Possible Links between Glucose-induced Changes in the Energy State of Pancreatic B Cells and Insulin Release

Unmasking by Decreasing a Stable Pool of Adenine Nucleotides in Mouse Islets

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Abstract

Whether adenine nucleotides in pancreatic B cells serve as second messengers during glucose stimulation of insulin secretion remains disputed. Our hypothesis was that the actual changes in ATP and ADP are obscured by the large pool of adenine nucleotides (ATP/ADP ratio close to 1) in insulin granules. Therefore, mouse islets were degranulated acutely with a cocktail of glucose, KCl, forskolin, and phorbol ester or during overnight culture in RPMI-1640 medium containing 10 mM glucose. When these islets were then incubated in 0 glucose + azide (to minimize cytoplasmic and mitochondrial adenine nucleotides), their content in ATP+ADP+AMP was decreased in proportion to the decrease in insulin stores. After incubation in 10 mM glucose (no azide), the ATP/ADP ratio increased from 2.4 to > 8 in cultured islets, and only from 2 to < 4 in fresh islets. These differences were not explained by changes in glucose oxidation. The glucose dependency (0–30 mM) of the changes in insulin secretion and in the ATP/ADP ratio were then compared in the same islets. In nondegranulated, fresh islets, the ATP/ADP ratio increased between 0 and 10 mM glucose and then stabilized although insulin release kept increasing. In degranulated islets, the ATP/ADP ratio also increased between 0 and 10 mM glucose, but a further increase still occurred between 10 and 20 mM glucose, in parallel with the stimulation of insulin release. In conclusion, decreasing the granular pool of ATP and ADP unmasks large changes in the ATP/ADP ratio and a glucose dependency which persists within the range of stimulatory concentrations. The ATP/ADP ratio might thus serve as a coupling factor between glucose metabolism and insulin release. (*J. Clin. Invest.* 1995. 96:1738–1745.) Key words: pancreatic islets • stimulus—secretion coupling • ATP • ADP • glucose metabolism

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Introduction

A peculiar feature of pancreatic B cells is that they probably use ATP not only as a primary source of energy as other cells, but also as an intracellular second messenger during stimulus–secretion coupling.

Stimulation of insulin secretion by glucose and most other nutrients requires metabolism of these stimuli by B cells (1–4). The exact biochemical nature of the signals that link the acceleration of metabolism to the exocytosis of insulin-containing granules has only been partially elucidated. Glucose entry in B cells is followed by an acceleration of glycolysis and glucose oxidation, with production of metabolic factors that close ATP-sensitive K^+ channels (K^+_{ATP} channels)[1] in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarization to a threshold potential where voltage-dependent Ca^{2+} channels open. Electrical activity then appears, which corresponds to an influx of Ca^{2+} that eventually increases the concentration of cytoplasmic free Ca^{2+} and triggers insulin release. When the concentration of glucose is raised above threshold values, the electrical activity augments, contributing to the increase in insulin release (4–9).

There is no dispute that the K^+ channels, which close in response to glucose metabolism, are sensitive to ATP and ADP (8–10), hence the hypothesis that they are controlled by variations of the ATP/ADP ratio in B cells. It has long been known that glucose increases ATP levels in islets, but this effect was restricted to concentrations of the sugar which are below the threshold for stimulation of insulin release (11, 12). Measurements of the ATP/ADP ratio have also yielded controversial results (reviewed in 13). Some studies have reported low ATP/ADP ratios (between 2 and 4), changing little (14–16) or not (17) in response to glucose. Other groups measured higher ratios and larger increases by glucose (18–20). However, these studies usually tested one single concentration of glucose and did not attempt to correlate the changes in the ATP/ADP ratio with those of insulin release. Such correlations have only been reported when the energy state of islet cells was decreased by mitochondrial poisons or changes in O_2 tension (12, 18, 21).

Two studies have reported that insulin granules isolated from pancreatic islets (22) and from a B cell line (23) contain substantial amounts of ATP and ADP in a ratio close to unity. This suggests that granular adenine nucleotides make up a substantial proportion of total ATP and an even larger proportion of ADP in intact B cells. Surprisingly, however, the hypothesis that this granular pool could mask the actual changes in ATP and ADP in the metabolically active pool has not been tested

1. *Abbreviation used in this paper:* K^+_{ATP} channels, ATP-sensitive K^+ channels.

directly. In the first part of this study, therefore, we examined the impact of degranulation on adenine nucleotide content in mouse islets. In the second part, we evaluated the effects of glucose on adenine nucleotide levels in degranulated islets. Our aim was to determine whether, under these conditions, correlations exist between insulin release and the ATP/ADP ratio.

Methods

Solutions. The medium used was a bicarbonate-buffered solution which contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM Na_2HPO_4, and 24 mM $NaHCO_3$. It was gassed with O_2/CO_2 (94:6) to maintain pH 7.4, and was supplemented with bovine serum albumin (1 mg/ml).

