Rat Islet Mitochondrial Adenine Nucleotide Translocase and the Regulation of Insulin Secretion

R. B. L. EWART, S. Y. K. YOUSUFZAI, M. W. BRADFORD, AND E. SHRAGO

SUMMARY

Employing a preparation of rat islet mitochondria, phosphoenolpyruvate has been shown to interact with the mitochondrial adenine nucleotide translocase. Thus, phosphoenolpyruvate inhibited mitochondrial uptake of [^{14}C]ADP and exchanged with intramitochondrial [^{14}C]ATP. A concentration-dependent inhibition of islet mitochondrial $^{45}Ca^{2+}$ accumulation was seen when mitochondria were exposed to phosphoenolpyruvate with half-maximal inhibition observed at a phosphoenolpyruvate concentration of 0.2 mM.

In experiments employing whole islets, phosphoenolpyruvate content was shown to be significantly elevated at both 1 and 30 min after an increase in the medium glucose concentration from 2 to 20 mM. In these experiments, the estimated islet concentrations of phosphoenolpyruvate fell in the range of maximal sensitivity of the islet adenine nucleotide translocase to phosphoenolpyruvate-induced inhibition of Ca^{2+} accumulation. It is concluded that increased concentrations of islet phosphoenolpyruvate resulting from increased extracellular glucose concentration may act to trigger or promote glucose-stimulated insulin secretion by modifying the distribution of Ca^{2+} between the islet cytosolic and mitochondrial compartments in a transport reaction catalyzed by the adenine nucleotide translocase. DIABETES *32*:793–797, September 1983.

The mechanism by which high concentrations of glucose stimulate rates of pancreatic islet insulin release remains obscure. Both glucoreceptor and glucose metabolic theories, first proposed by Randle and his colleagues[1] as early as 1968, remain current.[2,3] The glucose metabolic hypothesis suggests that increases in the concentration of extracellular glucose lead to alterations in rates of glucose metabolism resulting in increased rates of formation of a key metabolite, which in turn acts as the primary intracellular stimulus for augmented insulin secretion. The glycolytic intermediate, phosphoenolpyruvate (PEP), has been proposed as a metabolite fulfilling many of the requirements for such a secretory stimulant.[4] Alternatively, some investigators[5] consider PEP to potentiate rather than induce insulin secretion. Since ionized calcium (Ca^{2+}) is known to be required for the release of insulin by exocytosis, it has been proposed that PEP may exert its stimulatory effect by altering the distribution of Ca^{2+} between mitochondria and cytosol.[6,7] Thus, Sugden and Ashcroft[6] have reported that PEP prevented accumulation of Ca^{2+} by islet mitochondria, although the mechanism of the effect was not clarified by these workers.

Previous reports indicate that PEP alters the distribution of Ca^{2+} across the inner mitochondrial membrane in liver and heart.[8–10] In these tissues, this action of PEP has been shown to be mediated by an exchange between external PEP and intramitochondrial ATP in a transport reaction catalyzed by the adenine nucleotide translocase.[10,11] Recent work in our laboratory has demonstrated the existence of an active mitochondrial adenine nucleotide translocase in islet tissue and has characterized its properties in terms of its sensitivity to inhibition by atractylate, bongkrekic acid, and palmitoyl CoA.[12] It seemed possible, therefore, that the effect of PEP upon islet mitochondrial Ca^{2+} handling might be attributed to a mechanism similar to that observed in liver and heart. In the present communication, we present data that show that, like the ADP/ATP carrier in liver and heart, the islet translocase can catalyze the exchange of PEP with adenine nucleotides across the mitochondrial membrane, a process that is associated with a significant inhibition of islet mitochondrial Ca^{2+} accumulation similar to that seen in other tissues.[8–10] Since this effect would lead to increased availability of Ca^{2+} in the B-cell cytosol, these results support a possible role for PEP as the metabolic trigger for or promoter of insulin secretion[4–7] and may provide an explanation for the mechanism by which the effect is produced.

