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The Stimulus-Secretion Coupling of Glucose-Induced Insulin Release

XXXV. The Links between Metabolic and Cationic Events

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Summary. When isolated rat islets were exposed to glucose, the concentrations of NADH and NADPH, and the NADH/NAD^+ and NADPH/$NADP^+$ ratios were increased. The dose-response curve resembled that characterising the glucose-induced secondary rise in ^{45}Ca efflux, displaying a sigmoidal pattern with a half-maximal value at glucose 7.5 mmol/l. The glucose-induced increase in NAD(P)H was detectable within 1 min of exposure to the sugar. Except for the fall in ATP concentration and ATP/ADP ratio found at very low glucose concentrations (zero to 1.7 mmol/l) no effect of glucose (2.8–27.8 mmol/l) upon the steady-state concentration of adenine nucleotides was observed. However, a stepwise increase in glucose concentration provoked a dramatic and transient fall in the ATP concentration, followed by a sustained increase in both O_2 consumption and oxidation of exogenous + endogenous nutrients. This may be essential to meet the energy requirements in the stimulated B-cell. Although no significant effect of glucose upon intracellular pH was detected by the 5,5-dimethyloxazolidine-2,4-dione method, the net release of H^+ was markedly increased by glucose, with a hyperbolic dose-response curve (half-maximal response at glucose 2.9 mmol/l) similar to that characterising the glucose-induced initial fall in ^{45}Ca efflux. It is proposed that the generation of both NAD(P)H and H^+ participates in the coupling of glucose metabolism to distal events in the secretory sequence, especially the ionophoretic process of Ca^{2+} inward and outward transport, and that changes in these parameters occur in concert with an increased turn-over rate of high-energy phosphate intermediates.

Key words: ATP, ADP, AMP, NAD(P)H, pH, Ca, islets, insulin release.

In previous publications we have emphasised the idea that, in the stimulus-secretion coupling for glucose-induced insulin release, the generation of reduced pyridine nucleotides may play a key role in coupling metabolic to subsequent cationic events, as already postulated by several authors [1–3]. This concept was apparently supported by the existence of a tight correlation between the concentration of NADH and NADPH in the islets and the net uptake of ^{45}calcium under a variety of experimental conditions, including exposure of the islets to either agents known to reduce the concentration of NAD(P)H, such as menadione [4, 5] and NH_4^+ [6], or insulinotropic nutrients found to increase the concentration of NAD(P)H, such as glucose itself [5], pyruvate [7], and lactate [8]. The present work was primarily aimed at establishing the dose-response and time-course for the effect of glucose upon the concentration of adenine and pyridine nucleotides in isolated rat islets. The experimental data call for a more careful interpretation of the role of reduced pyridine nucleotides in the coupling between metabolic and cationic events in the secretory sequence.

Material and Methods

All experiments were performed with islets isolated from fed female Wistar albino rats (150–200 g body weight). The methods for measuring release of insulin [9], oxidation of exogenous glu-

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Table 1. Effect of glucose upon the concentration of pyridine nucleotides in the islets

Glucose (mmol/l)	Nil	2.8	5.6	8.3	11.1	16.7	27.8
NADH (fmol/islet)	169±21	170±16	175±16	242±19	276±39	299±22	347±56
NAD^+ (fmol/islet)	1403±46	1641±88	1516±93	1527±69	1642±168	1811±101	1775±120
NADH + NAD^+ (fmol/islet)	1572±71	1811±94	1691±102	1769±80	1918±174	2110±79	2122±162
NADH/NAD^+ (ratio)	0.106±0.007	0.093±0.007	0.119±0.012	0.159±0.014	0.167±0.021	0.174±0.009	0.216±0.041
NADPH (fmol/islet)	210±21	226±41	194±32	249±38	266±37	247±25	260±23
$NADP^+$ (fmol/islet)	164±16	175±35	134±29	146±17	171±18	144±18	137±23
NADPH + $NADP^+$ (fmol/islets)	374±20	401±26	328±17	395±29	437±69	391±27	397±37
NADPH/$NADP^+$ (ratio)	1.311±0.069	1.339±0.087	1.448±0.094	1.624±0.084	1.681±0.274	1.797±0.115	1.890±0.118
NADH + NADPH (fmol/islet)	379±14	396±22	369±22	491±28	542±51	546±34	607±42
(n)	(92)	(20)	(12)	(33)	(30)	(33)	(22)

cose [10], oxidation of endogenous nutrients in islets prelabelled with (U-^{14} C) palmitate [7], concentration of adenine nucleotides [5], concentration of pyridine nucleotides by enzymatic cycling [5] and total concentration of reduced pyridine nucleotides by the luciferase technique [5] were all previously reported. In these experiments the incubation medium containing albumin (0.5 mg/ml; bovine albumin, fraction V; Sigma Chemical Co., St Louis, MO., USA) was equilibrated with 95% O_2/5% CO_2, and had the following ionic composition (mmol/l): Na^+ 139, K^+ 5, Ca^{2+} 1, Mg^{2+} 1, Cl^- 124, and CO_3 H^- 24, pH 7.4 (standard buffer).

Oxygen uptake studies were performed in a microperifusion system. Groups of 250–300 islets were perifused (0.1 ml/min) with standard buffer, but which had been equilibrated with 95% air/5% CO_2. The oxygen tension of the perifusate leaving the chamber was determined with a Clark-type O_2 electrode and continuously recorded. Correction was made for the dead space (66 µl).

The method used to measure the intracellular pH has been described elsewhere [6]. Briefly, after 30 min of preincubation in the absence of glucose, groups of 10 islets were incubated at various glucose concentrations in the presence of either 5,5-dimethyl [2-^{14}C]oxazolidine-2,4 dione (^{14}C-DMO; 1.17 mmol/l) in combination with ^{3}H-sucrose (1.0 mmol/l), or ^3H_2O in combination with ^{14}C-sucrose (1.0 mmol/l). After 30 min of incubation, the islets were separated from the incubation medium (75 µl) by centrifugation through a layer of di-*n*-butylphthalate and examined for their radioactive content.