Preparation. Islets were isolated by collagenase digestion (24) of the pancreas of fed female NMRI mice (25–30 grams) followed by hand picking. These islets were either immediately used for the experiments or first cultured for 18–20 h at 37°C in RPMI-1640 medium 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. Freshly isolated or cultured islets were preincubated for 60 min at 37°C in a control medium containing 15 mM glucose, a concentration that causes half-maximum stimulation of mouse islets. They were then distributed in batches of five and incubated in 1 ml medium containing various concentrations of glucose or test substance. Acute degranulation of fresh islets was achieved by incubation for 120 min in a medium containing 25 mM glucose, 30 mM KCl, 1 μM forskolin, and 50 nM PMA. The medium was then removed and the islets were carefully rinsed three times before being incubated for another 60 min in 1 ml of control medium. The other experiments, carried out with either fresh or cultured islets, consisted of only one incubation of 60 min. At the end of each incubation period, an aliquot of the medium was appropriately diluted for measurement of insulin by a double-antibody radioimmunoassay using rat insulin as a standard (Novo Research Institute, Bagsvaerd, Denmark).

Measurements of adenine nucleotides in incubated islets. The aliquot of medium used for insulin assay was taken while the incubation tubes remained at 37°C. The islets were then incubated for another 5 min before the incubation was stopped by addition of 0.6 ml of ice-cold trichloroacetic acid to a final concentration of 5%. The tubes were vortex-mixed, left on ice for 15 min and centrifuged for 5 min in a microfuge (Eppendorf Inc., Fremont, CA). A fraction (0.5 ml) of the supernatant was then mixed with 1.5 ml diethylether, and the ether phase containing trichloracetic acid was discarded. This step was repeated three times to ensure complete elimination of trichloracetic acid. The extracts were eventually diluted with 0.5 ml of a buffer containing 20 mM Hepes, 3 mM $MgCl_2$, and KOH to adjust pH to 7.75, and frozen at −70°C until the day of the assay.

ATP, ADP, and AMP were assayed in duplicates or triplicates by a luminometric method (25). For ATP measurement, a portion (50 μl) of each sample was mixed with 300 μl of the above buffer and 100 μl of a commercially available ATP monitoring reagent containing firefly luciferase and luciferin (BioOrbit, Turku, Finland). The emitted light was measured in a luminometer (1250 luminometer system; BioOrbit). For ADP measurement, another 50-μl portion of each sample was incubated for 40 min with 300 μl of buffer containing 1.5 mM phosphoenolpyruvate and 2.3 U/ml pyruvate kinase to convert ADP into ATP. For AMP measurement, a 50-μl portion of each sample was mixed with 300-μl buffer containing phosphoenolpyruvate, pyruvate kinase, and 36 U/ml adenylate kinase to convert AMP into ADP before conversion into ATP. ATP was then assayed as described above, and ADP and AMP contents were calculated by difference. Appropriate blanks, ATP, ADP, and AMP standards were run through the entire procedure, including the extraction step.

Measurements of adenine nucleotides in intact and permeabilized islet cells. Freshly isolated or overnight cultured islets were rinsed with the usual bicarbonate-buffered medium containing 10 mM glucose, but without $CaCl_2$ and with 0.1 mM EGTA. They were then incubated at 37°C for 2–3 min in the same medium supplemented with 0.1 mg trypsin/ml, with gentle pipetting through a siliconized glass pipette until the islets disappeared. 1 min later, trypsin digestion was stopped and the cells were rinsed twice with cold RPMI-1640 medium containing 10% fetal calf serum. The cells were then resuspended in 4 ml RPMI-1640 medium, preincubated for 45 min at 37°C in an atmosphere of 5% CO_2 in air, and divided in two batches.

Cells from the first batch were resuspended, counted, and aliquoted in control medium without glucose. After 60 min at 37°C, ice-cold trichloracetic acid was added and the samples were processed for measurement of adenine nucleotides as described above.

Cells from the other batch were washed twice with a Ca^{2+}-free medium containing 0.4 mM EGTA. They were then resuspended at a concentration of $2 \cdot 10^6$ cells/ml in a buffer containing 20 mM Hepes, 140 mM K glutamate, 5 mM NaCl, 2 mM $MgSO_4$, and 0.2 mM EGTA. This medium did not contain ATP. The pH was 7.0 and free Ca^{2+} ~ 0.1 μM. This cell suspension was mixed 1:1 with the same buffer supplemented with *Staphylococcus aureus* α-toxin (2,000 Hemolytic U/10^6 cells/ml) (26, 27), and incubated for 10 min at 37°C. After centrifugation at 4°C, the supernatant was discarded and the cells were rinsed twice with 1 ml buffer (5 min each) to remove diffusible adenine nucleotides. They were then suspended in 1 ml buffer, and processed as above for adenine nucleotide measurement. More than 95% of cells were permeable to trypan blue (mol. wt. = 950) and 100% of cells were permeable to eosin G (mol. wt. = 624). These permeabilized cells do not release insulin unless the concentration of free Ca^{2+} is raised above 1 μM and exogenous ATP is provided (27).