From the Departments of Medicine and Nutritional Sciences, University of Wisconsin Medical School, Madison, Wisconsin.
Address reprint requests to Dr. Robin Ewart, Medical Service, Veterans Administration Medical Center, 800 Zorn Avenue, Louisville, Kentucky 40202.
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TABLE 1
Distribution of cytochrome oxidase, adenine nucleotide translocase, and insulin in subcellular fractions of rat pancreatic islets

Fractions	Cytochrome oxidase (nmol/min/mg protein)	Adenine nucleotide translocase activity (nmol/min/mg protein)	Insulin (% of total)
Homogenate	ND	0.021	100
Nuclei + cell debris	0.072	—	—
Mitochondria	0.273	0.19	29
Secretory granules	0.105	—	71

ND = not determined.
Isolation of the islet cell fractions and assays for cytochrome oxidase, adenine nucleotide translocase, and insulin were determined as described in METHODS.

MATERIALS AND METHODS

Reagents. [^{14}C]ADP, [^{14}C]ATP, and $^{45}CaCl_2$ were purchased from New England Nuclear (Boston, Massachusetts) and insulin assay kits from Amersham (Arlington Heights, Illinois). Palmitoyl CoA, ADP, and ATP were supplied by PL Biochemicals (Milwaukee, Wisconsin). Atractylate and PEP were obtained from Sigma (St. Louis, Missouri) and collagenase (Type IV, 165 U/mg) from Worthington Biochemicals (Freehold, New Jersey).

Isolation and incubation of islets. Islets were isolated from pancreas of male Sprague-Dawley rats by the collagenase digestion technique of Lacy and Kostianovsky.[13] The animals were in the weight range 200–250 g and were allowed free access to food and water to the time of death. For determinations of rates of insulin release and of islet PEP content, groups of 25 islets were incubated in 50-μl volumes of a bicarbonate-buffered salt solution[14] containing 1 mg/ml bovine serum albumin, 2 mM Ca^{2+}, and either 2 or 20 mM glucose, gassed to pH 7.4 with O_2:CO_2 (95:5, vol/vol). After incubation at 37°C for 1 or 30 min, 2-μl samples of the media were taken for assay of their insulin content as previously described.[15] To each tube, 10 μl of 0.3 M HCl was then added and the islets were immediately sonicated for 10 s at setting 1.5 of a sonicator manufactured by Ultrasonics, Inc. (Plainview, New York) and 50 μl of 200 mM triethanolamine buffer (pH 7.4) containing 10 mM $MgSO_4$, 200 mM KCl, and 1 mM disodium EDTA was added. The incubation tubes were then heated in a boiling water bath for 5 min, neutralized by the addition of 2 μl of 1 M KOH, and stored at −20°C for estimation of their PEP content as described by Sugden and Ashcroft.[4] The procedure uses the luciferin-luciferase assay of ATP formed from PEP in the presence of pyruvate kinase after depletion of endogenous ATP by addition of hexokinase and an excess of glucose.

Preparation of mitochondria. Groups of 250–400 islets were rinsed three times in 500 μl of ice-cold homogenization medium (210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4) and then homogenized in 200 μl of the same medium using seven passes of an all-glass homogenizer (Kontes Glass Co., Vineland, New Jersey). Where mitochondria were prepared for Ca^{2+} transport studies, EDTA was omitted from the medium. Homogenates were subjected to differential centrifugation at 2°C as described by Howell et al.[16] Briefly, nuclei and cell debris were removed by centrifugation at 600 × *g* for 10 min. The supernatant was then centrifuged at 5500 × *g* for 10 min to give a mitochondrial-rich pellet containing 50–75 μg protein, which was resuspended in 500 μl of mannitol-sucrose Tris-HCl medium. For some experiments, 100–150 μg protein was obtained from 500–800 islets. A high-speed precipitate containing secretory granules was obtained by centrifuging the mitochondrial supernatant for 20 min at 24,000 × *g*.

