM glucose and continuing the incubation for various times. The time course of [γ-18O]phosphoryl appearance in ATP even in the presence of 0.2 mM glucose (minimally stimulatory) is relatively rapid, with almost 25% of the cellular ATP undergoing labeling within 15 s (Fig. 2). Isotopic equilibrium is nearly complete by 5 min with greater than 95% of the total cellular ATP labeled. In the presence of 2.8 mM glucose there is a small, but consistently observed (14 experiments), increase in the rate of [γ]ATP labeling signifying a greater rate of ATP metabolic flux with glucose-induced insulin secretion. The calculated rates of [γ]ATP 18O labeling at 0.2 and 2.8 mM glucose were 49.2 and 59.3 nmol of 18O min⁻¹ mg of protein⁻¹, respectively. The glucose-induced increase in the rate of [γ]ATP labeling exhibits a dependence on the concentration of glucose, with maximal stimulation occurring at 2.8 mM glucose (Fig. 2, inset). The transition from 0.2 to 2.8 mM glucose also increased the rate of appearance of [18O]P, from 102 to 127 nmol of 18O min⁻¹ mg of protein⁻¹ (not shown), confirming that overall ATP metabolic flux is increased by about 25%. This increased rate of ATP utilization is most probably related to the increased energy demand of the glucose-stimulated secretion. Nevertheless, this information would not coincide with any postulated in...

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**Fig. 1.** Characteristics of glucose-induced insulin secretion and lactate production in HIT cells. A, glucose concentration dependence for insulin release. HIT cells were incubated with the indicated glucose concentrations for 5 min after the standard preincubation protocol. Each value represents the mean ± S.D. of triplicate samples from three independent experiments. B, time course of glucose-induced insulin release. C, time course of lactate production in glucose-treated cells. Cells were incubated in 0.2 mM (open squares) or 2.8 mM glucose (filled squares) for the subsequent times indicated. Insulin was assayed in the media, whereas lactate is the sum represented by both the media and cell acid extracts. Panels B and C show a representative experiment of three identical experiments that yielded very similar results. The results in panels B and C were obtained from the same cells used for the 18O labeling experiment shown in Figs. 2 and 3. Each value represents the mean ± S.D. of three determinations.

**Fig. 2.** Time course and glucose concentration dependence (inset) for the appearance of [γ-18O]phosphoryls in ATP during glucose-induced insulin secretion from HIT cells. HIT cells were then incubated with either 0.2 mM (open squares) or 2.8 mM glucose (closed squares) in KRB medium enriched with 12.2% atom excess of [18O]water for the times indicated after the standard preincubation protocol. The media was removed and assayed for insulin release and lactate production. Data are expressed as the percentage of the γ-phosphoryls of total cellular ATP labeled with 18O. A representative experiment of three identical experiments that yielded very similar results is shown. Each value represents the mean ± S.D. The standard deviations are smaller than the symbols and therefore have been omitted. The results are from the same cells used to determine insulin secretion and lactate production shown in Fig. 1, B and C, and the appearance of [γ-18O]ATP and [γ-18O]ADP shown in Fig. 3. The inset shows the glucose concentration dependence of the enhanced appearance of 18O in the γ-phosphoryl of ATP during a 1-min incubation. The data in the inset is presented as the percentage of maximal stimulation, whereby the maximum difference in the fraction of [γ]ATP-labeled in cells incubated in 2.8 mM compared to 0.2 mM glucose has been equalized to 100% stimulation. The inset shows a representative experiment of two identical experiments from which very similar results were obtained.

