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Cytosolic ratios of free [NADPH]/[NADP$^+$] and [NADH]/[NAD$^+$] in mouse pancreatic islets, and nutrient-induced insulin secretion

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When the extracellular concentration of glucose was raised from 3 mM to 7 mM (the concentration interval in which β-cell depolarization and the major decrease in K^+ permeability occur), the cytosolic free [NADPH]/[NADP$^+$] ratio in mouse pancreatic islets increased by 29.5%. When glucose was increased to 20 mM, a 117% increase was observed. Glucose had no effect on the cytosolic free [NADH]/[NAD$^+$] ratio. Neither the cytosolic free [NADPH]/[NADP$^+$] ratio nor the corresponding [NADH]/[NAD$^+$] ratio was affected when the islets were incubated with 20 mM-fructose or with 3 mM-glucose + 20 mM-fructose, although the last-mentioned condition stimulated insulin release. The insulin secretagogue leucine (10 mM) stimulated insulin secretion, but lowered the cytosolic free [NADPH]/[NADP$^+$] ratio; 10 mM-leucine + 10 mM-glutamine stimulated insulin release and significantly enhanced both the [NADPH]/[NADP$^+$] ratio and the [NADH]/[NAD$^+$] ratio. It is concluded that the cytosolic free [NADPH]/[NADP$^+$] ratio may be involved in coupling β-cell glucose metabolism to β-cell depolarization and ensuing insulin secretion, but it may not be the sole or major coupling factor in nutrient-induced stimulation of insulin secretion.

INTRODUCTION

Glucose has to be metabolized in the pancreatic β-cell to elicit insulin release (Hedeskov, 1980). It has been shown that in intact β-cells glucose metabolism closes a plasma-membrane K^+ channel with a conductance of 50 pS, an event initiating depolarization (Ashcroft *et al.*, 1984). This leads to opening of voltage-dependent Ca^{2+} channels and influx of Ca^{2+}, which is thought to be the ultimate trigger of the exocytotic process (Matthews & Sakamoto, 1975).

A series of coupling factors, created by glucose metabolism, may link metabolic and cell membrane ionic events. It has thus been demonstrated that ATP directly blocks the 50 pS K^+ channel (Cook & Hales, 1984). However, the concentration giving half-maximal inhibition was determined to be 15 μM, whereas cytoplasmic free ATP concentrations generally are 1–3 mM. That the redox ratio [NAD(P)H]/[NAD(P)$^+$] may play a role as trigger of insulin release was first suggested by experiments on islet surface fluorescence (Panten *et al.*, 1973). Later, increases in whole-islet tissue concentrations of reduced nicotinamide–adenine dinucleotides in islets exposed to stimulatory concentrations of glucose (Trus *et al.*, 1978; Malaisse *et al.*, 1979*a*,*b*; Capito *et al.*, 1984), pyruvate (Sener *et al.*, 1978) and leucine (Malaisse *et al.*, 1982) have been demonstrated. The studies cannot, however, give information on the changes taking place in any particular compartment of the cell, and ratios of [NAD(P)H]/[NAD(P)$^+$] are known to differ by several orders of magnitude between cytosol and mitochondria. The same limitation applies to the studies (Ammon & Verspohl, 1979) which especially have stressed the importance of the [NADPH]/[NADP$^+$] couple. Another limitation of the above-mentioned studies of whole-islet tissue concentrations of these nucleotides is that in cells much NADH and NADPH is protein- or enzyme-bound, and what is really important to know is how the concentrations of the free nicotinamide–adenine dinucleotides vary during stimulation of insulin secretion, since it is the free concentrations of the nucleotides which determine mass-action ratios and reaction rates.