Measurements of islet insulin and DNA content. When the insulin content of the islets or of dispersed cells was to be measured, the incubation medium was completely removed and replaced by an acid/ethanol mixture (ethanol 95%: 750 ml; concentrated HCl: 15 ml; H_2O: 235 ml) (28). After sonication, the tubes were kept at −20°C for at least 24 h, and an aliquot of the extract was eventually diluted in assay buffer before measurement of insulin. For measurement of islet DNA, the tissue (here batches of eight islets) was sonicated in 0.8 ml of a buffer (pH 7.4) containing 10 mM Tris, 2 M NaCl, and 1 mM EDTA. DNA was then measured by a spectrofluorimetric method (29) using bis-benzimide as a dye and fetal calf thymus DNA as a standard.

Measurements of glucose oxidation. After preincubation as above, freshly isolated or cultured islets were distributed into batches of 10 and incubated, for 60 min at 37°C, in 50 μl medium supplemented with 1 μCi [U-^{14}C]glucose. The amount of glucose oxidized was determined from the production of [^{14}C]CO_2. Technical aspects of the method have been described previously (30).

Materials. Diazoxide was obtained from Schering-Plough Avondale (Rathdrum, Ireland); DNA, bis-benzimide and PMA were from Sigma Chemical Co. (St Louis, MO); ATP, ADP, AMP, phosphoenolpyruvate, pyruvate kinase and adenylate kinase were from Boehringer-Mannheim (Mannheim, Germany); clonidine was from Boehringer-Ingelheim (Ingelheim, Germany); forskolin was from Calbiochem-Behring Corp. (San Diego, CA); sodium azide and all other reagents were from Merck A.G. (Darmstadt, Germany). *S. aureus* α-toxin was purified and kindly provided by Dr. M. Palmer (University of Mainz, Germany).

Presentation of results. Results are presented as means±SEM for the indicated number of batches of islets. The statistical significance of differences between means was assessed by Student *t* test for unpaired data when only two groups were compared, or by an analysis of variance followed by a Newman-Keuls' test for comparison of more than two groups. Differences were considered significant at $P < 0.05$.

Results

Effects of acute B cell degranulation on islet adenine nucleotide levels. Freshly isolated islets were incubated for 2 h in a medium containing 10 mM glucose alone or a strongly stimulatory cocktail composed of 25 mM glucose, 30 mM KCl, 1 μM forskolin

Table I. Effects of Acute B Cell Degranulation on Adenine Nucleotide Levels in Islets Subsequently Incubated with a Mitochondrial Poison

	Experimental conditions during initial incubation without poison			
	Experiment A		Experiment B	
	Glucose 10 mM	Cocktail	Cocktail	Cocktail + Nimodipine
Insulin release (ng/islet per 180 min)	3.6±0.2*	89±3	81±2	6.0±0.3*
Final insulin content (ng/islet)	220±14*	138±11	—	—
Total insulin release + content (ng/islet)	223±14	226±12	—	—
Adenine nucleotides (pmol/islet)				
ATP	7.2±0.3*	4.1±0.3	3.2±0.1	5.1±0.2*
ADP	4.8±0.2*	2.7±0.2	2.3±0.1	3.5±0.2*
AMP	5.3±0.2*	3.6±0.3	—	—
ATP + ADP			5.5±0.2	8.6±0.3*
ATP + ADP + AMP	17.3±0.7*	10.4±0.7	—	—

Batches of five freshly isolated islets were incubated for 120 min in 1 ml medium containing 10 mM glucose or a stimulatory cocktail composed of 25 mM glucose, 30 mM KCl, 250 μM diazoxide, 1 μM forskolin, and 50 nM PMA. At the end of this incubation, the medium was removed and replaced, for 60 min, by 1 ml medium containing no glucose and 10 mM azide. At the end of this second incubation, certain batches were used for measurement of insulin content and others for measurement of adenine nucleotides. Values are means±SEM. Experiment A was repeated three times with eight batches of islets in each group, four of which were used for measurement of insulin content (total $n = 12$) and four for measurement of adenine nucleotides (total $n = 12$). In experiment B, $n = 8$. * $P < 0.001$ vs islets submitted to cocktail alone (t test).

and 50 nM PMA. As shown in Table I *(experiment A)*, the cocktail stimulated insulin release considerably more than did 10 mM glucose and this resulted in a 38% decrease in the islet insulin content. At the end of this incubation total adenine nucleotides averaged 31.5±2.0 pmol/islet (65% ATP, 17% ADP, 18% AMP) in control islets and 25.1±1.2 pmol/islet (66% ATP, 14% ADP, 20% AMP) in islets incubated with the cocktail ($P < 0.05$). To minimize the cytoplasmic and mitochondrial pools of adenine nucleotides and permit an estimation of the residual granular pool, some of the islets were then incubated for 1 h in a medium containing no glucose and 10 mM azide, a drug that inhibits ATP synthesis by blocking the electron transport chain (31). This second incubation revealed a 40% decrease in total adenine nucleotides, with a similar relative fall in ATP, ADP, and AMP, in islets submitted to the degranulating cocktail (Table I, *experiment A*).