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**Table I**

| Glucose (mM) | ATP (nmol/mg protein) | ADP (nmol/mg protein) | AMP (nmol/mg protein) | ATP/ADP |
|-------------|------------------------|------------------------|------------------------|---------|
| 0.2         | 16.5 ± 1.4             | 2.8 ± 0.4              | 0.46 ± 0.08            | 6.0 ± 0.2 |
| 0.8         | 18.6 ± 0.9             | 2.3 ± 0.2              | 0.20 ± 0.03            | 9.2 ± 0.6 |
| 1.6         | 15.4 ± 3.2             | 2.0 ± 0.8              | 0.23 ± 0.03            | 7.7 ± 1.0 |
| 2.8         | 18.5 ± 2.0             | 2.4 ± 0.4              | 0.27 ± 0.06            | 8.0 ± 0.3 |

* p < 0.005.
cose-induced insulin secretion and this decreased the rate of 
18O-labeled labeling, the transition to the higher glucose concentration ofPi labeling with 18O is indicative of an increased rate of ATP
crease in cellular ATP concentration because the increased rate ofP, labeling with 18O is indicative of an increased rate of ATP
hydrolytic consumption which is undoubtedly followed by a commensurately increased rate of ATP regeneration to provide for the constancy of the overall cellular ATP concentration.

In contrast to the increase in the rates of [γ]ATP and P, labeling, the transition to the higher glucose concentration decreased the rate of 18O-labeled β-phosphoryl appearance in both ATP and ADP (Fig. 3A and B). The decrease in [γ]ATP and [β]ADP 18O-labeling occurs within 45 s (Fig. 3) and precedes enhanced insulin secretion which occurs after 1 min (Fig. 1B) coincident with the increase in lactate production (Fig. 1C).

The appearance of 18O-labeled β-phosphoryls in ADP arises as a result of AK-catalyzed transfer of isotopically labeled γ-phosphoryls of endogenous ATP to AMP and then it can appear as the β-phosphoryl of ATP as a result of the subsequent phosphorylation of [β,18O]ADP to [β-18O]ATP. These results therefore demonstrate that the transition from 0.2 to 2.8 mM glucose decreases the rate of AK-catalyzed phosphotransfer involving the phosphorylation of AMP by ATP.

Since [γ]ATP labeling is increased in association with glucose-induced insulin secretion and this [γ-18O]ATP is the precursor of AK-catalyzed phosphorylation of AMP generating [β-18O]ADP and [β-18O]ATP this must be taken into account in modeling the data to estimate the absolute rate of AK catalysis within the cell. This rate was calculated to decrease from 27.7 to 14.3 nmol of [18O] min\(^{-1}\) mg of protein\(^{-1}\) or by 48% with a 2.8 mM glucose stimulus. These estimates of absolute phosphotransferase velocities require three successive temporally displaced measurements which were obtained in the experiment for which these velocities were calculated. AK catalysis in subsequent experiments were assessed by the percentage of the total cellular ADP and ATP with 18O-labeled β-phosphoryls without modeling the results and therefore without correcting for the enhanced 18O labeling of [γ]ATP. Therefore, the percentage 18O labeling values reported for the subsequent experiments minimize the magnitude of the decrease in AK catalyzed phosphotransfer induced by glucose.

Glucose-induced changes in the rate of appearance of 18O-labeled β-phosphoryls of ATP and ADP were further characterized by monitoring the appearance of 18O-labeled β-phosphoryls for 2-min labeling durations representing the initial 2 min, the second 2 min, or the third 2-min interval after the transition from 0.2 to 2.8 mM glucose. Table II shows that when HIT cells are incubated with 0.2 mM glucose the percentage of the total ADP and ATP in which 18O-labeled β-phosphoryls appear is relatively constant for each 2-min interval examined. However, when the cells are incubated in 2.8 mM glucose the relative rate of 18O appearance is decreased within the first 2 min and this suppression of [β,18O]phosphoryl appearance is sustained at nearly the same or at a further diminished level during the next 2–4- and 4–6-min time intervals. The results in Table II also confirm that this glucose-induced suppression of [β,18O]phosphoryl appearance in ATP and ADP occurs very early (i.e. between 0 and 2 min) after 2.8 mM glucose addition and precedes enhanced insulin secretion which was not detectable in this experiment until the 2–4-min interval.