In the present study we have examined the variation in the cytosolic free [NADPH]/[NADP$^+$] ratio not only with glucose as stimulant but also during stimulation of insulin secretion with low glucose + fructose, leucine, and leucine + glutamine. We have furthermore examined whether also changes in the other cytosolic free nicotinamide–adenine dinucleotide couple, [NADH]/[NAD$^+$], could be demonstrated during stimulation with the above-mentioned secretagogues. During stimulation with glucose we have paid special attention to explore possible changes in the free [NADPH]/[NADP$^+$] ratio on raising the glucose concentration from 3 mM to 7 mM, which depolarizes the β-cell membrane to a threshold value from which the slow waves of the membrane potential start (Henquin & Meissner, 1982) and where the major decrease in K^+ permeability occurs (Henquin, 1980). The same glucose concentration interval is also the one in which the steepest increase in rate of insulin biosynthesis can be demonstrated (Ashcroft *et al.*, 1978). Our studies suggest that the cytosolic free [NADPH]/[NADP$^+$] ratio, but not the corresponding [NADH]/[NAD$^+$] ratio, may play a role in the coupling of islet glucose metabolism to insulin-secretory and biosynthetic events by being involved in depolarization of the β-cell plasma membrane. The cytosolic free [NADPH]/[NADP$^+$] ratio is, however, not obligatorily coupled to nutrient-induced stimulation of insulin secretion.

MATERIALS AND METHODS

Materials

^{125}I-labelled pig insulin, rat and mouse insulin standards and insulin antibodies were generous gifts from Dr. Lise Heding, Novo Research Institute, Copenhagen, Denmark. Luciferase from *Achromobacter fischerii* and glutamate decarboxylase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Human serum albumin was from Behringwerke A.G., Marburg/Lahn, W. Germany. Calf thymus DNA was from BDH Chemicals, Poole, Dorset, U.K. Pyruvate dehydrogenase was kindly given by Dr. R. M. Denton, Department of Biochemistry, University of Bristol, U.K. The enzyme was free from contaminating NADH oxidase, α-oxoglutarate dehydrogenase and branched-chain α-oxo acid dehydrogenase activity. Other enzymes, including collagenase, substrates and coenzymes were from Boehringer, Mannheim, W. Germany. 2-Oxo[1-^{14}C]glutaric acid (sodium salt) (sp. radioactivity 50–60 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

At present the only way to estimate free cytosolic [NAD(P)H]/[NAD(P)$^+$] ratios in islets is to use the 'metabolite indicator' method. The principle and limitation of the approach have been discussed in detail by Williamson *et al.* (1967) and by Veech *et al.* (1969). Measurement of whole tissue concentrations of substrate and product of a nicotinamide–adenine dinucleotide-linked dehydrogenase can be used to compute the ratio of free [NAD(P)H]/[NAD(P)$^+$] in a particular cellular compartment, provided that certain conditions are fulfilled: (*a*) the equilibrium constant of the reaction should be known; (*b*) the dehydrogenase in question should preferably be specifically localized to one cellular compartment, and should be present in such high activity that it would catalyse a near-equilibrium reaction; and (*c*) measurement of whole-tissue concentrations of the substrate and product of the dehydrogenase reaction should reasonably reflect their concentration in the compartment of interest.

For computation of the ratio of free [NADH]/[NAD$^+$] in the cytosol of pancreatic mouse islets, we have used measurements of substrate and product concentrations of lactate dehydrogenase (EC 1.1.1.27).

For computation of the corresponding free [NADPH]/[NADP$^+$] ratio we have used measurements of substrate and product of malic enzyme (EC 1.1.1.40).

Cytosolic free [NADPH]/[NADP$^+$] ratios were calculated from the equation

$$\frac{[\text{NADPH}]}{[\text{NADP}^+]} = \frac{[\text{malate}]}{[\text{pyruvate}]} \times \frac{K'}{[\text{CO}_2]}$$

where K' is the apparent equilibrium constant for malic enzyme at pH 7. A value of 3.44×10^{-2} was taken for K' and [CO_2] was assumed to be 1.16 mM (Veech *et al.*, 1969).

Cytosolic free [NADH]/[NAD$^+$] ratios were calculated from the equation

$$\frac{[\text{NADH}]}{[\text{NAD}^+]} = \frac{[\text{lactate}]}{[\text{pyruvate}]} \times K''$$

where K'' is the apparent equilibrium constant for lactate dehydrogenase. K'' was assumed to be 1.11×10^{-4} (Williamson *et al.*, 1967). In each independent experiment, ratios were calculated from concentrations of malate, lactate and pyruvate measured in the same islets.