In a separate experiment, the islets were stimulated with the degranulating cocktail in the absence and presence of 5 μM nimodipine. As shown in Table I *(experiment B)*, the Ca^{2+}-channel blocker largely prevented the stimulation of insulin release (insulin stores were not measured) and the fall in adenine nucleotides. ATP and ADP levels were indeed 36% lower in islets submitted to the cocktail alone than in those incubated with nimodipine.

Effects of overnight culture on islet insulin and adenine nucleotide content. The insulin content of mouse islets cultured for 18–20 h in a medium containing 10 mM glucose was 57% lower than that of fresh islets obtained from the same animals (Table II). The amount of insulin released in the medium over the same period averaged 74% of the initial content. The sum of insulin remaining in the islets and secreted in the medium was thus higher than in fresh islets ($P < 0.01$). This indicates that the important secretion of insulin has been partially compensated for by an increase in insulin synthesis. When 10 μM clonidine (which activates α_2-adrenoceptors) or 250 μM diazoxide (which opens K^+_{ATP} channels) were added to the culture medium, insulin release was strongly inhibited, as expected (32, 33). Under these conditions, the islet insulin content was decreased by only 16% (clonidine) and 17% (diazoxide) as compared with untreated fresh islets (Table II). Compared to that in fresh islets, the DNA content of cultured islets averaged 96% in controls, and 94 and 100% after culture with clonidine and diazoxide, respectively. This shows that no cell loss occurred during the culture period.

Fresh and overnight cultured islets were then incubated for 1 h in a glucose-free medium before determination of their content in ATP and ADP. As shown in Table II, the sum ATP + ADP was decreased by 40% after culture and this decrease was attenuated by clonidine and diazoxide.

A more complete comparison of fresh and cultured islets was performed in another series of experiments carried out in a glucose-free medium alone or with azide (Fig. 1). After incubation for 60 min in the absence of glucose, total adenine nucleotides were ~ 49% lower in cultured islets than in fresh islets, but the relative proportions of ATP, ADP, and AMP were still similar: 55, 24, and 21%, respectively, in cultured islets; 48, 25, and 27%, respectively, in fresh islets. Poisoning with azide decreased total adenine nucleotides by ~ 5.7 pmol/islet in both fresh and cultured islets. This decrease was due to a fall in ATP and ADP levels only. There was no significant change in AMP levels (Fig. 1). After incubation with azide, the relative proportions of ATP, ADP, and AMP remained similar in cultured islets (39, 26, and 35%, respectively) and in fresh islets (35, 26, and 39%, respectively).

Effects of overnight culture on adenine nucleotides of permeabilized islet cells. In another set of experiments, fresh or cultured islets were dispersed into isolated cells. Half of the samples were then incubated in a glucose-free medium before measurement of adenine nucleotides, whereas the other half were permeabilized with *S. aureus* α-toxin. The aim of this permeabilization, which is restricted to the plasma membrane (34), was to allow diffusion of cytoplasmic nucleotides out of the cells, without releasing nucleotides from organelles (granules, mitochondria ...). Intact cells from cultured islets contained lower stores of insulin than cells from freshly isolated islets (230±12 vs 563±42 ng/10^4 cells, $n = 15$, $P < 0.01$). As shown in Fig. 2, their ATP, ADP, and AMP content was also decreased, with a fall of 37% ($P < 0.01$) in the sum of adenine nucleotides. Permeabilization of the cells did not affect their insulin content: 231±19 ng/10^4 cells from cultured islets and 559±16 ng/10^4 cells from freshly isolated islets. On the other hand, ATP, ADP, and AMP levels were substantially and similarly decreased after permeabilization. More importantly, the content of each adenine nucleotide was lower in permeabilized

Table II. Influence of Culture on Insulin and Adenine Nucleotide Contents in Mouse Islets

	Insulin (ng/islet)		ATP + ADP (pmol/islet)		ATP/ADP	
	Released during 18 h	Content	Glucose 0	Glucose 10 mM	Glucose 0	Glucose 10 mM
Fresh islets	—	168±4	15.4±0.3	19.0±0.5	2.00±0.04	3.74±0.19
Cultured islets						
Controls	125±4	73±5*	9.2±0.5*	17.5±0.8	2.76±0.08*	9.84±0.63*
+ Clonidine 10 μM	18±2‡	141±5*‡	13.2±0.4*‡	21.0±0.5‡	2.36±0.06*‡	5.73±0.24*‡
+ Diazoxide 250 μM	19±2‡	140±5*‡	12.5±0.9*‡	20.2±1.0‡	2.44±0.06*‡	5.55±0.21*‡