Glucose-concentration Dependence of Suppressed AK-catalyzed Phosphotransfer Activity Relative to Stimulated Insulin Release—The suppression of [β,18O]phosphoryl appearance in both ATP and ADP occurs over a very similar range of glucose concentrations that enhances insulin secretion. Fig. 4 shows that increasing the glucose concentration from 0.2 to 2.8 mM glucose, results in incrementally enhanced insulin secretion and correspondingly greater suppression in the appearance of 18O-labeled β-phosphoryls in ATP and ADP during a 5-min period of glucose stimulation. Glucose-induced suppression of AK-catalyzed phosphotransfer is shown in Fig. 4C to occur decrementally over a range of glucose concentrations up to at least 2.8 mM glucose during a 1-min period of stimulation. It is important to note that insulin secretion was not enhanced during this initial 1-min period of stimulation by this range of glucose concentrations (not shown for this experiment; also see Figs. 1B and 6).

32P Incorporation into Adenine Nucleotide β-Phosphoryls Is Also Attenuated by Glucose—The incorporation of 32P into cellular adenine nucleotides was monitored during glucose-induced insulin secretion from HIT cells as an independent, semiquantitative means to further investigate the glucose-induced changes in nucleotide metabolism uncovered by the 18O labeling procedure. The time-dependent studies of the incorporation of 32P into adenine nucleotides during glucose-induced insulin secretion were performed as described for the 18O labeling experiments except that the KRB media contained 0.25 mCi of 32P, in these experiments the transition from 0.2 to 2.8 mM glucose led to a 2–3-fold increase in both insulin release and lactate production by 5 min (not shown, but similar to the data shown in Fig. 1). Increasing the glucose concentration to 2.8 mM increased the rate of total 32P incorporation into cellular ATP (Fig. 5A). Analysis of the individual phosphoryls

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**Fig. 3. Time course of the appearance of [β-18O]phosphoryls in ATP (A) and ADP (B) during glucose-induced insulin secretion.**

HIT cells were incubated in KRB medium containing 0.2 mM (open squares) or 2.8 mM glucose (filled squares) and enriched with a 12.2% atom excess of [18O] water for the times indicated after the standard preincubation protocol. The medium was removed and assayed for insulin release and lactate production. The results are expressed as the percentage of cellular ATP and ADP with β-phosphoryls labeled with 18O. A representative experiment of three identical experiments that yielded very similar results is shown. Each value represents the mean ± S.D. The standard deviations are smaller than the symbols and therefore cannot be seen. The results in Fig. 3 were obtained from the same cells used to determine insulin secretion and lactate production shown in Fig. 1, B and C, and the appearance of [γ-18O]ATP in Fig. 2.

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**Adenylate Kinase Phosphotransfer and Insulin Secretion**

16547
Adenylate Kinase Phosphotransfer and Insulin Secretion

Intervals of the appearance of \(^{18}\text{O}\) in the \(\gamma\)-phosphoryls of ATP and \(\beta\)-phosphoryls of ATP and ADP during glucose-induced insulin secretion

HIT cells were incubated with either 0.2 or 2.8 mM glucose for either 2, 4, or 6 min. At the specified times the incubation media was removed and either 0.2 or 2.8 mM glucose in KRB medium containing 12% atom excess of \(^{18}\text{O}\) water was added for 2 min. At the end of the 2-min incubation the KRB medium was removed and the cells were acid extracted and the nucleotide phosphoryls examined for \(^{18}\text{O}\) content as described under "Experimental Procedures." Total insulin secretion is presented as the sum of insulin secreted in the first glucose incubation (2- or 4-min \(^{18}\text{O}\) water-containing KRB medium) and the second 2-min incubation with 0.2 or 2.8 mM glucose in \(^{18}\text{O}\) water-enriched KRB medium. Incorporation of \(^{13}\text{C}\) into the nucleotide phosphoryls is presented as the percentage of the total cellular nucleotide isotopically labeled. Each value represents the mean ± S.D. of triplicate samples.