In sections below it appears clearly that the above-mentioned conditions (*a*) and (*b*) are fulfilled in our experiments. As regards condition (*c*), it is assumed that whole-tissue concentrations of malate, pyruvate and lactate reflect their concentrations in the cytoplasm reasonably. It is known that a ΔpH of 0.3 unit prevailing across the mitochondrial membrane theoretically results in an equilibrium gradient of approx. 4 between mitochondria and cytosol for an electroneutrally transported anion such as malate with a charge of −2. For pyruvate, with a charge of −1, the corresponding gradient would be approx. 2. In β-cells the mitochondrial volume comprises 4.3% of the total cell volume (Dean, 1973). It can thus be calculated that under these conditions equalizing the total cellular content of malate with the cytosolic content of malate causes a 8.9% overestimation of the true cytosolic concentration. Pyruvate will be overestimated by 2.6% and the [malate]/[pyruvate] ratio by 6.1%. Under a series of different experimental conditions, Williamson (1976) found in rat liver mitochondria malate gradients lower than the ΔpH-determined equilibrium value of 4, namely 2–3. If these lower values apply to the β-cells, the overestimation of the cytosolic [malate]/[pyruvate] ratio will be less then 5%, which is a negligible deviation both in relation to the errors in the substrate assays and in relation to the changes in the [NADPH]/[NADP$^+$] ratios actually taking place when the islets are exposed to metabolic perturbation.

Isolation of islets

Islets were prepared by collagenase digestion (Coll-Garcia & Gill, 1969) from the pancreas of male mice (Theillers original strain; Tuck and Son, Rayleigh, Essex, U.K.), weighing approx. 26 g and fed *ad libitum* on a standard laboratory diet.

Insulin measurements

Batches of five islets were incubated in 600 μl of bicarbonate buffer (Krebs & Henseleit, 1932) containing human albumin (1 mg/ml) and additions as stated in Tables and the Figure for 60 min at 37 °C [after gassing with O_2/CO_2 (19:1) for 10 min] with gentle shaking. Samples of the incubation medium were diluted with 0.04 M-sodium phosphate buffer, pH 7.4, containing human serum albumin (1 mg/ml) and stored at −20 °C until assayed by a radioimmunoassay (Heding, 1966) with rat insulin as standard.

Cellular distribution of malic enzyme, lactate dehydrogenase and glutamate dehydrogenase

This was studied as follows. Islets (300) were collected in 300 μl of Tris/HCl buffer (5 mM, pH 7.5) containing 150 mM-KCl and 0.1% human serum albumin. They were homogenized at 4 °C in a small Potter/Elvehjem homogenizer with a loose Teflon pestle. A 150 μl portion of the homogenate was used for assay of lactate dehydrogenase, malic enzyme and glutamate dehydrogenase (a mitochondrial marker) as described below. To the remaining homogenate was added 200 μl of 5 mM-Tris/HCl buffer (pH 7.5) containing 150 mM-KCl and 0.1% albumin, and the mixture was centrifuged at 6000 *g* for 8 min. The supernatant was aspirated (= cytosol).

The pellet was taken up in 300 μl of the above Tris/HCl buffer, centrifuged at 6000 *g* for 8 min and the supernatant aspirated. This washing procedure was repeated twice, and the final pellet was taken up in 150 μl of the above Tris/HCl/albumin medium. The cytosol and washings were combined. The mitochondrial fraction and the homogenate were frozen and thawed three times to disrupt the mitochondria. Glutamate dehydrogenase was measured fluorimetrically at 22 °C in 10–25 μl samples of the homogenate and fractions. The reaction medium (1.0 ml) contained triethanolamine/HCl buffer (50 mM, pH 8.0), EDTA (2.5 mM), ammonium acetate (100 mM), NADH (0.02 mM) and α-oxoglutarate (7 mM). Malic enzyme was also assayed fluorimetrically at 25 °C in 55–120 μl portions of homogenate or fractions. The reaction mixture (2.42 ml) contained triethanolamine/HCl buffer (50 mM, pH 7.4), L-malate (1 mM), $NADP^+$ (0.1 mM), $MnCl_2$ (3 mM) and albumin (0.04%). In the assay for malic enzyme it was demonstrated that the homogenate did not catalyse reduction of NAD^+ under the prevailing assay conditions for malic enzyme, excluding the possibility that the activity observed was residual activity of malate dehydrogenase with $NADP^+$. It was further ascertained that NADPH and pyruvate were formed in equimolar amounts. Lactate dehydrogenase was assayed spectrophotometrically at 25 °C in 70–100 μl samples of homogenate or fractions. The medium (1.115 ml) contained sodium phosphate buffer (50 mM, pH 7.0), pyruvate (0.65 mM), NADH (0.03 mM) and albumin (0.04%).