To measure the output of H^+, groups of 100 islets were incubated in 60 µl of a 5 mmol/l Hepes buffer (pH 7.4) with the following ionic composition (mmol/l): Na^+ 137, K^+ 5, Ca^{2+} 1, Mg^{2+} 1 and Cl^- 144. The medium also contained penicillin (100 iu/ml) and streptomycin (0.1 mg/ml) and was equilibrated against ambient air. After 180 min of incubation, an aliquot of the medium (50 µl) was diluted with 250 µl of distilled water. Within a few minutes, the pH of this diluted solution was measured with a standard pH meter (Metrohm AG, Herisau, Switzerland). The output of H^+ was calculated by reference to a standard curve obtained under identical conditions, when the incubation medium was acidified with known amounts of HCl. The results were expressed as pmol of H^+/min per islet, after correction for the mean reading obtained with control media incubated in the absence of islets. When islets were incubated in the bicarbonate-free Hepes buffer, they oxidised (U-^{14}C) glucose (16.7 mmol/l) at a mean rate (± SEM) of 28.6 ± 4.7 pmol/120 min per islet (n = 10), which was not lower than that found in the usual bicarbonate-buffered incubation medium [23.8 ± 1.6 pmol/120 min per islet (n = 10)]. However, the release of insulin occurred at a lower rate in the bicarbonate-free medium, as previously reported [11]. In the bicarbonate-free medium the output of insulin averaged (mean ± SEM) 3.7 ± 8.6 and 110.9 ± 8.5 µU/90 min per islet (n = 12 in both cases) in the absence and presence of glucose (16.7 mmol/l), respectively; within the same experiment, the glucose-stimulated release of insulin averaged 262 ± 19 µU/90 min per islet (n = 12) when the incubation was carried out in the usual bicarbonate-buffered solution.

All results are expressed as the mean (± SEM) together with the number (n) of observations, each of which refers to an individual group of islets. When data from different experiments were pooled together, the experimental data were expressed relative to the mean appropriate control value (no glucose or glucose 16.7 mmol/l) found within the same experiment. Statistical comparison of data was performed using Student's *t* test, and is restricted to measurements collected within the same experiment(s).

Results

1. Effect of Glucose upon the Steady-State Concentration of Pyridine Nucleotides

Pyridine nucleotides were measured by enzymatic cycling in islets incubated for 30 min at increasing glucose concentrations (Table 1). Glucose increased the concentration of NADH, the NADH/NAD^+ ratio, the NADPH/$NADP^+$ ratio, and the total concentration of both diphosphopyridine nucleotides (NADH + NAD^+) and reduced pyridine nucleotides (NADH + NADPH). Glucose failed to affect the total concentration of triphosphopyridine nucleotides (NADPH + $NADP^+$). The threshold concentration for the stimulant effect of glucose was close to 5.6 mmol/l and a maximum response was seen at a glucose concentration of 11.1 mmol/l or more. The same trend was observed when the total concentrations of reduced pyridine nucleotides (NADH + NADPH) was measured by the luciferase technique. The results obtained after incubation for

30 min at glucose 2.8, 5.6, 8.3, 11.1 and 16.7 mmol/l averaged 102 ± 8 (n = 18), 124 ± 9 (n = 29), 160 ± 9 (n = 54), 171 ± 20 (n = 30) and 186 ± 8 (n = 109) % of the mean basal value (222 ± 9 fmol/islet; n = 156) in the same experiments. Thus, the dose-response curve was clearly sigmoidal with half of the maximal increment attributable to glucose being reached at a glucose concentration close to 7.5 mmol/l (Fig. 1, panel A). This pattern was similar to that characterising the glucose-induced secondary rise in ^{45}Ca efflux (Fig. 1, panel B).

2. Effect of Glucose upon the Steady-State Concentrations of Adenine Nucleotides

When islets are incubated in a glucose-free medium, the concentration of ATP falls [12, 13]. As little as 2.8 mmol/l glucose was sufficient to maintain the concentrations of ATP, ADP and AMP, the ATP/ADP ratio and the adenylate charge (i. e. [ATP + 0.5 ADP]/[ATP + ADP + AMP]) at values comparable to those seen at higher glucose concentrations, all measurements being performed after 30 min of incubation at the stated glucose concentration (Table 2). Glucose failed to affect the total concentration of adenine nucleotides.

3. Time-Course for the Effect of Glucose upon the Concentration of Pyridine and Adenine Nucleotides

In a first series of experiments, groups of 10 islets were preincubated for 0–30 minutes in the absence of glucose and then exposed to glucose 11.1 mmol/l for 1, 3, 10, 15, 30 or 120 min. The NADH concentration and/or NADPH/$NADP^+$ ratio were measured by the enzymatic cycling technique (Fig. 2, lower panel). Within 1 min of exposure to glucose, these variables were increased to 126 ± 8% (n = 30; $p < 0.02$) of the mean corresponding basal value (100 ± 7%, n = 36). Steady-state concentrations of reduced pyridine nucleotides were apparently reached within 3 min, no further significant changes being recorded between the third and 120th min of exposure to glucose. Over this period, the NADH concentration and NADPH/$NADP^+$ ratio plateaued at 136 ± 5% (n = 66) of the initial values (Fig. 2, lower panel). When the islets were maintained in the glucose-free medium, the concentration of reduced pyridine nucleotides slowly decreased below the initial readings (data not shown).

