Temporal sequence of metabolic and ionic events in glucose-stimulated clonal pancreatic β -cells (HIT)

Vildan N. CIVELEK*, Jude T. DEENEY*, Kari KUBIK*, Vera SCHULTZ†, Keith TORNHEIM*† and Barbara E. CORKEY*†‡

*Diabetes and Metabolism Unit, Evans Department of Medicine, and †Department of Biochemistry, Boston University Medical Center, Boston, MA 02118, U.S.A.

Stimulation of insulin release by glucose requires increased metabolism of glucose and a rise in cytosolic free Ca^{2+} in the pancreatic β -cell. It is accompanied by increases in respiratory rate, pyridine and flavin nucleotide reduction state, intracellular pH and the ATP/ADP ratio. To test alternative proposals of the regulatory relationships among free Ca^{2+} , mitochondrial metabolism and cellular energy state, we determined the temporal sequence of these metabolic and ionic changes following addition of glucose to clonal pancreatic β -cells (HIT). Combined measurements of the native fluorescence of reduced pyridine nucleotides and oxidized flavin, intracellular pH, and free Ca^{2+} were performed together with simultaneous measurement of O_2 tension or removal of samples for assay of the ATP/ADP ratio. The initial changes were detected in three phases. First, decreases

occurred in the ATP/ADP ratio (< 3 s) and increases in pyridine (2 ± 1 s) and flavin (2 ± 1 s) nucleotide reduction. Next, increases in the O_2 consumption rate (20 ± 5 s), the ATP/ADP ratio (29 ± 12 s) and internal pH (48 ± 5 s) were observed. Finally, cytosolic free Ca^{2+} rose (114 ± 10 s). Maximal changes in the ATP/ADP ratio, O_2 consumption and pyridine and flavin nucleotide fluorescence preceded the beginning of the Ca^{2+} change. These relationships are consistent with a model in which phosphorylation of glucose is the initial event which generates the signals that lead to an increase in respiration, a rise in the ATP/ADP ratio and finally influx of Ca^{2+} . Our results indicate that Ca^{2+} does not function as the initiator of increased mitochondrial respiration.

INTRODUCTION

Numerous metabolic and ionic changes accompany glucose-induced insulin secretion and may be part of or modulate the stimulus–secretion coupling mechanism. These include increases in cytosolic free Ca^{2+} [1], pyridine [2] and flavin [3] nucleotide reduction states and intracellular pH (pH_i) [4]. There are rises in the mitochondrial precursor substrates, α -glycerophosphate (α -GP) [5], which is linked to flavin, and pyruvate [6], which is linked to pyridine nucleotide oxidation state. The rise in pH_i may also reflect mitochondrial pyruvate metabolism, since it can be blocked by an inhibitor of mitochondrial pyruvate transport [7]. In addition, glucose stimulation causes increases in the ATP/ADP ratio (reviewed by Erecinska et al. [8]) and the rate of respiration [9–11], both of which generally appear to correlate with the secretory rate. The rise in the ATP/ADP ratio is thought to be important for closing ATP-sensitive K^+ channels (K_{ATP} channels), thereby depolarizing the cell and causing influx of Ca^{2+} through voltage-sensitive channels [12]. Moreover, an initial decrease in the ATP/ADP ratio has been suggested to occur on glucose phosphorylation, to stimulate glycolysis and mitochondrial metabolism [13–15] and to account for observed transient increases in K_{ATP} channel activity and hyperpolarization [16]. On the other hand, the observed rise in cytosolic free Ca^{2+} , in addition to stimulating exocytosis of secretory vesicles, has also been suggested to be responsible for stimulating respiration, by raising mitochondrial Ca^{2+} and activating the Ca^{2+} -sensitive mitochondrial dehydrogenases [17–19]. Such stimulation of oxidative phosphorylation could then lead to the rise in the ATP/ADP ratio. In light of these alternative suggestions, a key

unanswered question is whether the rise in Ca^{2+} is the cause or a consequence of increased mitochondrial metabolism and/or the rise in the ATP/ADP ratio. Determination of the temporal relationship of these changes would provide a test of putative causal relationships. However, to date there have been few detailed time-course studies, other than those showing that pyridine and flavin nucleotide fluorescence changes precede the rise in free Ca^{2+} [2,3,20] and our recent studies showing that the rise in pH_i precedes the rise in Ca^{2+} [7]. The current study was undertaken to compare the time course of changes in the ATP/ADP ratio and O_2 consumption with other documented changes and to assess the role of Ca^{2+} in regulating oxidative metabolism.