Determination of adenine nucleotide translocase and cytochrome oxidase. For most experiments, adenine nucleotide translocase was measured by the forward exchange technique as previously described.[12] A mixture containing 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 0.5 mM EDTA, and mitochondrial protein in a final volume of 200 μl was preincubated with or without inhibitor in an ice bath for 5 min. Approximately 30 nCi of [^{14}C]ADP was then added. After incubation at the temperature and for the time indicated, the reaction was terminated by the addition of 5 mM atractylate. The mixture was then filtered through prewetted Millipore filters (pore size 0.45 μm), immediately washed with 5 ml of ice-cold suspension medium, and the filters were dried and dissolved in 10 ml of toluene scintillation fluid[17] for deter-

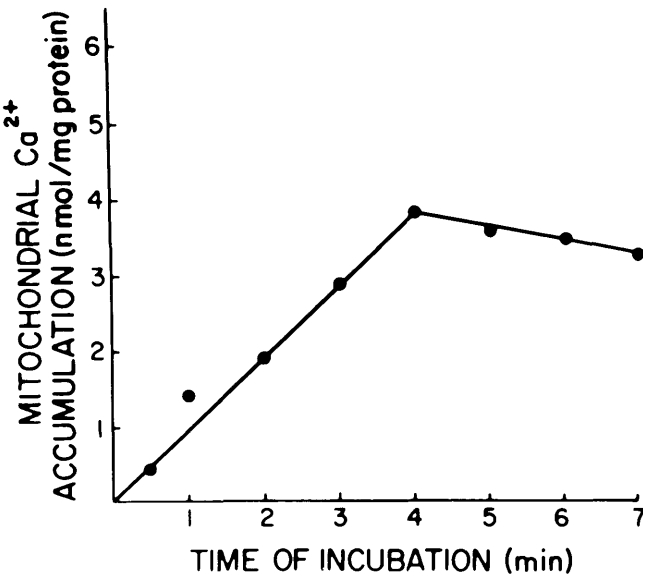

FIGURE 1. Time course of Ca^{2+} uptake by islet mitochondria. Mitochondria, equivalent to 75 μg protein, were preincubated at 28°C in a volume of 500 μl for 1 min, and the reaction was initiated by addition of 0.2 mM $^{45}CaCl_2$ (40,000 cpm/nmol). Aliquots (50 μl) were withdrawn at the times indicated, filtered through Millipore filters, and rapidly washed with 7 ml of suspension medium containing 2 mM EDTA after which the radioactivity on the filter papers was determined. Each point is an average of three experiments using different batches of islets.

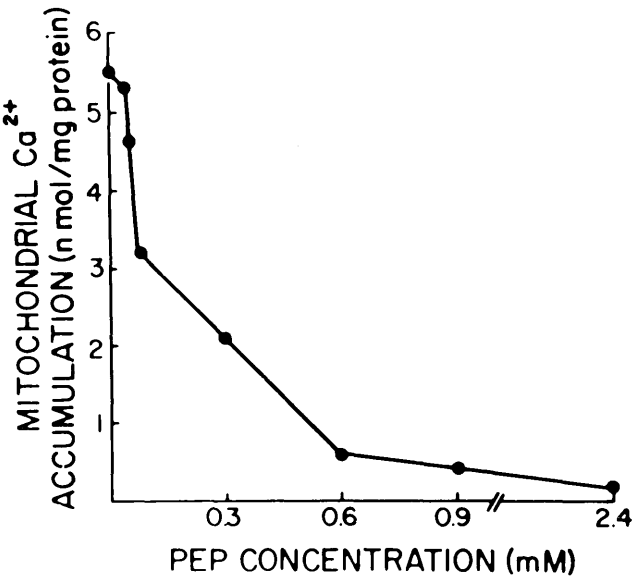

FIGURE 2. Effect of varying concentrations of PEP on islet mitochondrial Ca^{2+} accumulation. The experimental conditions were as described for Figure 1 using a 4-min incubation period except that different concentrations of PEP were added 1 min after initiating the reaction by addition of $^{45}CaCl_2$. Each point is an average of three experiments with separate islet preparations.

mination of radioactivity. A control sample containing atractylate added at zero time was run simultaneously to determine non-specific transport of adenine nucleotides and the values were subtracted from the experimental data. In other experiments, the back exchange assay was used.[17] In this procedure, islet mitochondria (150 μg protein) were loaded with [^{14}C]ATP (40 μCi) by incubation at 4°C for 1 h in a medium containing 250 mM sucrose, 4 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The mitochondria were then washed once and suspended in 500 μl of the above medium. The exchange reaction between the matrix [^{14}C]ATP and the cold external nucleotide or PEP was initiated by addition of 30 μg mitochondrial protein and allowed to proceed for 5 min at 25°C.