To study more carefully the initial time-course for changes in the concentration of pyridine and adenine nucleotides, groups of 8 islets were incubated for 60 min at a low glucose concentration (1.7 mmol/l) and then exposed to glucose 16.7 mmol/l (Table 3 and Fig. 3). The long preincubation was used to stabilise the concentrations of pyridine and adenine nucleotides prior to stimulation with glucose.

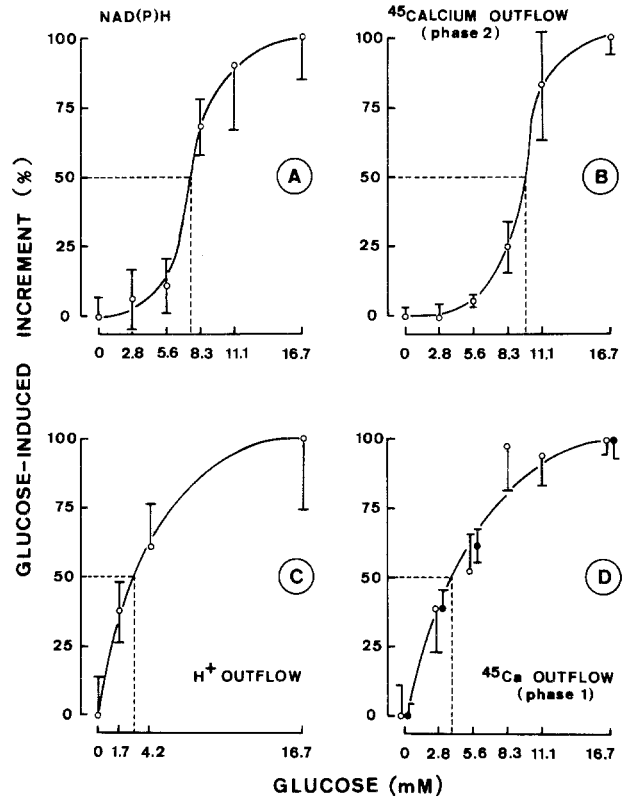

Fig. 1. Dose-response curves for the effect of glucose upon selected metabolic and cationic events in isolated islets. Glucose-induced increments (mean ± SEM) above basal value (no glucose) are expressed in % of the mean increment found at 16.7 mmol/l glucose within the same experiment(s). Also shown is the glucose concentration corresponding to & 50% increment. *Panel A.* Glucose-induced changes in the total concentration of reduced pyridine nucleotides (pooled data obtained by the enzymatic cycling and luciferase techniques). *Panel B.* The difference in ^{45}Ca efflux between the nadir value seen shortly after introduction of glucose and the later zenith value was measured in islets first perifused without glucose and then exposed to glucose at the stated concentration. *Panel C.* Dose-response curve for the glucose-induced increment in H^+ output (see Table 9). *Panel D.* In islets first perifused in the absence of glucose, the introduction of the sugar provokes an initial fall in ^{45}Ca efflux (28). Open circles refer to the fall in ^{45}Ca efflux seen 3 min after introduction of glucose (at the stated concentration) as compared with control values found at the same time in islets perifused without glucose throughout. Closed circles illustrate the complementary phenomenon, in that the differences in ^{45}Ca efflux between control and experimental values refer to those seen 3 min after raising the glucose concentration from the stated level up to 16.7 mmol/l and are plotted with the zero point at the top and the 100% point at the bottom of the ordinates [44]

Within 1 min after raising the concentration of glucose from 1.7 to 16.7 mmol/l, the islets content of NADH and NADPH, which were measured together by the luciferase technique, increased ($p < 0.005$) to

Table 2. Effect of glucose upon the concentration of adenine nucleotides in islets

Glucose (mmol/l)	Nil	2.8	5.6	8.3	11.1	16.7	27.8
Total (pmol/islet)	12.3±0.4	13.8±0.3	13.9±0.7	13.1±0.8	13.6±1.0	14.1±0.6	13.8±0.9
ATP (% total)	55.4±0.9	69.9±1.7	63.1±1.8	68.4±1.5	70.2±1.5	68.1±1.1	63.7±2.4
ADP (%)	26.8±0.7	20.7±1.5	21.0±1.6	17.9±1.3	20.8±1.7	18.1±0.8	21.0±1.5
AMP (%)	17.8±0.8	9.4±1.3	15.9±1.5	13.7±1.1	9.0±1.4	13.8±0.8	15.3±1.9
ATP/ADP	2.40±0.11	3.91±0.46	4.35±0.53	4.77±0.51	3.80±0.32	4.73±0.32	4.05±0.53
Adenylate charge[a]	0.689±0.007	0.803±0.013	0.747±0.015	0.773±0.011	0.805±0.012	0.775±0.008	0.738±0.020
(n)	(148)	(18)	(47)	(33)	(22)	(89)	(38)

[a] Defined as [ATP + 0.5 ADP]/[ATP + ADP + AMP]. Incubations were for 30 min at the appropriate glucose concentration