Our data confirm that Ca^{2+} changes follow pyridine and flavin nucleotide and pH_i changes in response to stimulation by glucose. They show that a small decrease in the ATP/ADP ratio is among the earliest changes, like the increase in redox state, and that this is followed by an increase in O_2 consumption and the ATP/ADP ratio and then a rise in pH_i . They are consistent with a model in which phosphorylation of glucose is the initial event which generates the signals that lead to an increase in respiration and a rise in pH_i and finally Ca^{2+} , and inconsistent with a role for Ca^{2+} in initiating the increase in respiration or the measured metabolic events.

EXPERIMENTAL

Growth and incubation of cells

Clonal pancreatic β -cells (HIT-T 15) were cultured in RPMI 1640 medium supplemented with 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$

Abbreviations used: α -GP, α -glycerophosphate; K_{ATP} channel, ATP-sensitive K^+ channel; pH_i , intracellular pH; BCECF/AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester.

‡ To whom correspondence should be addressed.

streptomycin and 10% (v/v) fetal-calf serum, used between passages 64 and 80. The cells were harvested with PBS containing 0.02% EDTA and washed in Hank's Ca^{2+} - Mg^{2+} free buffer, pH 7.4 [21]. Cells were added at a final concentration of 0.14–2.4 mg of protein/ml.

Ca^{2+} and pH_i measurements

Cells were loaded in a modified Krebs buffer (109 mM NaCl, 4.6 mM MgSO_4 , 2 mM CaCl_2 , 0.15 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 5 mM NaHCO_3 , 10 mM Hepes, pH 7.4) containing $1\ \mu\text{M}$ 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy-methyl ester (BCECF/AM), $2\ \mu\text{M}$ fura-2/AM or fluo-3/AM for 30 min at 37 °C. For fluorescence recordings, cells were suspended in 1–2 ml at 30 °C with constant stirring. A computer-controlled Hitachi F-2000 spectrofluorimeter, cycle scanning up to four excitation and emission wavelengths, was employed to monitor fluorescence. The signals were stored in an IBM-compatible computer for further analysis. pH_i was measured using BCECF at excitation wavelengths of 440 and 495 nm and an emission wavelength of 535 nm. Ca^{2+} was measured using fura-2 at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. For measurement of Ca^{2+} with fluo-3 to avoid interference with native NAD(P)H fluorescence, wavelengths of 488 nm excitation and 530 nm emission were used.

Oxygen consumption

O_2 consumption was measured using a Clark-type electrode with an amplifier designed and built by the Bio-Instrumentation Group of the University of Pennsylvania.

Pyridine and flavin nucleotide fluorescence

Reduced pyridine and oxidized flavin nucleotide fluorescence was measured at excitation and emission wavelengths of 340 and 460 nm, and 460 and 540 nm respectively.

Assays and materials

For assay of ATP and ADP, samples of the cell suspension were deproteinized with trichloroacetic acid at various times before and after the glucose addition. After centrifugation, the supernatants were neutralized by ether extraction, lyophilized (SpeedVac) and stored at $-80\ ^\circ\text{C}$ until redissolved in water for assay. ATP and ADP were assayed by previously described bioluminescence methods [22]. Data are presented as the ATP/ADP ratio, which is unaffected by loss of sample volume in the ether extraction or numbers of cells.

RPMI 1640 medium, penicillin and streptomycin were from Gibco. Fetal-calf serum was obtained from Hyclone Laboratories. Biochemicals were from Sigma or Boehringer Mannheim.

Statistical evaluation

Data are presented as the means $\pm$ S.D. of the indicated number of separate experiments.