In the experiments designed to measure the insulin content, islets obtained from the same preparations were distributed in batches of eight and, either immediately processed for measurement of insulin content, or first cultured for 18 h in RPMI-1640 medium containing 10 mM glucose, with or without clonidine or diazoxide. At the end of the culture, the amount of insulin secreted in the medium and the insulin content of the islets were determined. In the experiments designed to measure the ATP/ADP ratio, fresh or cultured islets were incubated for 60 min, in batches of five, in a medium containing 0 or 10 mM glucose before being processed for assay of adenine nucleotides. Values are means±SEM for 17 to 24 batches of islets. * $P < 0.01$ or less vs fresh islets. ‡ $P < 0.01$ vs control cultured islets (ANOVA followed by Newman-Keuls test).

cells from cultured islets than from fresh islets (Fig. 2). Thus, in spite of major differences in their conceptual and technical basis, the permeabilization of dispersed islet cells and the poisoning of intact islets lead to a similar conclusion: islet culture decreases the size of a relatively stable pool of adenine nucleotides.

Effects of glucose on adenine nucleotide levels in fresh and cultured islets. We, therefore, evaluated to which extent the decrease of this background of adenine nucleotides might influence the apparent changes in the ATP/ADP ratio brought about by glucose (Table II). After incubation for 60 min in a glucose-free medium, the ATP/ADP ratio was low (2.00) in fresh islets. It was slightly higher in cultured islets, and more so when the culture medium did not contain diazoxide or clonidine. The differences were much larger after incubation in the presence of 10 mM glucose. The ATP/ADP ratio increased 3.6-fold in control cultured (degranulated) islets, but did not even double in fresh (nondegranulated) islets. The increase was intermediate when islets had been cultured in a medium supplemented with clonidine or diazoxide (Table II).

To determine whether changes in the rate of glucose metabolism by cultured islets might contribute to these differences, glucose oxidation by fresh and cultured islets was measured

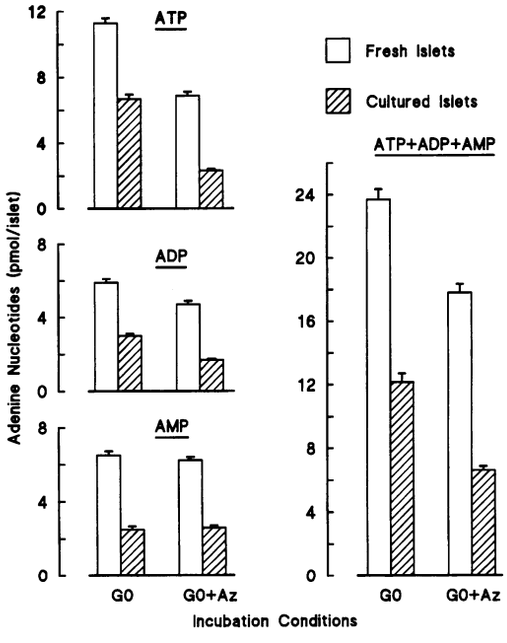

Figure 1. Adenine nucleotide levels in fresh and cultured mouse islets incubated under conditions of low metabolism. Fresh islets *(open columns)* and islets cultured for 18–20 h in a medium containing 10 mM glucose *(hatched columns)* were incubated for 60 min in 1 ml of glucose-free medium, without *(G0)* or with 10 mM azide *(G0 + Az)*. Their ATP, ADP, and AMP content was then determined at the end of this incubation. Values are means±SEM for 22 batches of islets from six separate experiments.

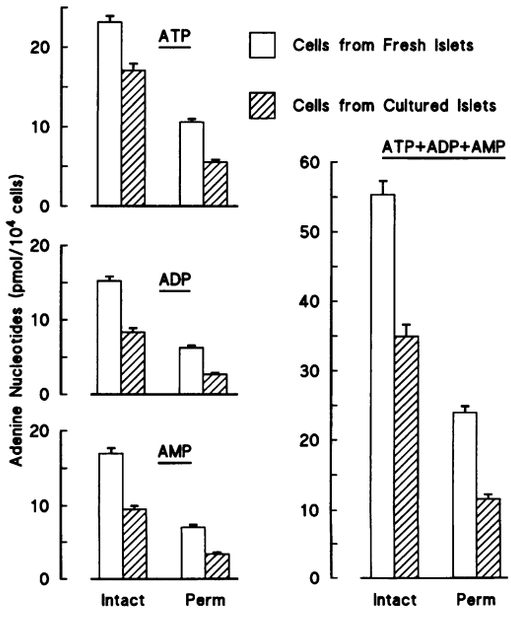

Figure 2. Adenine nucleotide levels in intact and permeabilized islet cells. Dispersed cells from fresh islets *(open columns)* or from islets cultured for 18–20 h in a medium containing 10 mM glucose *(hatched columns)* were incubated for 60 min in a glucose-free medium *(Intact)* or permeabilized with *S. aureus* α-toxin *(Perm)*. Their ATP, ADP, and AMP content was then determined. Values are means±SEM for 16–19 batches of cells from five to six separate preparations.