| Glucose | Length of glucose incubation | Time of \(^{18}\text{O}\)-KRB addition | Insulin release | \(\%[^{18}\text{O}]\text{ATP}\) | \(\%[^{18}\text{O}]\text{ADP}\) | \(\%[^{13}\text{C}]\text{ADP}\) |
|---------|----------------------------|-------------------------------|----------------|----------------|----------------|----------------|
| 0.2     | 0.0–2.0                    | 0.0–2.0                       | 110.9 ± 13.3  | 69.1 ± 0.1    | 50.9 ± 0.6    | 47.4 ± 0.3    |
| 0.2     | 0.0–4.0                    | 2.0–4.0                       | 165.0 ± 15.1  | 70.3 ± 0.4    | 50.5 ± 0.6    | 48.2 ± 0.2    |
| 0.2     | 0.0–6.0                    | 4.0–6.0                       | 159.0 ± 8.3   | 69.8 ± 0.4    | 50.4 ± 0.1    | 48.1 ± 0.2    |
| 2.8     | 0.0–2.0                    | 0.0–2.0                       | 100.9 ± 5.3   | 73.2 ± 0.1    | 31.9 ± 0.6    | 30.5 ± 0.3    |
| 2.8     | 0.0–4.0                    | 2.0–4.0                       | 283.1 ± 20.2  | 73.8 ± 0.5    | 25.6 ± 0.4    | 24.1 ± 1.7    |
| 2.8     | 0.0–6.0                    | 4.0–6.0                       | 388.9 ± 33.3  | 72.9 ± 0.6    | 28.1 ± 0.6    | 21.5 ± 0.6    |

showed that increasing the glucose concentration to 2.8 mM glucose resulted in increased incorporation of \(^{32}\text{P}\) into the \(\gamma\)-phosphoryls of ATP but diminished incorporation of \(^{32}\text{P}\) into the \(\beta\)-phosphoryls of ATP. This confirms the observations made with the \(^{18}\text{O}\) labeling procedure with respect to both the greater rate of \(\gamma\)-ATP turnover and suppression of AK-catalyzed phosphorylation of AMP by ATP. The addition of 2.8 mM glucose also inhibited the incorporation of \(^{32}\text{P}\) into the cellular pool of ADP (Fig. 5B). The inhibition of \(^{32}\text{P}\) labeling of the \(\beta\)-phosphoryls in both ATP and ADP is detectable within 1 min after glucose addition and precedes enhanced insulin release (not shown). No \(^{32}\text{P}\) incorporation was detected in the \(\alpha\)-phosphoryls of ATP or ADP. These results confirm the \(^{18}\text{O}\)phosphoryl labeling results showing that glucose induces a suppression of AK-catalyzed phosphotransfer.

Glucose Uptake and Metabolism Are Required for Both Glucose-induced Insulin Secretion and the Suppression of AK-catalyzed Phosphotransfer—Cytochalasin B, a glucose transport inhibitor, was used to further characterize the glucose-induced suppression of AK catalysis associated with stimulating insulin secretion. As observed previously, upon the addition of 2.8 mM glucose, HIT cells respond with a greater than 2-fold increase in both insulin secretion and lactate production, with accompanying suppression of the appearance of \(^{18}\text{O}\)-labeled \([\beta]\text{ATP}\) and \([\beta]\text{ADP}\) (Table III). The addition of 10 \(\mu\text{M}\) cytochalasin B was sufficient to lower lactate production below the level observed with 0.2 mM glucose and this same level was also achieved with 2.8 mM glucose in cells treated with cytochalasin B (Table III). This inhibition of glucose transport by cytochalasin B also prevented the stimulated insulin release ordinarily observed with 2.8 mM glucose (Table III). Cytochalasin B also completely blocked the ability of 2.8 mM glucose to suppress \(^{18}\text{O}\)-labeled \(\beta\)-phosphoryl appearance in ATP and ADP. Cytochalasin B treatment caused no significant changes in the cellular nucleotide concentrations (not shown).