RESULTS

The ATP/ADP ratio

Addition of glucose at 37 °C caused a rapid decrease in the ATP/ADP ratio (Figure 1A) that was significant and maximal at 2 s, the earliest time point obtainable. After the decrease, an increase in the ATP/ADP ratio occurred which was evident between 5 and 10 s and maximal by about 40 s (Figure 1A). The

Figure 1 Time course of changes in the ATP/ADP ratio in clonal pancreatic β -cells following glucose addition at 37 °C (A) and 30 °C (B)

Cells (0.14 mg/ml) were incubated in Krebs-Hepes buffer in a stirred temperature-controlled cuvette. Glucose (10 mM) was added at zero time. At the indicated times aliquots were removed and extracted for ATP and ADP analysis as described in the Experimental section. (A) shows the changes in ATP/ADP ratio from a single preparation that is representative of studies that were repeated three times with different preparations. The insert to (A) shows the early changes with standard deviation from a single preparation in which the experiment was repeated three times. The changes in the ATP/ADP ratio shown in (B) are representative of studies that were repeated three times with different preparations. The average basal value for the ATP/ADP ratio was 4.2 ± 0.2 ($n = 9$).

early changes are shown in the insert to Figure 1(A). The rapidity of the changes and accompanying difficulty in comparing changes in this ratio with other parameters led us to evaluate the time course at the lower temperature of 30 °C where secretion still occurs [23]. Figure 1(B) illustrates a more sustained decrease that was significant and maximal at 3 s, the earliest time point tested, and remained low for at least 10 s. This was followed by a slower increase that was not evident until about 20 s and did not peak until 150 s. In a series of three separate experiments, basal ratios were 4.2 ± 0.2 ($n = 9$). The ratio decreased to 3.6 ± 0.3 ($n = 6$) during the interval between 3 and 11 s, began to rise at 20 s to 4.4 ± 0.1 ($n = 3$) and rose to the maximal value of 8.1 ± 0.8 ($n = 6$) by 150 s. Since basal values did not differ significantly and qualitatively similar changes occurred at 30 °C and 37 °C, subsequent experiments were performed at 30 °C to slow the processes and permit clearer comparisons among the parameters measured.

Simultaneous time courses of changes in NAD(P)H, FAD^+ and O_2 consumption

Figure 2 illustrates a typical experiment monitoring changes in these parameters in response to a stimulatory glucose concentration. The broken line indicates the time of glucose addition in this and subsequent Figures. A rapid increase in reduced pyridine nucleotide and decrease in oxidized flavin fluorescence occurred with mean delay times of 2 s, whereas the increase in O_2 consumption began at about 20 s.

Figure 2 Simultaneous measurements of changes in NAD(P)H, FAD⁺ and O₂ consumption

Glucose (10 mM) was added at zero time. Traces shown are from a representative experiment that was repeated three times.

Figure 3 Simultaneous measurements of changes in O₂ consumption, pH_i and cytosolic free Ca²⁺

Cells were loaded with the fluorescent indicators BCECF (for pH_i) and fura-2 (for Ca²⁺). Traces shown are from a representative experiment that was repeated three times.

Simultaneous time courses of changes in O₂ consumption, pH_i and Ca²⁺

Figure 3 shows that the increases in O₂ consumption and pH_i preceded the rise in intracellular free Ca²⁺. In a series of 10 experiments, the time between the increase in O₂ consumption and the increase in Ca²⁺ was 85 ± 12 s, while that between the increase in pH_i and Ca²⁺ was 66 ± 7 s.

Simultaneous time courses of changes in NAD(P)H and Ca²⁺

Figure 4 illustrates that both the initial increase and half-maximal change in pyridine nucleotide fluorescence preceded the increase in Ca²⁺. It should be noted that little further increase in the NAD(P)H signal followed the rise in Ca²⁺. The temporal difference between the half-maximal rise in NAD(P)H and the start of the Ca²⁺ increase was 93 ± 10 s ($n = 10$). These experiments indicate that the rise in NAD(P)H is not a consequence of the rise in Ca²⁺. Ca²⁺ is here measured with fluo-3, to avoid the

Figure 4 Simultaneous measurements of changes in pyridine nucleotide oxidation state and cytosolic free Ca²⁺

Cells were loaded with the Ca²⁺ indicator fluo-3 instead of fura-2 to avoid interference with native NAD(P)H fluorescence. Traces shown are from a representative experiment that was repeated three times.