Meaurements of islet contents of lactate, malate and pyruvate

This was accomplished as follows. Batches of 40 islets were incubated in 100 μl of bicarbonate buffer (Krebs & Henseleit, 1932) containing albumin (1 mg/ml) and glucose, fructose, leucine and glutamine and combinations of these in concentrations as given in the Figure and Tables. The incubation vessels were NUNC (Roskilde, Denmark) Microfuge tubes. The incubation medium was layered on top of 50 μl of oil [a 10:7 (v/v) mixture of phthalic acid dibutyl ester and phthalic acid didecyl ester, sp. gr. 1.02], which again was layered on top of 20 μl of 5% (w/v) $HClO_4$. The tubes were gassed with O_2/CO_2 (19:1) and incubated for 30 min at 37 °C with shaking. After incubation the islets were spun (Beckman Microfuge, 10 s) through the oil layer into the $HClO_4$. The incubation medium was removed and the vessel space above the oil washed with 2×200 μl of distilled water. Control experiments showed that the islets were completely rinsed free of extracellularly added [^{14}C]inulin by this procedure. After removal of incubation medium, the tubes were frozen at −80 °C before assay. The tubes were then heated at 60 °C for 30 min to extract the islet content of metabolites and to destroy endogenous reduced nicotinamide nucleotides. It was duly verified that this extraction procedure was as effective as addition of ice-cold $HClO_4$ to a final concentration of 5%, followed by sonication with a Branson Sonifier B 12, at 50 W for 15 s. The $HClO_4$ extract was neutralized with 5 μl of 2 M-KOH in 0.5 M-triethanolamine, and the tubes were centrifuged on a Beckman Microfuge for 20 s to sediment the precipitate of $KClO_4$. The neutralized extract was now less dense than the oil layer and became the upper phase. Pyruvate, malate and lactate in the neutralized extract were assayed as follows.

Lactate was assayed as $^{14}CO_2$ in the following reactions

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{pyruvate} + \text{NADH} \quad \text{(i)}$$

$$\alpha\text{-Oxo[1-}^{14}\text{C]glutarate} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{Glutamate dehydrogenase}} [^{14}\text{C]glutamate} + \text{NAD}^+ \quad \text{(ii)}$$

$$[^{14}\text{C]Glutamate} \xrightarrow{\text{Glutamate decarboxylase}} \gamma\text{-aminobutyrate} + {}^{14}CO_2 \quad \text{(iii)}$$

The reaction vessel was a small NUNC Microfuge tube. The reaction medium had a final volume of 27 μl and

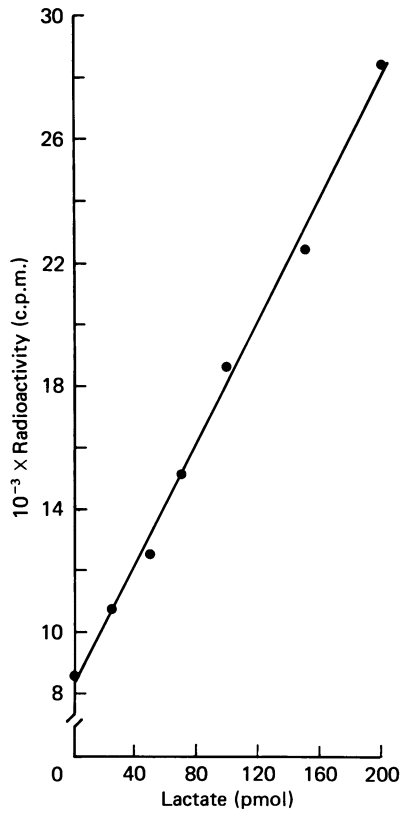

Fig. 1. Typical standard curve for the determination of lactate in the 20–200 pmol range, obtained as described in the Materials and methods section