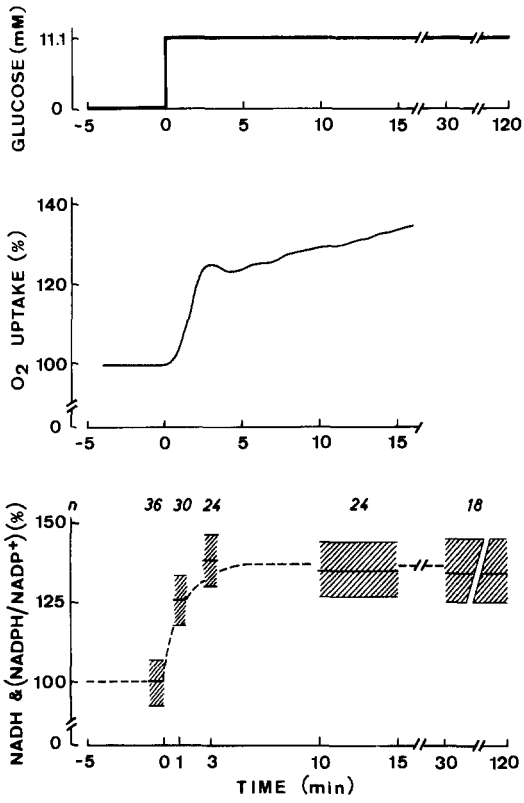

Fig. 2. Time-course for the effect of glucose upon the uptake of O_2 and concentration of reduced pyridine nucleotides in the islets. The concentration of glucose was raised from zero to 11.1 mmol/l at time zero (upper panel). Uptake of O_2 is expressed in % of the basal value and refers to a representative experiment (middle panel). The glucose-induced changes in the concentration of reduced pyridine nucleotides was judged from either the NADH absolute values (fmol/islet) or NADPH/$NADP^+$ ratio, both parameters being expressed in percent of the mean control value (time zero) found within the same experiments. In the present series of experiments the relative magnitude of such changes being similar for both parameters, the data obtained for each parameter were eventually pooled and shown as the mean (± SEM; shaded area) together with the number (n) of individual observations (lower panel)

141 ± 10% (n = 28) of the mean steady-state value measured after 60 min of preincubation at glucose 1.7 mmol/l (100 ± 8%; n = 28). The mean initial peak value was reached after 2 min of stimulation with glucose. Thereafter, i. e. between the third and sixth min inclusive, the values for NAD(P)H were significantly lower ($p < 0.02$) than would be expected if the transition to the new steady-state had occurred in an exponential manner with a time constant defined by the increase in NAD(P)H observed during the first minute of exposure to glucose 16.7 mmol/l. Thus, at the 6th min of exposure to glucose, when the output of insulin reached its nadir between the early secretory peak and later secondary rise [14, 15], the concentration of NAD(P)H represented only 68 ± 6% (n = 14; $p < 0.05$) of the mean value at the 10th min in the same experiments (100 ± 13%; n = 14). In contrast, in the simple exponential model, the value reached at the 6th min should represent 98% of that at the 10th min. Thus, the changes in NAD(P)H with time suggested the existence of a biphasic (or multiphasic) regulatory process. It made little difference whether the concentration of reduced pyridine nucleotides was expressed in absolute value (fmol/islet) or relative to the total amount of adenine nucleotides in order to minimise any variability due to fluctuation in the mean size of the islets in each sample (Table 3).

The total concentration of adenine nucleotides remained fairly stable throughout these experiments, with a mean value of 17.7 ± 0.5 pmol/islet (n = 96). When comparing the steady-state values observed at glucose 1.7 mmol/l (time zero) and 16.7 mmol/l (min 10 in Fig. 3), respectively, there was a significant increase ($p < 0.01$ or less) in the concentration of ATP, the ATP/ADP ratio and the adenylate charge at the highest glucose concentration. This indicates that the 1.7 mmol/l glucose concentration used during the preincubation period was not quite sufficient, as distinct from a 2.8 mmol/l glucose concentration (Table 2), to maintain the adenylate charge of the

Table 3. Concentration of adenine and reduced pyridine nucleotides in islets incubated for 60 min at 1.7 mmol/l glucose and thereafter exposed for different periods of time to glucose 16.7 mmol/l

Time (min)	0	1	2	3	4.5	6	10
ATP + ADP + AMP (pmol/islet)	16.6±0.9	18.1±0.8	18.1±1.0	18.2±1.7	17.3±0.9	18.8±2.0	17.7±1.2
ATP (% of total)	60.7±1.0	52.0±1.3	62.3±2.0	60.1±2.3	64.0±1.6	65.4±1.4	66.3±1.3
ADP (% of total)	20.1±1.1	26.7±1.1	20.3±1.7	19.8±2.0	21.5±2.2	14.4±0.8	16.7±0.9
AMP (% of total)	19.2±1.2	21.3±1.2	17.4±1.8	20.1±1.4	14.5±2.1	20.2±1.2	17.0±1.2
ATP/ADP (ratio)	3.29±0.22	2.07±0.12	3.56±0.34	3.35±0.42	3.75±0.35	4.29±0.34	4.32±0.28
Adenylate charge (ratio)	0.707±0.009	0.652±0.010	0.714±0.016	0.711±0.016	0.735±0.014	0.737±0.014	0.746±0.011
NADH + NADPH (fmol/islet)	176±15	247±18	264±16	242±24	239±16	258±20	349±33
NAD(P)H/(ATP + ADP + AMP) (fmol/pmol)	9.9±0.6	13.3±0.6	14.1±0.7	14.5±0.8	13.1±0.6	14.9±0.9	18.5±1.0
(n)	(26–28)	(28)	(13)	(14)	(14)	(14)	(28)

islet cells at its physiological level. Nevertheless, within 1 min of raising the glucose concentration from 1.7 to 16.7 mmol/l, there was a dramatic fall in the concentration of ATP, the ATP/ADP ratio and the adenylate charge ($p<0.001$ in all cases). This phenomenon (also seen in other tissues; 16, 17) was rapidly reversed. After 2 min of exposure to the high glucose concentration, the islet content of adenine nucleotides had recovered its initial value. Over the late period (min 3 to 10), the concentration of adenine nucleotides re-equilibrated at the level usually found at glucose 16.7 mmol/l; the apparent half-life for such a readjustment did not exceed 1.1–1.2 min (Fig. 3, lower panel).