Table 1 Temporal sequence of initial and half-maximal changes in metabolic and ionic parameters following glucose addition

Data are means $\pm$ S.D. ($n = 6-12$) obtained from experiments of the type illustrated in Figures 1-4. The following maximal changes were obtained in response to glucose: cytosolic free Ca²⁺ increased from a basal value of 58 ± 10 to 123 ± 19 nM; O₂ consumption increased from a basal value of 20 ± 1 to 40 ± 2 nmol/min per mg of protein; and glucose reduced nucleotide fluorescence by $11 \pm 3\%$ of the maximum KCN-induced reduction.

Parameter	Initial change (s)	Half-maximal change (s)
NAD(P)H	2 ± 1	21 ± 7
FAD ⁺	2 ± 1	53 ± 8
ATP/ADP decrease	< 3	< 3
ATP/ADP increase	20 ± 5	44 ± 9
O ₂	29 ± 12	29 ± 12
pH _i	48 ± 5	175 ± 22
Ca ²⁺	114 ± 10	325 ± 20

overlap of fura-2 excitation with that of NAD(P)H. Similar time courses were seen with the two Ca²⁺ indicators [initial increase at 106 ± 5 s ($n = 3$) with fluo-3 and 114 ± 10 s with fura-2].

Summary of the temporal sequence of changes in glucose-induced signals

A summary of the times for initial (column 2) and half-maximal changes (column 3) in three to 10 experiments is shown in Table 1. Changes following glucose addition were detected in the following sequence: first of all, parallel changes in flavin and pyridine nucleotides began at 2 ± 1 and 2 ± 1 s respectively, while the maximal decrease in the ATP/ADP ratio had already occurred by 3 s. Next, the rise in the ATP/ADP ratio began at 20 ± 5 s and O₂ consumption increased maximally by 29 ± 12 s. These changes coincided with half-maximal changes in pyridine nucleotide oxidation state (21 ± 7 s). Subsequently, pH_i increased (48 ± 5 s) and the half-maximal increase in the ATP/ADP ratio occurred (44 ± 9 s). Finally, the Ca²⁺ increase began at 114 ± 10 s, while the pH_i increase reached its half-maximal value at 175 ± 22 s. After all the other changes, Ca²⁺ reached its half-maximal value at 325 ± 20 s.

DISCUSSION

The studies presented here delineate the temporal relationships of metabolic and ionic changes that occur on glucose stimulation of clonal pancreatic β -cells. We show that among the earliest events is a small fall in the ATP/ADP ratio. This occurs at about the same time as the rise in pyridine and flavin nucleotide reduction state. It is consistent with a model in which the initial event is glucose phosphorylation via glucokinase, although the importance of this small decrease in the ATP/ADP ratio is not clear. If, as we have previously proposed [14,15], metabolism occurs in an oscillatory manner, the lowering of the ATP/ADP ratio may play a role in permitting substrate-driven increases in pathway flux, since high values of this ratio inhibit both respiration and flux through phosphofructokinase. It is also possible that the apparent magnitude of the transient decrease in the ATP/ADP ratio is attenuated by asynchrony in the cell population. Our recent documentation of the oscillatory isoform of phosphofructokinase (muscle-type) in β -cells [24] and coordinate oscillations in cytosolic free Ca^{2+} and the ATP/ADP ratio in glucose-stimulated mouse β -cells [25] support this concept.

The very rapid changes in the pyridine and flavin nucleotide oxidation state probably reflect increased production of mitochondrial substrate. The FAD-linked enzyme, α -GP dehydrogenase, is very active in β -cells [26], and increased α -GP production occurs with glucose stimulation [5]. Hence, increased α -GP levels from glycolytically generated dihydroxyacetone phosphate and NADH are a likely cause of the decreased FAD (increased FADH_2) signal. The equilibrium among the different oxidation sites within the respiratory chain could then explain the simultaneous rise in pyridine nucleotide fluorescence, which is probably primarily a reflection of mitochondrial NAD(P)H because of the enhancement of fluorescence by mitochondrial proteins [27].