Iodoacetate was used to examine whether impairment of glucose metabolism would interfere with glucose-stimulated insulin secretion as well as the suppression of \([\beta-^{18}\text{O}]\)phosphoryl appearance in adenine nucleotides. Iodoacetate (0.9 mM) inhibited the ability of 2.8 mM glucose to stimulate insulin secretion, which coincided with an iodoacetate-induced decrease in glycolytic rate as indicated by the suppression of lactate production (Table IV). Iodoacetate also prevented the suppression of \(^{18}\text{O}\)-labeled phosphoryl appearance in \([\beta]\text{ATP}\) and \([\beta]\text{ADP}\) ordinarily seen with stimulation by 2.8 mM glucose, although the blockade by iodoacetate was not complete in the case of the \(\beta\)-phosphoryl of ATP (Table IV). Iodoacetate also reduced the ATP concentration to 60 and 30% of the control levels in cells stimulated with 0.2 and 2.8 mM glucose, respectively (not shown). These decreases in cellular ATP concentrations are undoubtedly related to the inhibitory effect of iodoacetate on glycolysis but the reason for the greater decrease with the higher glucose concentration was not established.

Effect of Leucine or Arginine on Insulin Secretion and AK-catalyzed Phosphotransfer—Leucine is the most potent physiological non-glucose stimulator of insulin release. With 0.2 mM glucose in the medium, the addition of 20 mM leucine induced a 2-fold increase in insulin release without altering the rate of lactate production (Table IV). The addition of leucine also caused a decrease in AK-catalyzed phosphotransfer as indicated by the decreased appearance of \([\beta-^{18}\text{O}]\)ATP and \([\beta-^{13}\text{C}]\)ADP that is qualitatively similar to the effect produced by 2.8 mM glucose (Table IV). Unlike leucine, the amino acid arginine, which is a potentiator of insulin release with stimulatory concentrations of glucose greater than 0.2 mM, did not stimulate insulin release or decrease the appearance of \([\beta-^{18}\text{O}]\)ATP and \([\beta-^{13}\text{C}]\)ADP in the presence of 0.2 mM glucose (Table IV). These results suggest that only the metabolizable (i.e. glycolysis and/or tricarboxylic acid cycle) insulin secretagogues, such as glucose and leucine, lead to suppression of AK-catalyzed phosphotransfer manifest as a reduction in \([\beta-^{18}\text{O}]\)ATP and \([\beta-^{13}\text{C}]\)ADP generation.

Glucose-induced Suppression of AK Catalysis Is Independent of Membrane Depolarization and Extracellular Calcium—The effect of high concentrations of \(\text{K}^+\), sulfonylureas, and extracellular calcium on the appearance of \([\beta-^{18}\text{O}]\)ATP and \([\beta-^{13}\text{C}]\)ADP were examined to determine if a relationship between AK-catalyzed phosphotransfer and membrane depolarization and/or calcium influx existed. In control cells stimulated with 2.8 mM glucose for only 1 min, there was no enhanced insulin release, but at this early interval of stimulation, there was a measurable decrease in the appearance of \([\beta-^{18}\text{O}]\)ATP and \([\beta-^{13}\text{C}]\)ADP (Fig. 6). The addition of 40 mM KCl even after 1-min increased insulin release strikingly, presumably as a result of its action to directly depolarize the HIT cell plasma membrane causing an influx of extracellular calcium. This \(\text{K}^+\)-induced release of insulin occurred without affecting AK-catalyzed phosphotransfer (Fig. 6). These results indicate that the suppression of AK-catalyzed phosphotransfer elicited by a metabolizable secretagogue does not result from and therefore probably occurs prior to membrane depolarization.

The addition of the sulfonylurea, glipizide (1 \(\mu\text{M}\)), stimulated insulin secretion both in the absence and presence of stimulatory concentrations of glucose (Table III). The mechanism of glipizide-induced insulin secretion is thought to involve a direct inhibition of the \(\text{K}^+_{\text{ATP}}\) channel, leading to \(\beta\) cell membrane
depolarization and a subsequent influx of extracellular calcium. As observed with 40 mM KCl, the addition of glipizide in the presence of a minimally stimulatory concentration of glucose resulted in enhanced secretion of insulin but did not alter the magnitude of $[^{18}O]ATP$ and $[^{18}O]ADP$ appearance.

However, when glipizide was augmented by 2.8 mM glucose suppression of AK-catalyzed $[^{18}O]ATP$ and $[^{18}O]ADP$ labeling occurred, even though this concentration of glucose only enhanced glipizide-induced insulin release to a relatively small extent (17%).