Table III. Glucose Oxidation by Fresh or Cultured Mouse Islets

	Glucose oxidized (pmol/islet per 60 min)	
	Glucose 3 mM	Glucose 10 mM
Series 1		
Fresh islets	12.8±0.3	48.3±1.8
Cultured islets	16.1±0.4*	57.7±2.5*
Series 2		
Cultured islets		
Controls	17.2±0.8	51.3±1.4
+ Clonidine 10 μM	—	50.4±1.5
+ Diazoxide 250 μM	—	59.9±1.9‡

In experimental series 1, half of the islets were tested immediately after isolation, whereas the other half were cultured for 18 h in RPMI-1640 medium with 10 mM glucose before being tested. In experimental series 2, all islets were cultured for 18 h in RPMI-1640 medium containing 10 mM glucose, without or with clonidine or diazoxide. Values are means±SEM for 11 batches of islets. * $P < 0.01$ vs fresh islets (t test). ‡ $P < 0.01$ vs control cultured islets (ANOVA followed by Newman-Keuls test).

(Table III). Control cultured islets oxidized glucose at a slightly higher rate (~ 20%) than did fresh islets. However, culture in the presence of clonidine or diazoxide did not impair glucose metabolism. Glucose oxidation was even slightly increased after culture in the presence of diazoxide (Table III). Partial inhibition of B cell degranulation, therefore, attenuated the effect of glucose on the ATP/ADP ratio in islets without decreasing glucose metabolism.

Concentration dependence of glucose-induced changes in islet adenine nucleotide levels and insulin release. When fresh islets were incubated in the presence of increasing concentrations of glucose, their ATP content augmented (Fig. 3). A significant ($P < 0.01$) increase by ~ 2.6 pmol/islet was noted in 3 mM glucose and the largest change (~ 6 pmol/islet) was recorded in 10 mM glucose. However, there was no significant difference between ATP levels from 6 to 30 mM glucose. Concomitantly, ADP levels decreased slightly (Fig. 3). This decrease reached ~ 1.2 pmol/islet ($P < 0.01$) at 10 mM glucose and remained stable (~ 1.5 pmol/islet) at higher glucose concentrations. Since ATP levels increased more than ADP levels decreased, the sum ATP + ADP slightly augmented with the concentration of glucose. This increase was maximal (~ 3.8 pmol/islet) at 10 mM glucose ($P < 0.05$), but there was no significant difference in the sum ATP + ADP from 3 to 30 mM glucose.

In cultured islets, the ATP content was increased by 3 and 6 mM glucose and then plateaued at higher glucose concentrations (Fig. 3). The ADP content slightly (by ~ 0.5 pmol) decreased in the presence of 6 mM glucose ($P < 0.01$). This decrease then stabilized at ~ 1.1 pmol/islet between 10 and 30 mM glucose (Fig. 3). As a result, the sum ATP + ADP increased between 0 and 6 mM glucose, and then remained stable within the experimental errors.

From these ATP and ADP contents, the ATP/ADP ratio in islets incubated in the presence of various glucose concentrations was calculated (Fig. 4). In fresh islets, this ratio increased from 1.97±0.04 in 0 glucose to 3.66±0.18 ($P < 0.01$) in 10 mM glucose and then remained stable as the concentration of

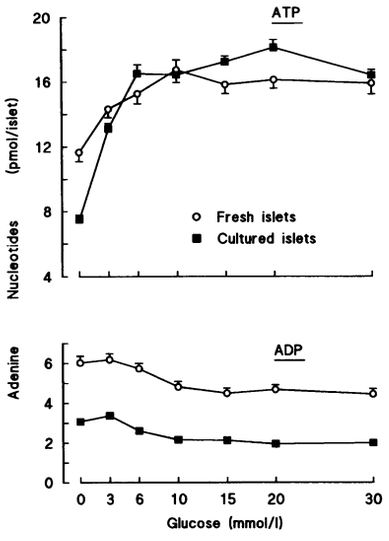

Figure 3. Effects of various glucose concentrations on ATP and ADP levels in fresh or cultured mouse islets. Fresh islets (○) or islets cultured for 18–20 h in a medium containing 10 mM glucose (■) were first preincubated for 60 min in 1 ml medium containing 15 mM glucose. They were then incubated for 60 min in the presence of the indicated glucose concentrations. Values are means±SEM for 25–30 batches of islets from six to seven separate experiments.

glucose was raised further. In cultured islets, the ATP/ADP ratio sharply increased from 2.44±0.06 in 0 glucose to 6.66±0.45 ($P < 0.01$) in 6 mM glucose. A slower increase was then observed between 6 and 20 mM glucose (9.51±0.37; $P < 0.01$). When the concentration of glucose was raised to 30 mM, however, the ATP/ADP ratio fell slightly to 8.48±0.35.