The increase in O_2 consumption is initiated after and is probably a direct consequence of the rises in both ADP and reduction state. The changes in respiration as well as oxidation state are maximal or near maximal before the rise in cytosolic Ca^{2+} , and there is thus little if any additional change in response to Ca^{2+} . This excludes the possibility that respiration is initially stimulated through Ca^{2+} activation of mitochondrial dehydrogenases, such as NAD^+ -isocitrate dehydrogenase, and the pyruvate and 2-oxoglutarate dehydrogenase complexes. However, Ca^{2+} may still serve to activate these dehydrogenases during the sustained phase of secretion.

The temporal sequence shown here, especially in the experiments simultaneously measuring NAD(P)H fluorescence and Ca^{2+} with fluo-3, supports earlier indications that the change in oxidation state precedes the rise in Ca^{2+} [2,3,20]. Those earlier studies used the Ca^{2+} indicator fura-2, which overlaps with the NAD(P)H signal; therefore, measurements of the two parameters were usually performed separately, or inferences made on the basis of relative direction of changes at 340 and 380 nm excitation. Glucose-stimulated changes in flavoprotein fluorescence were also reported (but not shown) by Duchen et al. [3], but no timing was given because of the smallness of the signals. Although our time for the beginning of the Ca^{2+} rise (114 s) is in the range of those earlier studies (95–129 s), the rapid rise in reduction state seen here appears sooner than the 27–46 s reported in those studies. This may indicate more rapid glucose metabolism in the HIT cells; alternatively, it may reflect a broader distribution of response times in the larger population of the suspensions used here, compared with results obtained from a small number of single cells.

The stimulation of both respiration (oxidative phosphorylation) and glycolysis is presumably responsible for the rise in the ATP/ADP ratio, which begins almost simultaneously with the increase in respiration. The decrease in ADP and rise in ATP should cause closure of K_{ATP} channels, leading to membrane depolarization and influx of Ca^{2+} through voltage-sensitive channels. This sequence of events is consistent with the observation here that the rise in cytoplasmic Ca^{2+} follows the rise in the ATP/ADP ratio. The temporal data clearly show that the initial rise in the ATP/ADP ratio cannot be a consequence of the rise in Ca^{2+} , such as by stimulation of the Ca^{2+} -sensitive mitochondrial dehydrogenases. These data are consistent with the observations of Duchen et al. [3] that closure of K_{ATP} channels occurs earlier than the Ca^{2+} rise.

As also shown here, we recently demonstrated that the rise in cytoplasmic pH precedes the rise in Ca^{2+} in single or small clusters of glucose-stimulated mouse β -cells [7]. Furthermore, in most cases the rise in pH_i (but not Ca^{2+}) was prevented by 3-hydroxycyanocinnamate, an inhibitor of mitochondrial pyruvate transport. This suggested the involvement of pyruvate co-transport with H^+ and/or metabolism to CO_2 and water. Such a mechanism would require consumption of pyruvate before its replenishment from glycolysis. The observations here that the pH_i rise follows the increase in pyridine nucleotide fluorescence would suggest either that another alkalization mechanism is dominant in HIT cells [7], that cytosolic NAD(P)H makes a large contribution to the fluorescent signal, or that other mitochondrial substrates than pyruvate are responsible directly or indirectly for the pyridine nucleotide redox change (e.g. α -GP; see above). The rise in pH_i may be important to the stimulus–secretion mechanism, by activating phosphofructokinase [28,29] and hence glycolysis and ATP production, as well as through effects on ion channels [30–32].

The Ca^{2+} rise occurs late and does not overlap with any changes except in pH_i . The continued rise in pH_i after the start of the Ca^{2+} rise could perhaps be due in part to Ca^{2+} activation of pyruvate dehydrogenase and a further stimulation of pyruvate entry into mitochondria. Although the Ca^{2+} increase does not cause further changes in other metabolic parameters measured here, high Ca^{2+} levels may be important for the formation of other coupling factors such as citrate and malonyl-CoA [33,34], as well as for exocytosis of the secretory granules.

Various of the parameters monitored here, in particular intracellular free Ca^{2+} and pyridine nucleotide fluorescence, have been observed to oscillate when studied at the single-cell or single-islet level [15,20,35–43]. Furthermore, oxygen consumption oscillates in perfused islets, as does insulin secretion *in vivo* and *in vitro* [15,36,44–48]. We have proposed that these oscillations with similar periods of minutes may be due to oscillatory behaviour of glycolysis and the ATP/ADP ratio [13–15]. Such oscillations were not seen in the present study, most likely because of a lack of synchrony of the large number of cells in the suspensions and perhaps also because of the limited time duration of the observations.