Extracellular calcium is required for glucose induced-insulin release but its involvement is believed to be manifest distal to stimulus-induced membrane depolarization. Extracellular calcium concentrations of 0, 2.5, and 5 mM were examined to determine whether the influx of extracellular calcium was responsible for the suppression of AK-catalyzed production of $[^{18}O]ATP$ and $[^{18}O]ADP$ inducible by 2.8 mM glucose. In the presence of either 2.5 or 5.0 mM Ca$^{2+}$, the addition of stimulatory glucose concentrations (i.e. 0.8 and 2.8 mM) led to increased insulin release and a glucose concentration-dependent suppression of the appearance of AK-generated $[^{18}O]ATP$ and $[^{18}O]ADP$ (Table V). When the cells were incubated with calcium-free KRB supplemented with 5 mM EGTA the cells no longer secreted insulin in response to 0.8 or 2.8 mM glucose, however, the effect of glucose to suppress the appearance of $[^{18}O]$-labeled AK-catalyzed nucleotides in ATP and ADP in a glucose concentration-dependent manner was preserved. The higher levels of insulin found in the Ca$^{2+}$-free medium (EGTA supplemented) probably results from this Ca$^{2+}$-free condition to permeabilize the cells. These results show that the effect of glucose to suppress AK-catalyzed phosphotransfer is not dependent on the influx of extracellular Ca$^{2+}$.

**DISCUSSION**

The experimentation described here provides the first information about the dynamics of high energy phosphoryl metabolism in intact insulin secreting cells. Our results show that stimulatory concentrations of glucose cause a relatively rapid and graded suppression of AK-catalyzed phosphotransfer closely corresponding to the magnitude of the glucose stimulus.
Adenylate Kinase Phosphotransfer and Insulin Secretion

HIT cells were incubated for 3 min with 0.2 or 2.8 mM glucose in KRB medium enriched with 12.4% atom excess of $^{18}$O water, in the absence or presence of 10 μM cytochalasin B. The media were removed and the cells were acid extracted. Insulin release, lactate production, and the incorporation of $^{18}$O into the nucleotide phosphorus were determined as described under “Experimental Procedures.” Data are expressed as the percentage of cellular ATP and ADP with $^{18}$O-labeled phosphates labeled with $^{18}$O. A representative experiment of two similar experiments that yielded similar results is shown. Each value represents the mean ± S.D. from triplicate determinations.

| Glucose | Additions | Insulin release | Lactate production | Percent $[^{18}\text{O}]\text{ATP}$ | Percent $[^{18}\text{O}]\text{ADP}$ |
|---------|-----------|-----------------|-------------------|-----------------------------|-----------------------------|
| 0.2 | Control | 60.4 ± 2.9 | 7.8 ± 0.2 | 70.8 ± 0.5 | 70.6 ± 0.7 |
| 2.8 | Control | 156.5 ± 5.3 | 16.6 ± 0.5 | 44.6 ± 0.1 | 38.6 ± 0.1 |
| 0.2 | Cytochalasin B | 66.7 ± 8.3 | 2.5 ± 0.1 | 70.0 ± 0.1 | 64.7 ± 0.2 |
| 2.8 | Cytochalasin B | 58.1 ± 3.0 | 2.4 ± 0.1 | 70.8 ± 0.2 | 65.8 ± 0.1 |
| 0.2 | Glipizide | 215.7 ± 15.3 | 4.0 ± 0.5 | 69.2 ± 0.1 | 68.5 ± 0.8 |
| 2.8 | Glipizide | 261.7 ± 14.8 | 13.1 ± 2.0 | 43.9 ± 0.1 | 39.1 ± 0.4 |

The effect of iodoacetate, leucine, or arginine on insulin secretion, lactate production, and the appearance of $^{18}$O-labeled β ATP and β ADP