To measure lactate at this very low concentration, it is absolutely necessary to use gloves, pincette and acid-washed glassware. Even the best distilled water may contain lactate concentrations of 0.5–1.0 μM, so also very small reaction volumes will have to be applied to minimize the blank value.

contained Tris/HCl buffer (0.1 M, pH 9.0), EDTA (1 mM), NAD^+ (0.7 mM), NH_4Cl (80 mM), α-oxo[1-^{14}C]-glutarate (0.08 mM, 0.1 μCi), lactate dehydrogenase (0.6 unit), glutamate dehydrogenase (1.0 unit) and 10 μl of islet extract or lactate standards (in the range 10–200 pmol). The reaction mixture was incubated for 75 min at 25 °C. Then 4 μl of 1 M-HCl was added, and exactly 2 min later 10 μl of 3 M-sodium acetate buffer (pH 5) containing 20 mM-glutamate carrier was added. The reaction vessel was placed in a liquid-scintillation vial together with a somewhat bigger plastic tube containing 200 μl of Hyamine, and the vial was closed by a rubber serum cap.

Through the rubber cap and into the reaction vessel was then injected 10 μl (0.1 unit) of glutamate decarboxylase. After incubation with shaking for 60 min at 30 °C, the rubber caps and reaction vessels were removed and $^{14}CO_2$ radioactivity was counted in a Packard liquid-scintillation counter. Fig. 1 shows a lactate standard curve displaying linear proportionality between amount of lactate standard and $^{14}CO_2$ formed in the range 20–200 pmol of lactate. Incubation medium without islets was also taken through the whole procedure to serve as blanks.

Pyruvate and malate were assayed essentially as described by Ashcroft & Christie (1979).

RESULTS

Table 1 shows that 7 mM-glucose stimulated insulin release, which was further enhanced by 20 mM-glucose. Leucine (10 mM) also stimulated insulin release, whereas 20 mM-fructose had no effect. However, in combination with 3 mM-glucose, 20 mM-fructose elicited insulin release, and 10 mM-glutamine enhanced the effect of 10 mM-leucine considerably.

In the crude homogenate, malic enzyme activity averaged 1.12±0.12 pmol/min per islet ($n = 4$), lactate dehydrogenase activity averaged 82.3±9.9 pmol/min per islet ($n = 4$), and glutamate dehydrogenase activity was 7.92±0.61 pmol/min per islet ($n = 5$). Lactate dehydrogenase and malic enzyme were predominantly localized in the extramitochondrial compartment, with only 11% and 17%, respectively, of their total activities confined to the mitochondrial fraction, which, however, contained more than 85% of the total glutamate dehydrogenase activity. The islets used have a mean dry weight of 0.67 μg (Hedeskov *et al.*, 1972). This is equivalent to a mean wet weight of 2.7 μg, since the water content of mouse islets is 75% (Matschinsky & Ellerman, 1968). The activities of lactate dehydrogenase and malic enzyme are therefore 30.5 and 0.42 μmol/min per g wet wt. at 25 °C. These activities are of the same order of magnitude as the corresponding enzyme activities in the liver (approx. 180 and 1.27 μmol/min per g) (Veech *et al.*, 1969), which have been shown to catalyse near-equilibrium reactions. We have therefore concluded that these two enzymes were suitable for estimation of the cytosolic free [NADH]/[NAD^+] and [NADPH]/[$NADP^+$] ratios in mouse pancreatic islets.

On raising the glucose concentration from 3 mM to 7 mM (Tables 2 and 3), the mean islet content of pyruvate was unaltered and the malate content increased, resulting in a significant 29.5% increase in the [malate]/[pyruvate] ratio and consequently the same increase in the cytosolic free [NADPH]/[$NADP^+$] ratio.