This work was supported by United States Public Health Service grants DK35914 (B.E.C.), DK46200 (B.E.C.) and DK31559 (K.T.).

REFERENCES

- 1 Prentki, M. and Matschinsky, F. M. (1987) *Physiol. Rev.* **67**, 1185–1248
- 2 Pralong, W. F., Bartley, C. and Wollheim, C. B. (1990) *EMBO J.* **9**, 53–60
- 3 Duchen, M. R., Smith, P. A. and Ashcroft, F. M. (1993) *Biochem. J.* **294**, 35–42

- 4 Juntti-Berggren, L., Tornheim, K., Corkey, B. E. and Berggren, P.-O. (1994) in *Frontiers of Pancreatic β -Cell Research* (Flatt, P. R., ed.), pp. 145–151, Elsevier, Amsterdam
- 5 Sener, A. and Malaisse, W. J. (1992) *J. Biol. Chem.* **267**, 13251–13256
- 6 Ashcroft, S. J. H. and Christie, M. R. (1979) *Biochem. J.* **184**, 697–700
- 7 Juntti-Berggren, L., Civelek, V. N., Berggren, P. O., Schultz, V., Corkey, B. E. and Tornheim, K. (1994) *J. Biol. Chem.* **269**, 14391–14395
- 8 Erecinska, M., Bryla, J., Michalik, M., Meglasson, M. D. and Nelson, D. (1992) *Biochim. Biophys. Acta* **1101**, 273–295
- 9 Welsh, M., Hellerström, C. and Andersson, A. (1982) *Biochim. Biophys. Acta* **721**, 178–184
- 10 Panten, U. and Klein, H. (1982) *Endocrinology* **111**, 1595–1600
- 11 Hutton, J. C. and Malaisse, W. J. (1980) *Diabetologia* **18**, 395–405
- 12 Hopkins, W. F., FATHERAZI, S., Peter-Riesch, B., Corkey, B. E. and Cook, D. L. (1992) *J. Membr. Biol.* **129**, 287–295
- 13 Corkey, B. E., Deeney, J. T., Glennon, M. C., Matschinsky, F. M. and Prentki, M. (1988) *J. Biol. Chem.* **263**, 4247–4253
- 14 Corkey, B. E., Tornheim, K., Deeney, J. T., Glennon, M. C., Parker, J. C., Matschinsky, F. M., Ruderman, N. B. and Prentki, M. (1988) *J. Biol. Chem.* **263**, 4254–4258
- 15 Longo, E. A., Tornheim, K., Deeney, J. T., Varnum, B. A., Tillotson, D., Prentki, M. and Corkey, B. E. (1991) *J. Biol. Chem.* **266**, 9314–9319
- 16 Arkhammar, P., Nilsson, T., Rorsman, P. and Berggren, P.-O. (1987) *J. Biol. Chem.* **262**, 5448–5454
- 17 McCormack, J. G., Longo, E. A. and Corkey, B. E. (1990) *Biochem. J.* **267**, 527–530
- 18 Rutter, G. A., Pralong, W.-F. and Wollheim, C. B. (1992) *Biochim. Biophys. Acta* **1175**, 107–113
- 19 Rutter, G. A., Theler, J. M., Murgia, M., Wollheim, C. B., Pozzan, T. and Rizzuto, R. (1993) *J. Biol. Chem.* **268**, 22385–22390
- 20 Gilon, P. and Henquin, J. C. (1992) *J. Biol. Chem.* **267**, 20713–20720
- 21 Deeney, J. T., Tornheim, K., Korchak, H. M., Prentki, M. and Corkey, B. E. (1992) *J. Biol. Chem.* **267**, 19840–19845
- 22 Schultz, V., Sussman, I., Bokvist, K. and Tornheim, K. (1993) *Anal. Biochem.* **215**, 302–304
- 23 Kikuchi, M., Wollheim, C. B., Cuendet, G. S., Renold, A. E. and Sharp, G. W. (1978) *Endocrinology* **102**, 1339–1349