Cytochrome oxidase was assayed in subcellular fractions spectrophotometrically at 550 nm.[18]

Determination of mitochondrial calcium uptake. The uptake of $^{45}Ca^{2+}$ by islet mitochondria was measured as described by Peng et al.[9] as previously carried out in this laboratory.[10] The reaction was initiated by the sequential addition to the mitochondrial suspension of $^{45}CaCl_2$ (approximately 40,000 cpm/nmol) and the appropriate concentration of PEP. At the times designated in the figures or tables, the reaction mixtures were filtered by vacuum through Millipore filters (pore size, 0.45 μm). The filters were washed immediately with 7 ml of ice-cold suspension medium, dried, and placed in 10 ml of toluene scintillation fluid to determine radioactivity in a scintillation counter equipped with a ^{45}Ca-channel.

Protein concentration. Mitochondrial protein was determined by the method of Lowry et al.[19] using bovine serum albumin as standard.

Statistical treatment of results. Statistical significance of differences between observed means was determined by use of Student's *t* test.

RESULTS

The distribution of adenine nucleotide translocase, immunoreactive insulin, and cytochrome oxidase in subcellular fractions is shown in Table 1. The results indicate as previously demonstrated[12] that the total adenine nucleotide translocase activity is located in the mitochondrial fraction, whereas the major portion of the immunoassayable insulin is associated with the secretory granule fraction.

The time course of accumulation of Ca^{2+} in islet mitochondria is shown in Figure 1. Uptake of $^{45}Ca^{2+}$ by the mitochondria was complete in 4 min at 28°C, the observed time course being similar to that described for rat liver[8,9,20] and heart[10] mitochondria. The effect of PEP upon the $^{45}Ca^{2+}$ accumulation by islet mitochondria is shown in Figure 2. The inhibition of Ca^{2+} accumulation observed was related to the concentration of PEP with 50% and 96% inhibitions being seen at PEP concentrations of approximately 0.2 and 2.0 mM, respectively.

To establish that the observed effect of PEP upon islet mitochondrial Ca^{2+} accumulation was mediated by the adenine nucleotide translocase, an additional series of experiments was performed. As shown in Table 2, PEP (1 mM) prevented the accumulation of Ca^{2+} by the mitochondria and this effect was abolished by the site-specific inhibitor of the adenine nucleotide translocase, atractylate, at a concentration of 10 μM. The PEP-induced inhibition of mitochondrial Ca^{2+} accumulation was also prevented by 10 μM ATP, the natural substrate for the translocase. In liver[11] and heart[10] mitochondria, PEP is known to be transported on the adenine nucleotide translocase in exchange for ADP and ATP, but at a much slower rate and with a higher K_m than those characteristic for the adenine nucleotides.

In further experiments, the direct effect of PEP on [^{14}C]ADP transport into islet mitochondria was examined (Table 3). Addition of unlabeled ADP to the system led to an 88% inhibition of [^{14}C]ADP uptake due to the expected isotope dilution effect. Under the same conditions of incubation, 0.4 mM PEP led to an 83% inhibition of [^{14}C]ADP uptake into the mitochondria.