On raising the glucose concentration from 3 mM to 20 mM (Tables 2 and 3) the mean islet content of pyruvate was unchanged, whereas the malate content was more than doubled. Thus in this concentration interval there was a 117% increase in the [malate]/

Table 1. Insulin release in mouse islets exposed to glucose, fructose, leucine and glutamine

Values were obtained as described in the Materials and methods section and are given as means±S.E.M., with the numbers of experiments in parentheses. The indices $^dP < 0.005$ and $^eP < 0.001$ refer to the statistical significance of the difference from basal values at 3 mM-glucose.

Treatment	Insulin release (μunits/five islets per h)
3 mM-Glucose	105±11 (21)
7 mM-Glucose	209±28 (6)^d
20 mM-Glucose	388±22 (21)^e
3 mM-Glucose+20 mM-fructose	187±14 (14)^e
20 mM-Fructose	108± 9 (16)
10 mM-Leucine	197±19 (20)^d
10 mM-Leucine+10 mM-glutamine	334±23 (20)^e

Table 2. Intracellular concentrations of lactate, pyruvate and malate in mouse pancreatic islets after different addition to the medium

Values are given as means±S.E.M., with the numbers of experiments in parentheses. The indices $^bP < 0.02$, $^cP < 0.01$, $^dP < 0.005$ and $^eP < 0.001$ refer to the statistical significance of the difference from basal values at 3 mM-glucose; N.D., not determined.

Addition	Concn. (pmol/islet)		
	Lactate	Pyruvate	Malate
3 mM-Glucose	6.95±0.79 (33)	0.93±0.07 (33)	0.67±0.08 (32)
7 mM-Glucose	N.D.	0.91±0.06 (31)	1.07±0.10 (31)^d
20 mM-Glucose	7.20±0.63 (31)	1.08±0.09 (33)	1.61±0.12 (32)^e
3 mM-Glucose+20 mM-fructose	8.56±1.15 (20)	0.80±0.07 (24)	0.54±0.06 (23)
20 mM-Fructose	8.57±0.98 (22)	0.77±0.06 (24)	0.43±0.05 (24)
10 mM-Leucine	9.30±1.56 (19)	0.79±0.11 (20)	0.39±0.09 (15)^c
10 mM-Leucine+10 mM-glutamine	19.52±2.63 (21)^e	1.27±0.12 (19)^b	2.17±0.12 (19)^e

Table 3. Cytosolic free [NADPH]/[NADP+], [NADH]/[NAD+], [malate]/[pyruvate] and [lactate]/[pyruvate] ratios in mouse pancreatic islets

Values are given as means ± S.E.M., with the numbers of experiments in parentheses. The indices [a]$P < 0.05$, [b]$P < 0.02$, [c]$P < 0.01$, [d]$P < 0.005$ and [e]$P < 0.001$ refer to the statistical significance of the difference from the basal value at 3 mM-glucose; N.D., not determined.

Addition	Ratio			
	[Malate]/[pyruvate]	[Lactate]/[pyruvate]	Cytosolic free [NADPH]/[NADP+]	Cytosolic free [NADH]/[NAD+]
3 mM-Glucose	0.91 ± 0.14 (31)	8.43 ± 0.88 (30)	26.8	9.4×10^{-4}
7 mM-Glucose	1.18 ± 0.13 (31)[a]	N.D.	34.7[a]	N.D.
20 mM-Glucose	1.95 ± 0.27 (32)[d]	8.58 ± 1.17 (29)	57.8[d]	9.5×10^{-4}
3 mM-Glucose + 20 mM-fructose	0.75 ± 0.10 (23)	11.40 ± 1.57 (20)	22.3	12.6×10^{-4}
20 mM-Fructose	0.66 ± 0.10 (23)	12.69 ± 1.70 (22)	19.5	14.1×10^{-4}
10 mM-Leucine	0.52 ± 0.09 (13)[c]	14.33 ± 2.58 (19)	15.5[c]	15.9×10^{-4}
10 mM-Leucine + 10 mM-glutamine	2.09 ± 0.25 (18)[e]	18.92 ± 3.21 (19)[e]	62.0[e]	21.0×10^{-4}[e]

[pyruvate] ratio, and it would be calculated that the cytosolic free [NADPH]/[NADP+] ratio rose from 26.8 to 57.8.