4. Effects of Glucose or Metabolic Inhibitors on Adenine Nucleotides and Oxygen Consumption

When islets were incubated in the presence of glucose 16.7 mmol/l and then suddenly exposed to a mixture of rotenone (2,0 μmol/l), antimycin A (10.0 μmol/l) and potassium cyanide (KCN; 1.0 mmol/l), the ATP concentration (Fig. 4), ATP/ADP ratio and adenylate charge (data not shown) fell precipituously. Within about 1 min, the ATP concentration was decreased to half its initial value. The total concentrations of AMP + ADP + ATP also decreased from 23.0 ± 2.2 to 18.6 ± 0.6 pmol/islet (n = 7) after 160 sec of exposure. However, even after 30 min of exposure to glucose and such inhibitors, the ATP content of the islets still represented 20.9 ± 1.6% of its initial value, suggesting the possible existence of a compartmentalised pool of ATP (12, 18) characterised by a low fractional turn-over rate.

The true turn-over rate of high-energy phosphate

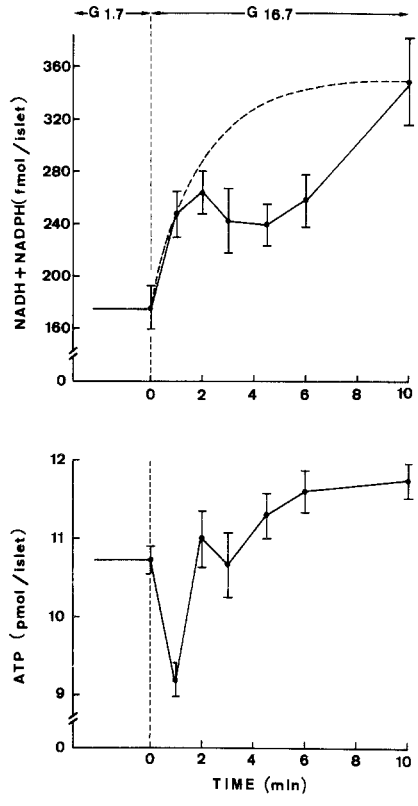

Fig. 3. Time-course for the effect of glucose upon the concentration of reduced pyridine nucleotides (upper panel) and ATP (lower panel) in the islets. Mean values (± SEM) refer to 14–28 individual experiments. The dotted line in the upper panel illustrates the theoretical pattern based on the assumption of an exponential shift from the initial (time zero) to final (10th min) concentration with a time constant defined by the rate of increase in NAD(P)H observed during the first min of exposure to the high glucose concentration. The islets were preincubated for 60 min in 1.7 mmol/l glucose, prior to being exposed to 16.7 mmol/l glucose

intermediates cannot be estimated from the data in Figure 4, since the effect of the drugs upon ATP expenditure, glycolytic flux and creatine-phosphate concentration during these short-term experiments was not assessed. That glucose causes a rapid and sustained increase in ATP generation rate is suggested by the time-course for O_2 consumption illustrated in Figure 2 (middle panel). The glucose-induced increment in O_2 uptake was slightly (but not significantly) smaller than that expected from the rate of exogenous (U-^{14}C) glucose oxidation (Table 4, lines 1 & 2). This difference could reflect a modest sparing action of glucose upon the oxidation of endogenous nutrients (Table 4, line 3). When islets had been labelled with (U-^{14}C) palmitate, the output of $^{14}CO_2$ during subsequent incubation was significantly reduced at high glucose concentrations (Table 5).

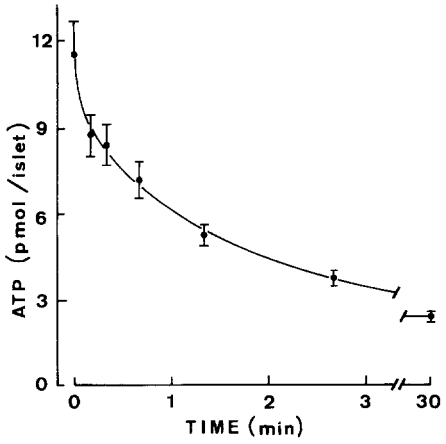

Fig. 4. Time-course for the fall in ATP concentration in islets incubated with 16.7 mmol/l glucose and exposed from time zero onwards to a mixture of rotenone (2.0 μmol/l), antimycin A (10.0 μmol/l) and KCN (1.0 mmol/l). Each value (± SEM) refers to 7 individual determinations

5. Effect of Metabolic Inhibitors upon Other Metabolic and Functional Events

Mannoheptulose decreased glucose oxidation, antagonised the effect of glucose to increase the concentration of reduced pyridine nucleotides, lowered the ATP/ADP ratio and adenylate charge and abolished glucose-induced insulin release in islets exposed to 16.7 mmol/l glucose (Table 6). In the absence of glucose, mannoheptulose failed to affect the relative (Table 7) or absolute (not shown) concentration of adenine nucleotides.

In the presence of glucose, anoxia, although maintaining a high concentration of reduced pyridine nucleotides, lowered the ATP/ADP ratio and adenylate charge, and inhibited glucose oxidation [19] and insulin release (Table 6). A comparable state charac-

Table 4. Effect of glucose upon metabolic indices of islets

Line Nr.	Metabolic index (pmol/min per islet)	Basal value (no glucose)	Glucose-induced increments	
			(glucose 2.8–4.2 mmol/l)	(glucose 16.7–18.2 mmol/l)
1	O_2 consumption	8.10±0.78 (13)	+0.48±0.13 (7)	+1.92±0.24 (7)
2	CO_2 from exogenous glucose[a]	0.00	+0.61±0.07 (16)	+2.59±0.20 (81)
3	CO_2 from endogenous fuel[b]	6.89±0.66 (13)	−0.03±0.20 (31)	−0.99±0.23 (26)
4	Lactate & pyruvate output[c]	0.37±0.03 (11)	+0.77±0.06 (25)	+1.79±0.07 (102)

[a] [8, 13]
[b] Calculated from the basal value in line 1 and the "normalised" $^{14}CO_2$ output in Table 5, assuming a basal R. Q. of 0.85
[c] [43] for lactate output; [5, 8] for pyruvate output