HIT cells treated with iodoacetate were preincubated with 0.9 mM iodoacetate for the last 10 min of the 71-min standard preincubation protocol prior to the addition of $[^{18}$O]water-enriched KRB medium. Cells were then incubated for 5 min with either 0.2 or 2.8 mM glucose in the absence or presence of 0.9 mM iodoacetate or in 0.2 mM glucose in the absence or presence of 10 mM arginine or 20 mM leucine in KRB medium enriched with 11.3% atom excess of $[^{18}$O]water. The media was then removed and the cells were acid extracted. Insulin release, lactate production, nucleotide levels and $^{18}$O incorporation into the nucleotide phosphorus were determined as described under “Experimental Procedures.” Data are expressed as the percentage of cellular ATP and ADP with $^{18}$O-labeled phosphates labeled with $^{18}$O. A representative experiment of two experiments that yielded similar results is shown. Each value represents the mean ± S.D. of triplicate determinations.

| Glucose | Additions | Insulin release | Lactate production | Percent $[^{18}\text{O}]\text{ATP}$ | Percent $[^{18}\text{O}]\text{ADP}$ |
|---------|-----------|-----------------|-------------------|-----------------------------|-----------------------------|
| 0.2 | Control | 63.8 ± 10.1 | 1.6 ± 0.3 | 82.4 ± 0.2 | 74.8 ± 0.2 |
| 2.8 | Control | 238.9 ± 3.5 | 26.6 ± 0.9 | 60.0 ± 0.5 | 56.4 ± 0.2 |
| 0.2 | Iodoacetate | 57.9 ± 4.3 | 2.2 ± 1.5 | 82.4 ± 0.2 | 75.7 ± 0.2 |
| 2.8 | Iodoacetate | 56.4 ± 0.9 | 3.2 ± 1.6 | 75.7 ± 0.6 | 75.7 ± 0.3 |
| 0.2 | Leucine | 127.8 ± 13.8 | 1.7 ± 0.4 | 68.6 ± 0.3 | 60.1 ± 0.4 |
| 0.2 | Arginine | 62.2 ± 0.9 | 2.4 ± 0.6 | 81.4 ± 0.1 | 75.1 ± 0.1 |

The mechanism by which glucose causes a change in the composition of adenine nucleotides so they may serve as effectors of the $K_{ATP}$ channel is poorly understood and highly controversial. Although it is tacitly assumed that the determinant is a change in the cellular ATP and/or ADP concentration there are numerous arguments that oppose this view. Whether glucose even effectively alters total ATP concentrations in the β cell is controversial. For example, it has been shown that stimulatory concentrations of glucose increase ATP concentrations in islets only if they have been first attenuated by restricting glucose from the islet (25–27). However, when islets are maintained in non-stimulatory concentrations of glucose, and then stimulated with higher concentrations of glucose, there are marginal or no changes detected in total cellular ATP concentration (9, 27, 28). In addition, there are no changes in ATP/ADP ratios in response to high glucose stimulation (9, 27–30) unless HIT cells (31) or islets were previously fuel restricted (27, 29, 32, 33). Our observations that HIT cells maintained in low glucose concentrations and exposed to stimulatory concentrations of glucose do not significantly change their intracellular levels of ATP and only marginally alter their ADP levels, is consistent with the latter observations cited (9, 27–30) and do not support the concept that increases in the concentration of intracellular ATP or the ATP/ADP ratio serves as the signal for regulating the $K_{ATP}$ channel. As in islets (28), the only adenine nucleotide in HIT cells to undergo a significant change in response to increased glucose concentration was AMP. Although this decreased AMP level corresponds with and may be a critical determinant of the observed glucose-induced decrease in AK-catalyzed phosphotransfer, two important aspects of this altered AMP level remain undefined. What metabolic alteration underlies this decrease in AMP and why is the decrease a relatively constant 50% irrespective of the stimulatory glucose concentration, when suppression of AK catalysis is graded relative to the glucose concentration? Only speculation can be offered at this point. The only source of AMP that could account for its relatively rapid rate of AK-catalyzed phosphorylation is...
its generation from ADP by AK-catalyzed phosphotransfer (2ADP → 2AMP + ATP). This ADP according to the current operational model of the AK phosphotransfer system (19, 34) derives from ATP consumed by specific cellular ATPases. The observed decrease in AMP concentration could, therefore, have resulted from a glucose-induced decrease in a specific ATPase as suggested by Levin et al. (38) or an increase in the rate of ADP processing by a metabolic system competing with AK (i.e. creatine kinase, glycolytic enzymes, etc.). The apparent disparity between the constant decrease in AMP concentration and decremental suppression of AK catalysis may arise because a rate, determined in this instance by net accumulation of 18O atoms possibly arising intermittently during a fixed time does not equate with the measurement of a steady-state metabolite level.