When the glucose concentration was increased to 20 mM it had no effect on the mean islet content of lactate and pyruvate, and consequently no effect on either the [lactate]/[pyruvate] ratio or the cytosolic free [NADH]/[NAD+] (Tables 2 and 3).

Also, neither 20 mM-fructose nor the insulin-stimulatory condition 3 mM-glucose + 20 mM-fructose had any significant effect on the [malate]/[pyruvate] ratio or on the [lactate]/[pyruvate] ratio, and the cytosolic free [NADPH]/[NADP+] ratio and the corresponding [NADH]/[NAD+] ratio were therefore unchanged under these conditions (Table 3).

Leucine (10 mM) decreased the malate content and the free cytosolic [NADPH]/[NADP+] ratio of the islets (Tables 2 and 3). However, the combination of 10 mM-leucine and 10 mM-glutamine increased the mean islet contents of lactate, malate and pyruvate and enhanced the [malate]/[pyruvate] ratio and the [lactate]/[pyruvate] ratio. In this case we found more than a doubling of both the cytosolic free [NADPH]/[NADP+] and [NADH]/[NAD+] ratios (Tables 2 and 3).

DISCUSSION

In liver the redox couple of free [NADPH]/[NADP+] is much more reduced in the cytosol than in the mitochondria (Tischler *et al.*, (1977). Our values for mouse islet cytosolic free [NADPH]/[NADP+] and cytosolic free [NADH]/[NAD+] ratios are very close to those reported for liver, and are clearly markedly different from the whole-islet ratios of [NADPH]/[NADP+] (approx. 1.2) and of [NADH]/[NAD+] (approx. 0.17) measured by ourselves (Capito *et al.*, 1984). This emphasizes the compartmentation of the nicotinamide–adenine nucleotides and underlines the necessity of the type of approach used in the present study. Still it must be emphasized that we are assuming equilibria in the lactate dehydrogenase reaction and the malic enzyme reaction in mouse islets. However, the fact that Sener *et al.* (1984), using the NADP+-linked isocitrate dehydrogenase system, found exactly the same cytosolic [NADPH]/[NADP+] ratio in control islets as we did with the malic enzyme system is convincing evidence that both dehydrogenases do in fact establish equilibria between their substrates and the free nicotinamide–adenine dinucleotides.

The data demonstrate that the cytosolic free [NADPH]/[NADP+] ratio in pancreatic islets is increased by 29.5% on raising the extracellular glucose concentration from 3 mM to 7 mM, and by 117% when the glucose concentration is increased from 3 mM to 20 mM. Furthermore, both 7 mM-glucose and 20 mM-glucose stimulated insulin release. Assuming that the data are representative for the β-cells, which comprise 88% of the total mouse islet volume (Dean, 1973), the results support the concept that an increase in the cytosolic free [NADPH]/[NADP+] ratio may be involved in coupling β-cell glucose metabolism to insulin-secretory and biosynthetic events. They also confirm and extend previous reports (Ashcroft & Christie, 1979; Sener *et al.*, 1984) that glucose increases the [NADPH]/[NADP+] ratio in the cytosolic compartment of islet cells. However, Sener *et al.* (1984) also reported an increase in the cytosolic [NADPH]/[NADP+] ratio in leucine-stimulated rat islets, whereas we observed a fall in the ratio in mouse islets. The reason for this discrepancy is not clear. It may be due to a species difference in β-cell metabolism or to the different composition of rat and mouse islets as regards content of α- and β-cells.

It can also be concluded that the cytosolic free [NADH]/[NAD+] ratio is not a coupling factor in glucose-mediated insulin release.