The effects of PEP concentration on the outward transport of preloaded [^{14}C]ATP from islet mitochondria are shown in Table 4. In these studies the reaction was carried out at 25°C to ensure a rapid rate of PEP-ATP exchange. It is seen that under these circumstances, the exchange of external cold

TABLE 2
Effect of PEP on islet mitochondrial Ca^{2+} accumulation and its reversal by inhibitors of adenine nucleotide transport

Additions to reaction mixture	Mitochondrial Ca^{2+} concentration (nmol/mg protein)
None (Control)	5.85 ± 0.09
Atractylate (10 μM)	5.10 ± 0.16
PEP	1.09 ± 0.02
PEP + atractylate (10 μM)	5.35 ± 0.24
ATP (10 μM)	5.42 ± 0.69
PEP + ATP (10 μM)	5.38 ± 0.37

Approximately 150 μg mitochondrial protein suspended in 500 μl buffer was divided into eight equal aliquots and mitochondrial Ca^{2+} uptake was determined at 5 min as described in METHODS. Other additions were made 45 s before addition of 1 mM PEP. Results are expressed as means ± SEM of three separate experiments.

TABLE 3
Effect of PEP on [^{14}C]ADP transport into islet mitochondria

Additions	Mitochondrial [^{14}C]ADP uptake (dpm/0.1 mg protein)
None	4850 ± 376
ADP (4 μM)	580 ± 27*
PEP (0.4 mM)	820 ± 47*

Mitochondrial adenine nucleotide translocase activity was determined as described in METHODS. Incubations containing about 25 μg mitochondrial protein were carried out for 1 min at 37°C. Results are expressed as the means ± SEM of three separate experiments.
*$P < 0.01$ when compared with the corresponding value in the absence of any addition to the medium.

ATP with internal or matrix [^{14}C]ATP can be mimicked by external PEP, albeit at higher concentrations.

In a final series of experiments, the effects of varying extracellular concentrations of glucose upon islet concentrations of PEP and rates of insulin release were examined. As shown in Table 5, increase in the medium glucose concentration from 2 to 20 mM led to a significant increase in the rate of insulin release ($P < 0.001$) during a 30-min period of incubation at 37°C while a significant increase in the islet content of PEP ($P < 0.01$) was observed under the same conditions. Since stimulation of rates of insulin secretion by glucose is known to occur rapidly, it seemed important to determine whether the increase in the islet content of PEP could be documented to occur with similar promptness. An experiment was therefore conducted in which the PEP content of islets was determined exactly 1 min after their exposure to 2 or 20 mM glucose. In these studies, the measured islet PEP contents were 1.27 ± 0.10 and 1.60 ± 0.11 pmol/islet in the presence of 2 and 20 mM glucose, respectively (means ± SEM of six observations in each case). These values are statistically significantly different ($P < 0.05$) and suggest that the alteration of intracellular PEP concentrations resulting from changes in extracellular glucose concentration may occur with sufficient rapidity to allow changes in islet PEP concentration to account for the observed increase in rates of insulin secretion.

TABLE 5
Effect of glucose concentration on islet PEP content and rates of insulin secretion

Addition to medium	Islet PEP content (pmol/islet)	Rate of insulin release (μU/25 islets/30 min)
Glucose (2 mM)	0.97 ± 0.07	128 ± 24
Glucose (20 mM)	1.31 ± 0.08*	1040 ± 99*

Groups of 25 islets were incubated in vitro for 30 min in the presence of the glucose concentrations indicated. Islet phosphoenolpyruvate content and rates of islet insulin release were then determined as described in METHODS. Results are expressed as means ± SEM of seven observations.
*$P < 0.01$ when compared with the values in the presence of 2 mM glucose.

DISCUSSION

Arguments in favor of the hypothesis that a key glucose metabolite, the concentration of which rises in the B-cell in response to elevated extracellular glucose concentrations, acts as the trigger for glucose-stimulated insulin secretion have previously been well summarized.[21,22] Sugden and Ashcroft[4] have shown that PEP concentrations rise in islets exposed to stimulatory glucose concentrations and that the glycolytic intermediate leads to inhibition of mitochondrial Ca^{2+} accumulation.[6]

These results have been confirmed in other laboratories[5,7] and in the present communication. Similar effects of PEP in modifying mitochondrial Ca^{2+} accumulation by an action mediated by the adenine nucleotide translocase have previously been described in liver,[8,9,23] heart,[10] and hepatoma[24] mitochondria.