- 24 Yaney, G. C., Schultz, V., Cunningham, B. A., Dunaway, G. A., Corkey, B. E. and Tornheim, K. (1995) *Diabetes* **44**, 1285–1289
- 25 Nilsson, T., Schultz, V., Berggren, P.-O., Corkey, B. E. and Tornheim, K. (1996) *Biochem. J.* **314**, 91–94
- 26 MacDonald, M. J. (1981) *J. Biol. Chem.* **256**, 8287–8290
- 27 Avi-Dor, Y., Olson, J. M., Doherty, M. D. and Kaplan, N. O. (1962) *J. Biol. Chem.* **237**, 2377–2383
- 28 Trivedi, B. and Danforth, W. H. (1966) *J. Biol. Chem.* **241**, 4110–4112
- 29 Ui, M. (1966) *Biochim. Biophys. Acta* **124**, 310–322
- 30 Misler, S., Gillis, K. and Tabcharani, J. (1989) *J. Membr. Biol.* **109**, 135–143
- 31 Kaibara, M. and Kameyama, M. (1988) *J. Physiol. (Camb)* **403**, 621–640
- 32 Takahashi, K.-I., Dixon, D. B. and Copenhagen, D. R. (1993) *J. Gen. Physiol.* **101**, 695–711
- 33 Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T. and Corkey, B. E. (1992) *J. Biol. Chem.* **267**, 5802–5810
- 34 Corkey, B. E., Glennon, M. C., Chen, K. S., Deeney, J. T., Matschinsky, F. M. and Prentki, M. (1989) *J. Biol. Chem.* **264**, 21608–21612
- 35 Kindmark, H., Kohler, M., Nilsson, T., Arkhammar, P., Wiechel, K. L., Rorsman, P., Efendic, S. and Berggren, P. O. (1991) *FEBS Lett.* **291**, 310–314
- 36 Gilon, P., Shepherd, R. M. and Henquin, J. C. (1993) *J. Biol. Chem.* **268**, 22265–22268
- 37 Grapengiesser, E., Gylfe, E. and Hellman, B. (1988) *Biochem. Biophys. Res. Commun.* **151**, 1299–1304
- 38 Grapengiesser, E., Gylfe, E. and Hellman, B. (1989) *Arch. Biochem. Biophys.* **268**, 404–407
- 39 Santos, R. M., Rosario, L. M., Nadal, A., Garcia, S. J., Soria, B. and Valdeolmillos, M. (1991) *Pflügers Arch.* **418**, 417–422
- 40 Theler, J. M., Mollard, P., Guérineau, N., Vacher, P., Pralong, W. F., Schlegel, W. and Wollheim, C. B. (1992) *J. Biol. Chem.* **267**, 18110–18117
- 41 Valdeolmillos, M., Santos, R. M., Contreras, D., Soria, B. and Rosario, L. M. (1989) *FEBS Lett.* **259**, 19–23
- 42 Wang, J.-L. and McDaniel, M. L. (1990) *Biochem. Biophys. Res. Commun.* **166**, 813–818
- 43 Zhang, A. Q., Gao, Z. Y., Gilon, P., Nenquin, M., Drews, G. and Henquin, J. C. (1991) *J. Biol. Chem.* **266**, 21649–21656
- 44 Stagner, J. I., Samols, E. and Weir, G. C. (1980) *J. Clin. Invest.* **65**, 939–942
- 45 Chou, H.-F. and Ipp, E. (1990) *Diabetes* **39**, 112–117
- 46 Bergsten, P., Grapengiesser, E., Gylfe, E., Tengholm, A. and Hellman, B. (1994) *J. Biol. Chem.* **269**, 8749–8753
- 47 Bergstrom, R. W., Fujimoto, W. Y., Teller, D. C. and de Haën, C. (1989) *Am. J. Physiol.* **257**, E479–485
- 48 Polonsky, K. S., Given, B. D., Hirsch, L. J., Tillil, H., Shapiro, E. T., Beebe, C., Frank, B. H., Galloway, J. A. and Van Cauter, E. (1988) *New Engl. J. Med.* **318**, 1231–1239