It is possible that in islets transport of reducing equivalents from NADPH to GSSG takes place, since it has been demonstrated that glucose increases the islet content of GSH (Ammon *et al.*, (1984) and that the glutathione reductase inhibitor 1,3-bis-(2-chloroethyl)-1-nitrosourea decreases insulin release as stimulated by glucose and by leucine + glutamine (Malaisse *et al.*, (1985). It is further possible that GSH may reduce disulphide bridges in plasma-membrane proteins. The amount of protein thiol groups in islets has been shown to be enhanced after glucose stimulation (Anjaneyulu *et al.*, (1982). The reducing equivalents from GSH could be conveyed to the protein disulphides by thioredoxin,

the presence of which also has been demonstrated in islets (Täljedal, 1981). Since presumed increases in islet NAD(P)H after pentobarbital treatment inhibit $^{86}Rb^+$ and $^{42}K^+$ efflux from islets, it is possible that the reducing equivalents ultimately are transferred to a protein disulphide group in a K^+ channel and thereby change its conductance. It has also been shown that a 'gated-pore' mechanism for K^+ loss from uncoupled heart mitochondria can be rapidly closed under conditions that reduce $NAD(P)^+$ (Jung & Brierley, 1982). However, the NADPH–glutathione system may also affect a Ca^{2+} channel.

It seems very reasonable to assume that the 'triggering' of insulin release by glucose- and amino acid-induced changes in islet metabolism is a multifactorial process involving several parameters, and among these, in the case of insulin release stimulated by glucose and by leucine + glutamine, possibly an increase in the cytosolic free [NADPH]/[$NADP^+$] ratio. In contrast, leucine may utilize other triggering factors, since leucine elicited a clear-cut decrease in the cytosolic free [NADPH]/[$NADP^+$] ratio. Also, insulin secretion in the presence of low glucose + fructose was not accompanied by any change in either the [NADPH]/[$NADP^+$] or the [NADH]/[NAD^+] ratio. Other factors may include changes in pH, changes in the phosphate potential in the plasma membrane and an increase in the islet content of cyclic AMP (Hedeskov, 1980). Forskolin, an activator of adenylate cyclase, has been shown, at 10 mM-glucose, to cause a marked increase in the β-cell time spent at a depolarized level with electrical spike activity (Henquin *et al.*, 1983). This effect of forskolin was blocked when Ca^{2+} influx was prevented by Co^{2+}. It has also been shown that glucose and carbacholin elicit a phosphatidylinositol response in rat islets (Montague *et al.*, 1985; Best & Malaisse, 1983). It is thus possible that some of the changes mentioned above may substitute for each other or act in concert and that more than one 'pathway' may lead from islet glucose and amino acid metabolism to depolarization and ensuing insulin secretion.

So far there has only been indirect evidence that the [NAD(P)H]/[$NAD(P)^+$] ratio could play a role in decreasing the K^+ permeability of the β-cells and thereby elicit depolarization of the plasma membrane, Ca^{2+} influx and insulin secretion. Thus pentobarbital, which may increase NAD(P)H concentrations in islet cells (Panten *et al.*, 1973), inhibited the $^{86}Rb^+$ efflux, and Methylene Blue, which may oxidize islet NAD(P)H, increased $^{86}Rb^+$ efflux (Henquin, 1980). Our observation that the cytosolic free [NADPH]/[$NADP^+$] ratio is increased when the glucose concentration is raised from 3 mM to 7 mM gives more direct evidence that this redox couple may in fact be involved in depolarization of the β-cell plasma membrane. However, the relationship is not simple, since depolarization and the decrease in K^+ efflux are most marked for changes of glucose concentration between 3 and 7 mM (Henquin, 1978; Henquin & Meissner, 1982), and we found that the largest increase in the cytosolic free [NADPH]/[$NADP^+$] ratio occurred above 7 mM-glucose. It thus seems unlikely that the sole or major role of this increase in the free [NADPH]/[$NADP^+$] ratio was to regulate the K^+ permeability of the β-cell membrane.

The threshold for stimulation of (pro)insulin biosynthesis by glucose is 2.5 mM, and the steepest increase in the rate of insulin biosynthesis is observed in the glucose concentration range 2.5–8 mM (Ashcroft *et al.*, (1978). An increase in the cytosolic free [NADPH]/[$NADP^+$] ratio may therefore also be involved in the initiation and regulation of (pro)insulin biosynthesis.

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