Table 5. Effect of glucose upon $^{14}CO_2$ output from islets prelabelled with (U-^{14}C) palmitate

Glucose (mmol/l)	Antimycin A (mmol/l)	$^{14}CO_2$ output (cpm/120 min per islet)	^{14}C final content (cpm/islet)	$^{14}CO_2$ output/^{14}C content		(n)
				(%)	(normalised)[a]	
Nil	–	187±10	3171±128	5.91±0.22	100.0±1.9	74
5.6	–	228±17	3449±207	6.78±0.39	99.5±2.9	31
8.3	–	182±20	3599±249	4.95±0.31	88.0±3.3	20
16.7	–	137± 8	3174±156	4.47±0.23	85.6±3.3	26
Nil	0.01	67± 5	3445±156	1.95±0.11	33.6±1.2	60

[a] Normalised relative to mean control value (no glucose) found in the same experiment. The amount of [U-^{14}C] palmitate incorporated in the islets during preincubation averaged 2.0 pmol/islet

Table 6 A–C. Effect of metabolic inhibitors in islets exposed to 16.7 mmol/l glucose

A Concentration of adenine nucleotides (30th min)[a, b]

Drug (concentration)	ATP (%)	ADP (%)	AMP (%)	ATP/ADP	Adenylate charge
Nil	72.5±1.3	17.2±1.2	10.3±1.3	4.76±0.37	0.841±0.013
Mannoheptulose (14.3 mmol/l)	63.7±1.4	24.4±2.3	11.9±1.4	2.86±0.38	0.759±0.008
N_2 (95%) – CO_2 (5%)	64.7±2.7	19.4±1.1	15.9±1.9	3.44±0.36	0.704±0.022
KCN (1.0 mmol/l)	32.9±1.1	29.6±1.7	37.5±2.3	1.13±0.06	0.477±0.016
Antimycin A (10.0 μmol/l)	27.8±0.8	27.0±1.1	45.2±1.3	1.04±0.06	0.412±0.010
Oligomycin (5.0 μmol/l)	31.7±2.0	32.4±1.1	35.9±1.9	0.99±0.08	0.478±0.019
CCCP (5.0 μmol/l)	74.3±2.8	17.8±2.1	7.9±1.7	4.75±0.82	0.832±0.021

B Concentration of pyridine nucleotides (30th min)[a]

Drug (concentration)	NADH	NAD^+	NADPH	$NADP^+$	$NADH/NAD^+$	$NADPH/NADP^+$
	(fmol/islet)					
Nil	323±39	2260±140	378±34	298±38	0.142±0.014	1.485±0.205
Mannoheptulose (14.3 mmol/l)	217±28	1679±124	218±31	230±32	0.129±0.008	1.168±0.198
N_2 (95 %) – CO_2 (5%)	428±44	2240±328	347±56	302±51	0.212±0.031	1.406±0.115
KCN (1.0 mmol/l)	673±92	1971±228	368±66	239±30	0.341±0.018	1.762±0.148

C Glucose oxidation and insulin release

Drug (concentration)	Glucose oxidation (pmol/120 min per islet)	Insulin release[c] (μU/90 min per islet)
Nil	49.4±3.2 (100)	317.7±12.8 (42)
Mannoheptulose (14.3 mmol/l)	18.5±3.6 (12)	33.0± 6.3 (4)
N_2 (95%) – CO_2 (5%)	6.3±0.8 (8)	22.6± 9.5 (18)
KCN (1.0 mmol/l)	6.0±1.1 (8)	43.8± 8.9 (20)
Antimycin A (10.0 μmol/l)	− 1.4±0.4 (9)	20.0± 7.9 (10)
Oligomycin (5.0 μmol/l)	9.7±2.9 (10)	56.2±15.6 (12)
CCCP (5.0 μmol/l)	42.4±3.0 (19)	104.8± 8.6 (20)

[a] Mean values (± SEM) refer to 8 or more individual observations

[b] The total concentration of adenine nucleotides was reduced from a mean control value of 8.4±0.8 pmol/islet to 6.4±0.6, 4.9±0.4 and 4.6±0.4 pmol/islet in the presence of KCN, antimycin A and oligomycin, respectively. The other drugs failed to affect significantly the total concentration of adenine nucleotides

[c] In the absence of glucose, the basal release of insulin averaged 21.0±9.8 μU/90 min per islet (n = 20)

terised by a high concentration of reduced pyridine nucleotides and a low ATP/ADP ratio was seen in the presence of KCN, which suppressed glucose oxidation and insulin release (Table 6). Other inhibitors of oxidation (antimycin A) and oxidative phosphorylation (oligomycin) exerted metabolic and functional effects comparable to those evoked by KCN. KCN and antimycin A reduced the ATP concentration both in the absence and presence of glucose (Tables 6 and 7). In islets exposed to glucose, antimycin A, like KCN, did not decrease the concentration of NAD(P)H, which averaged 116 ± 5% of the mean control value found in the sole presence of glucose 16.7 mmol/l (n = 8). All these findings indicate that an elevated concentration of reduced pyridine nucleotides is not sufficient *per se* to maintain an adequate secretory behaviour.

Unexpectedly [12], carbonyl cyanide m-chlorophenyl hydrazone (CCCP) failed to affect the oxidation of glucose and the concentration of adenine nucleotides in islets exposed to glucose 16.7 mmol/l. CCCP augmented by 97 ± 22% ($p < 0.05$) the output of lactic acid from the glucose-stimulated islets (data not shown). Nevertheless, CCCP partially inhibited glucose-induced insulin release, as if the mitochondrial and extramitochondrial generation of ATP was not sufficient to match fully the energy demand of the stimulated islet cells.