Until recently there has been little or no regulatory importance attributed to AK-catalyzed phosphotransfer. In intact skeletal muscle, evidence has been provided that AK operates as a high energy phosphoryl transfer system that also regulates the rate of glycolytic ATP generation so that it does closely correspond with the rate of ATP utilization by specific energy consuming processes (19, 34). The feature common to the control of muscle glycolysis and nutrient-stimulated insulin secretion is that both processes are regulated by ATP and its metabolic product ADP. In the case of muscle glycolysis, specific enzymes including phosphofructokinase, aldolase, and glyceraldehyde phosphate dehydrogenase (35-37) are allosterically inhibited by ATP and this inhibition is relieved or enzyme activities are stimulated by ADP. The adenine nucleotide-sensitive counterpart in the insulin secretory system is the $K_{ATP}$ channel; it is also inhibited (i.e. closed) as a result of ATP liganding and stimulated (i.e. opened) by ADP.

Within this frame of reference AK could function in the $\beta$ cell as a high energy phosphoryl transfer system that could also regulate the composition of adenine nucleotide species at the $K_{ATP}$ site. The glucose-induced changes in AK phosphoryl transfer in the HIT cell temporarily correlate with the closure of the $K_{ATP}$ channel. In the HIT cell, 2.8 mM glucose decreased AK activity within 45 s. Eddlestone et al. (14) have reported that in the HIT cell closure of the $K_{ATP}$ channel commences within 1-3 min and a new steady-state of $K_{ATP}$ activity is reached within 2-9 min after the addition of glucose (14). In addition, the changes in AK activity occur within the same glucose concentration range required for insulin secretion and for closure of the $K_{ATP}$ channel (14). Eddlestone et al. (14) have reported that in HIT cells there is a 50% reduction in $K_{ATP}$ channel closure in response to 0.45 mM glucose and a maximum number of closures with 8.0 mM glucose. The link between suppression of AK-catalyzed phosphotransfer and alterations in $K_{ATP}$ channel conductance is further supported by the observations that leucine stimulates insulin release, decreases AK-catalyzed phosphotransfer, and leads to the closure of $K_{ATP}$ channel in the HIT cell (14). That AK is physically close to the $K_{ATP}$ channel can be concluded from the recent report that AMP addition to an ATP-inhibited $K_{ATP}$ channel in an isolated inside-out patch of $\beta$ cell membrane results in rapid opening of the channel (8). In addition, AK activity has been measured in isolated plasma membranes from HIT cells and isolated islets.²

The activity of AK at the channel site could be envisioned to regulate the duration of the ATP- or ADP-liganded state by the rate of its catalytic action to transform ATP to ADP with AMP serving as the critical reactant (i.e. ATP + AMP → ADP). The duration of the ATP-liganded state would depend on the rate that AMP is generated and made available, probably through the AK catalyzed transfer system, to the site or the microenvironment of the $K_{ATP}$ channel. The decreased rate of AK-cata-

² L. K. Olson, P. Dzeja, T. F. Walseth, and N. D. Goldberg, unpublished results.
alyzed phosphotransfer observed in the experiments reported here that precedes stimulation of secretion would coincide with a diminished rate of generation of AMP (i.e. $2 \text{ADP} \rightarrow \text{AMP} + \text{ATP}$), transfer of AMP, and/or subsequent conversion of ATP to (2)ADP at the channel site. This would extend the duration of the ATP-ligated state and decrease the duration of the ADP-ligated state, which would result in $K_{\text{ATP}}$ channel closure, membrane depolarization, calcium influx, and insulin secretion.

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