Fig. 4 also shows the effect of glucose on insulin release from the same islets. The relationship between the rate of release and the glucose concentration was sigmoidal and similar for both fresh and cultured islets. It is noteworthy that 6 mM glucose did not increase insulin release in either fresh or cultured islets (103±10 and 106±10% of control values in 0 glucose, respectively).

The relationship between insulin release and the ATP/ADP ratio in the same islets incubated in the presence of various glucose concentrations is shown in Fig. 5. No correlation was found in fresh islets. From 0 to 10 mM glucose, the ATP/ADP ratio increased whereas insulin release hardly changed. Higher glucose concentrations then stimulated insulin release without changing the ATP/ADP ratio. In cultured islets, low glucose

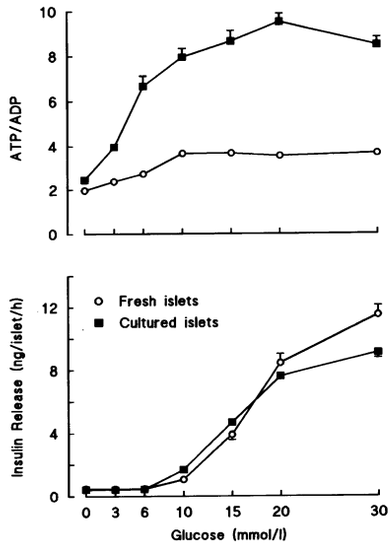

Figure 4. Effects of various glucose concentrations on the ATP/ADP ratio in fresh or cultured mouse islets and on insulin release from the same islets. The results were collected in the same experiments as those of Fig. 3. Values are means± SEM for 25–30 batches of islets from six to seven separate experiments.

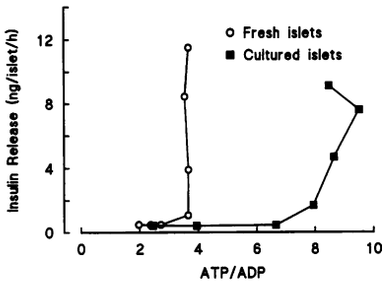

Figure 5. Relationship between the ATP/ADP ratio in fresh or cultured mouse islets and insulin release from the same islets. The different points, along each curve correspond to results obtained in the presence of 0, 3, 6, 10, 15, 20, and 30 mM glucose (from left to right). The results are taken from Fig. 4

concentrations also increased the ATP/ADP ratio without affecting release. However, between 6 and 20 mM a linear correlation was disclosed between both parameters (Fig. 5).

The above findings with cultured islets were verified in a smaller, but totally independent series of experiments. The results again showed that, after 60 min of incubation in the presence of 10, 20, and 30 mM glucose, the ATP/ADP ratio increased from 9.11±0.59 to 11.87±1.31 and then declined to 11.01±0.84 (n = 10), respectively. The relationship between the ATP/ADP ratio and insulin release was similar to that shown in Fig. 5. When all results are pooled, a statistically significant difference ($P < 0.01$) is found between the ATP/ADP ratio in islets incubated in 10 mM glucose (8.3±0.3) and in 20 mM glucose (10.2±0.5).

Discussion

This study was a test of the hypothesis that adenine nucleotides present in insulin secretory granules (22, 23) make up a background that masks the actual changes in ATP and ADP occurring in the metabolically active pool of pancreatic B cells. We, therefore, investigated whether degranulation of mouse islets would decrease this background and permit better characterization of the relationship between the changes in insulin release and in the ATP/ADP ratio during glucose stimulation.

The model. The fractional rate of insulin secretion is low (35). Degranulation of normal islets cannot be achieved acutely by using a physiologic stimulus only. With a cocktail of high glucose, high KCl, forskolin, and PMA, an ~ 38% decrease in insulin stores could be produced within 2 h. However, islets submitted to such a stimulation cannot reliably be used for a subsequent study of the effects of glucose alone. We, therefore, cultured the islets overnight in usual RPMI-1640 medium containing 10 mM glucose. This decreased the insulin content by 57% as compared with freshly isolated islets. A stronger degranulation (e.g. with a higher glucose concentration) was purposely avoided, because of a possible desensitization of the secretory process under such conditions (35), and because we wished to maintain enough insulin reserves to permit reliable measurements of insulin secretion without the insulin content becoming rate limiting.