6. Effect of Glucose upon Intracellular pH and H^+ Output

After 30 min of incubation, glucose failed to affect the estimated intracellular water space, which aver-

Table 7. Effect of metabolic inhibitors upon the concentration of adenine nucleotides in islets incubated for 30 min in the absence of glucose[a]

Drug (concentration)	ATP	ADP	AMP	ATP/ADP	Adenylate charge
	% total adenine nucleotides				
Nil	61.4±2.0	24.0±2.4	14.6±1.2	2.87±0.49	0.734±0.011
Mannoheptulose (14.3 mmol/l)	62.9±2.7	22.1±1.8	15.0±1.8	3.05±0.38	0.739±0.021
N_2 (95%) − CO_2 (5%)	54.5±2.7	28.2±2.7	17.4±2.5	2.09±0.26	0.685±0.022
KCN (1.0 mmol/l)	18.7±2.5	23.9±1.4	57.4±2.1	0.82±0.15	0.306±0.021
Antimycin A (10.0 μmol/l)	21.4±2.0	22.5±2.0	56.1±2.8	0.99±0.11	0.325±0.022
CCCP (5.0 μmol/l)	52.9±6.6	24.9±2.4	22.2±6.0	2.35±0.77	0.646±0.062

[a] The total concentration of adenine nucleotides was reduced from a mean control value of 9.5±0.8 pmol/islet to 6.3±0.5 and 6.3±0.7 pmol/islet in the presence of KCN and antimycin A, respectively. The other drugs failed to affect significantly the total concentration of adenine nucleotides (n = 8 in all cases)

Table 8. The ^3H_2O and ^{14}C-DMO apparent distribution spaces (in excess of that found with ^{14}C- and ^{3}H-sucrose, respectively) and intracellular pH (range corresponding to the mean ± SEM) was measured in isolated rat islets incubated for 30 min at increasing glucose concentrations

Glucose (mmol/l)	^3H_2O (nl/islet)	^{14}C-DMO (nl/islet)	pH (range)
Nil	3.05±0.24 (31)	1.64±0.20 (30)	6.99−7.11
2.8		1.69±0.23 (16)	7.00−7.13
8.3		2.01±0.21 (15)	7.10−7.20
16.7	3.08±0.23 (31)	1.94±0.25 (31)	7.07−7.19

Table 9. Effect of glucose upon H^+ output from isolated islets. Mean values (±SEM) for the net output of H^+, paired increment in H^+ output above basal value (Δ) and fractional increment relative to that seen at glucose 16.7 mmol/l (Δ in %) are shown together with their statistical significance ([a]$p < 0.05$; [b]$p < 0.025$) and the number of individual observations (n)

Glucose (mmol/l)	H^+(pmol/min per islet)		Δ (%)	(n)
	output	Δ		
Nil	1.10±0.18	−	0.0	(8)
1.7	1.60±0.19	0.46±0.16	37.4±11.0[a]	(4)
4.2	1.80±0.10	0.78±0.20[b]	60.9±15.6[b]	(5)
16.7	2.35±0.33	1.28±0.39[b]	100.0	(6)

aged 3.07 ± 0.17 nl/islet (n = 62). Glucose also failed to affect significantly the ^{14}C-DMO space of distribution in excess of that found with ^{3}H-sucrose (Table 8). Hence, the estimated intracellular pH was not significantly affected by glucose. For the group as a whole, it averaged 7.10 ± 0.03 (n = 92) as distinct from an extracellular pH of 7.35–7.40 ($p < 0.001$).

As shown in Table 9, glucose at high concentrations (16.7 mmol/l) doubled the H^+ output relative to basal values ($p < 0.025$). As little as 1.7 mmol/l glucose was sufficient to increase H^+ output by 0.46 ± 0.16 pmol/min per islet (paired comparison) such an increment representing 37.4 ± 11.0% ($p < 0.05$) of that seen within the same experiment at glucose 16.7 mmol/l. Relative to the latter reference value, the dose-response curve for H^+ output at increasing glucose concentrations was hyperbolic, with a half-maximum value being reached at a glucose concentration close to 2.9 mmol/l. Figure 1 (panels C and D) illustrates the close similarity between the dose-response curves for H^+ output and the initial fall in $^{45}Ca^{2+}$ fractional outflow (phase 1), respectively.

In order to explore the possible cause-and-effect relationship between the two latter events, we investigated the effect of valinomycin, which was recently found to inhibit and delay the glucose-induced initial fall but not secondary rise in ^{45}Ca efflux [20]. In the presence of glucose 16.7 mmol/l but absence of valinomycin, the output of H^+ averaged 2.24 ± 0.30 pmol/min per islet (n = 6), a value significantly higher ($p < 0.05$) than the basal output and almost identical to that found under the same experimental conditions in the first series of experiments (see Table 9). Valinomycin (0.1 μmol/l) caused a partial inhibition of glucose-stimulated H^+ output, the valinomycin-induced paired reduction averaging 0.41 ± 0.15 pmol/min per islet (n = 6; $p < 0.05$). At the concentration used here (0.1 μmol/l), valinomycin augments lactic acid output and only provokes a modest decrease in the oxidation of either glucose (− 12 ± 5%) or endogenous nutrients (− 10 ± 4%), suggesting that the observed fall in H^+ output may not be solely due to a decreased generation of acid metabolites [20].

Discussion

Previous studies suggest that (i) the process of glucose identification as a stimulus for insulin release entirely depends on the metabolism of glucose in the

islet cells [21], (ii) a redistribution of ionophoretic ionic fluxes leads to the accumulation of Ca^{2+} in a critical site of the B-cell [22], and (iii) Ca^{2+} acts as the trigger of insulin release [22]. The data presented in this report suggest that two major factors, i. e. the NAD(P)H concentration and H^+ output, may be responsible for the modification of Ca fluxes. These changes occur in concert with an increased generation of ATP, which may be essential to meet the energy requirement in the stimulated B-cell.