Previous studies in our laboratory have demonstrated that islets possess an active mitochondrial adenine nucleotide translocase.[12] The data presented here show that PEP apparently acts as a substrate for this transporter in islets as it does in liver and heart. Moreover, since atractylate is a site-specific inhibitor of the ADP/ATP carrier, the observed inhibition by atractylate of the PEP-induced blockade of islet mitochondrial Ca^{2+} accumulation by this agent provides evidence that the PEP effect is a function of the adenine nucleotide translocase. While we recognize that the mitochondrial preparation employed in these studies is contaminated with secretory granules, this fact does not confound our conclusions. Thus, any uptake of calcium by the contaminating granules would not demonstrate atractylate sensitivity. Furthermore, the pore size of the filters employed in these experiments was chosen so that mitochondria, but not secretory granules, would be retained during the filtration process.

The results presented show that PEP leads to a highly significant inhibition of [^{14}C]ADP transport into mitochondria at times as short as 1 min, when glucose-stimulated insulin release is well established. Furthermore, increasing concentrations of PEP progressively promote the adenine nucleotide translocase-catalyzed transport of ATP out of mitochondria. Together, these results, like those obtained in liver[8,9] and heart,[10] provide further confirmation that the PEP effect on the calcium accumulation by mitochondria is catalyzed by the adenine nucleotide translocase.

The principal transport mechanism for PEP in liver mitochondria is an exchange for citrate on the tricarboxylate carrier.[25] Although neither this carrier nor any other metabolite transport system except the adenine nucleotide translocase has been identified in islet mitochondria, it is likely that others are present. An active PEP carboxykinase has been demonstrated in rat islet cytosol.[5] The requirement for

TABLE 4
Effect of PEP concentration on the outward transport of matrix [^{14}C]ATP from islet mitochondria

Additions to reaction mixture	Mitochondrial [^{14}C]ATP content (dpm/0.1 mg protein)
None (Control)	1665 ± 183
ATP (25 μM)	270 ± 17*
PEP (0.5 mM)	840 ± 38*
PEP (1 mM)	625 ± 41*
PEP (2 mM)	390 ± 32*

Mitochondrial ATP transport measured by back exchange was determined at 25°C as described in METHODS with approximately 30 μg mitochondrial protein containing radioactive ATP.
*$P < 0.01$ when compared with the control.

a cytosolic dicarboxylic acid or amino acid precursor (malate or aspartate) of oxalacetate, the substrate for the enzyme, would be met by specific mitochondrial transport carriers.

In a final series of experiments the effects of variation in extracellular glucose concentration upon islet content of PEP and rates of insulin release were investigated. Increase in glucose concentration from 2 to 20 mM led to significant increases in both islet PEP concentration and in the observed rate of insulin release. These values were obtained after 30 min of islet incubation and confirm the observations of Sugden and Ashcroft.[4] However, since glucose-stimulated insulin secretion is known to occur within approximately 1 min of the initiation of the glucose signal, it seemed necessary to show that the observed change in the islet content of PEP occurred with equal rapidity after islet exposure to 20 mM glucose. Our results are consistent with this requirement (see text). A further requirement that must be fulfilled before a physiologic role for PEP-induced changes in islet mitochondrial Ca^{2+} handling can be claimed, relates to the range of islet concentrations of glycolytic intermediates. In the present studies, no attempt was made to establish the precise volume of distribution of PEP within the islet cells. It is, however, generally accepted that rat islets are in the range of 10 μg wet weight. Assuming an intracellular volume of 10 nl/islet, the present results indicate that islet PEP concentrations are in the range of 0.1–0.16 mM, where the sensitivity of the translocase to variation in PEP concentration is maximal (Figure 2).

In conclusion, we have confirmed the previous reports[4,5,7] of increased islet PEP content in response to increased extracellular glucose concentration and have provided evidence that this glycolytic intermediate can act as a substrate for the islet mitochondrial adenine nucleotide translocase. Inhibition of mitochondrial Ca^{2+} uptake by PEP shows a concentration dependence that is consistent with a physiologic role for PEP as a trigger for glucose-stimulated insulin secretion.

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