Decrease in adenine nucleotides in degranulated islets. Adenine nucleotides and insulin are cosecreted from pancreatic B cells (22). However, comparison of the changes in adenine nucleotide and insulin content of the islets is complicated by the contamination with nucleotides present in non-B cells, which make up 15–20% of normal mouse islets (1). Estimation of the adenine nucleotide content of different cellular compartments is even more difficult. In chromaffin cells which, like pancreatic B cells (22, 23) contain large amounts of nucleotides in secretory granules (36), two approaches have been used successfully to characterize the relatively stable granular pool (37). First, various poisons were found to minimize the size of the metabolically active pool without affecting the granular pool. Second, the cytoplasmic pool could be selectively depleted by allowing emptying of its nucleotides through a permeabilized plasma membrane. When this second approach was applied to dispersed islet cells, adenine nucleotides decreased by ~ 50%, in agreement with a previous report which, however, equated the resistant pool with a mitochondrial pool only (15). In our experiments, previous stimulation of insulin secretion and decrease in insulin reserves similarly reduced the total and permeabilization-resistant pools. According to the first approach, we poisoned intact islets with azide after degranulation or not. Two interesting sets of observations were made. First, the fall in the more stable pool of adenine nucleotides was commensurate with the decrease in insulin content both after acute degranulation (40 vs 38%) and after culture (63 vs 57%). Second, the relative proportions of adenine nucleotides in the azide-resistant pool, that was decreased by degranulation, are compatible with those reported for insulin granules. An ATP/ADP ratio of 1.1 has been measured in insulin granules isolated from rat islets (22), and the relative proportions of ATP, ADP, and AMP were 30, 40, and 30% in granules from a rat insulinoma (23).

Taken together, our findings indicate that the decrease in adenine nucleotides in acutely stimulated or cultured islets is largely linked to insulin secretion and subsequent degranulation. The contribution of other mechanisms is not excluded, but would not invalidate the use of our model for the subsequent experiments.

Effects of glucose on adenine nucleotides in islets degranulated by culture. Our results confirm that ATP levels fall when the islets are incubated in a glucose-free medium, and that low glucose concentrations (3–6 mM) are sufficient to attenuate this fall (11–13, 38). They further show that increasing the glucose concentration above 3 mM brings about a decrease in ADP levels. As a result, the ATP/ADP ratio increased. The increase measured between 0 and 10 mM glucose was much larger in cultured islets (from ~ 2.5 to 8–10) than in fresh islets (from ~ 2.0 to ~ 3.7). We first verified whether the difference could simply be ascribed to a change in the responsiveness to glucose. In agreement with other reports (39), cultured islets were found to oxidize glucose at a higher rate than fresh islets. However, this difference is relatively small (20%) and cannot entirely account for the differences in energy state. When the degranulation was partially prevented by addition of clonidine or diazoxide to the culture medium, the rise in the ATP/ADP ratio between 0 and 10 mM glucose was much less (from ~ 2.4 to ~ 5.6) than in islets cultured in a control medium, although the rate of glucose oxidation was similar or slightly higher. There may thus exist two components in the larger rise in ATP/ADP ratio in islets degranulated by culture: the loss of a stable pool of adenine nucleotides with a low ratio (background) and an increase in glucose metabolism.

Significance for the control of insulin release. The relationship between insulin secretion and the ATP/ADP ratio, in the same islets, was characterized by a curve with two components, of which the first was, at least qualitatively, similar in fresh and cultured islets. For changes in the glucose concentration between 0 and 6–10 mM, the ATP/ADP ratio increased, while

insulin release was not or only slightly stimulated. A similar relationship has long been described between the decrease in K^+ efflux and the increase in insulin secretion from islets stimulated with different glucose concentrations (40). The decrease in K^+ efflux reflects closure of K^+_{ATP} channels. There is general agreement that this closure underlies the decrease in K^+ conductance which causes depolarization of the B cell membrane from the resting to a threshold potential, when the concentration of glucose is raised from 0 to 6–7 mM (4–10). The increase in ATP/ADP ratio occurring between 0 and 6 mM glucose may indeed subserve this effect.

There is, however, a major quantitative difference between fresh and cultured islets. The ATP/ADP ratio at all glucose concentrations was much higher after culture. It is particularly striking that the threshold was not changed (between 6 and 10 mM glucose) although 6 mM glucose increased the ATP/ADP ratio in degranulated islets 2.5 times more than did a maximally effective concentration of glucose (20–30 mM) in fresh islets. This might mean that the ATP/ADP ratio is irrelevant for the control of B cell membrane potential and of insulin release. The weight of the evidence (4–10) speaks against this trivial explanation. This apparent discrepancy more likely indicates that the ATP/ADP ratio measured in fresh islets is only a poor index of that in the cytosol because of the size of the granular pool of adenine nucleotides, in which the ratio is close to one. Moreover, the ATP/ADP ratio in the critical submembrane compartment could also be modified by local consumption of ATP (41). Cytosolic ADP, which remains difficult to measure precisely even when the granular pool has been partially decreased, could be the essential variable in the ratio. These variations could well occur within the micromolar range, where they effectively influence the membrane potential of B cells (10).

The major contribution of this study was to show that raising glucose levels above the threshold concentration leads to an increase in the energy state of B cells, that correlates with the increase in insulin secretion at least up to 20 mM glucose. Potential mechanisms of control by adenine nucleotides include the regulation of Ca^{2+} influx through an action on K^+_{ATP} channels (42) or Ca^{2+} channels (43), and the regulation of the efficacy of Ca^{2+} on the secretory process (44). The present observations do not prove that changes in the ATP/ADP ratio are directly involved in the regulation of insulin secretion by physiological glucose concentrations, but show that this long disputed hypothesis is plausible.

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