Several limitations of the present study should be kept in mind. First, our data do not refer to a pure B-cell population. Second, no information is given on the distribution of nucleotides between different cellular compartments, and no distinction is made between free and bound nucleotides. Third, in most experiments groups of eight of more islets were incubated in a small volume of medium (100 µl or less), so that a possible feedback effect of released insulin upon metabolic variables [23] cannot be ruled out. And last, the measurement of H^+ output was performed under unphysiological conditions, i. e. in a medium deprived of bicarbonate.

1. Possible Role of NAD(P)H

Any postulated coupling factor between metabolic and cationic events should be able to account for both the dose-response curve and rapidity of the cationic response to glucose. There are converging observations to imply reduced pyridine nucleotides as such a coupling factor [3–8]. In previous works [5, 24–26], it was already demonstrated that glucose increases the concentration of reducing equivalents in the islets, but the dose-response curve had not been established. The present data indicate that there is a close similarity between the dose-response curves for NAD(P)H concentration and secondary rise in ^{45}Ca efflux, respectively (Fig. 1, panels A and B). Since the glucose-induced secondary rise in ^{45}Ca may correspond to a Ca-Ca exchange process mediated by a native ionophoretic system [27], it may be that the redox state of the B-cell directly influences the affinity for Ca of such a system. The latter hypothesis is compatible with the finding that the dihydro-derivative of the ionophore X537A displays an increased capacity to translocate Ca across a hydrophobic phase [33]. It is also known that the secondary rise in ^{45}Ca efflux, the magnitude of which apparently depends on the rate of Ca inward transport in the islet cells [27], is exquisitely sensitive to agents selectively affecting the concentration of NAD(P)H (see Fig. 9 in [4]).

The data in Figure 2 & 3 confirm [24, 45] that the glucose-induced changes in the concentration of reduced pyridine nucleotides occur rapidly enough to account for the changes in cationic fluxes, which are first detected during the second minute of exposure to glucose [28–30]. We were eager to find out whether oscillations in metabolic variables may represent the pacemaker for the biphasic pattern of the bioelectrical and secretory responses to glucose [31, 32]. For reasons already mentioned (see Results, section 3), we feel that our data neither confirm nor rule out such a hypothesis.

2. Possible Role of H^+

The present findings suggest that, whenever glucose at low concentrations (<4.0–5.0 mmol/l) exerts a sustained stimulant action upon physiological processes in the islets, this is unlikely to be due to a change in the concentration of reduced pyridine nucleotides (Table 1). Glucose at these low concentrations affects a number of functional variables, for instance the efflux of $^{86}Rb^+$ and $^{45}Ca^{2+}$ (phase 1) [33, 34] and biosynthesis of proinsulin. Moreover, glucose 2.8 mmol/l causes a sustained enhancement of insulin release evoked either by high concentrations of extracellular Ca^{2+} [35] or by the combination of Ba^{2+} and theophylline in Ca^{2+}-depleted media (Sener & Malaisse, unpublished observation).

It is unlikely that changes in the concentration of adenine nucleotides are solely responsible for these effects of glucose. The magnitude of certain of these effects is markedly increased when the glucose concentration is raised from 2.8 to 5.6 mmol/l. Yet, no significant change in ATP concentration, ATP/ADP ratio or adenylate charge occurs within this range of glucose concentrations (Table 2).

Some of the functional responses seen at low glucose concentrations may instead be coupled to an increased generation and efflux of H^+. Since the measurement of intracellular pH by the DMO method may be affected by inhomogeneity of pH in subcellular areas [36], the failure to detect any significant effect of glucose upon the apparent intracellular pH (Table 8, [37]) does not rule out the possibility of a modest or localised change in cytosolic pH. Anyhow, Table 9 clearly indicates that glucose increases H^+ output from the islets. The magnitude and dose-response curve for the latter effect suggest that it could reflect, in part at least, the release of acidic products derived from the metabolism of nutrients in the islet cells.

The close analogy (Fig. 1, panels C and D) between the dose-response curves for H^+ output and ^{45}Ca efflux (Phase 1) could suggest a cause-and-effect relationship between the two phenomena. The latter concept is compatible with the following obser-

vations. First, in an artificial system for the study of calcium ionophoresis across a hydrophobic phase, a decrease of pH in the chamber mimicking the intracellular compartment indeed causes a decrease outflow of Ca^{2+} from such a chamber [33]. Second, valinomycin, which inhibits and delays the glucose-induced initial fall in ^{45}Ca efflux without suppressing the secondary rise in effluent radioactivity, inhibits glucose-stimulated H^+ output. Third, the postulated link between the glucose-induced changes in H^+ and Ca^{2+} outflow may explain why the initial fall in ^{45}Ca (and ^{86}Rb) efflux is more resistant than the secondary rise in ^{45}Ca efflux to agents (e. g. menadione) which influence islet function by lowering the concentration of NAD(P)H rather than by altering glycolytic or oxidative fluxes (see Fig. 9 in [4]).

3. Energy Metabolism

It has been repeatedly claimed that insulin release represents an energy-requiring process [38, 39]. The data here obtained with metabolic inhibitors are compatible with such a postulate, as are the observed increments in the consumption of O_2 [40] and the oxidation of (exogenous + endogenous) nutrients (Fig. 3 and Table 4). The present values for basal O_2 consumption are higher than those reported in the literature [40–42], but previous measurements were performed in a bicarbonate-free medium.

In conclusion, the present findings invite us to postulate that, in the secretory sequence, the coupling of metabolic to cationic events corresponds to a multifactorial process, in which changes in the availability of reduced pyridine nucleotides and H^+, coupled with an increased generation of ATP, may play an essential role.

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