Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca^{2+} in glucose-stimulated pancreatic β-cells

Thomas NILSSON*, Vera SCHULTZ†, Per-Olof BERGGREN*, Barbara E. CORKEY† and Keith TORNHEIM†‡

*Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institute, Karolinska Hospital, S-17176 Stockholm, Sweden, and
†Diabetes and Metabolism Unit, Evans Department of Medicine, and Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, U.S.A.

Closure of ATP-sensitive K^+ (K_{ATP}) channels is part of the stimulus–secretion coupling mechanism in the pancreatic β-cell, leading to membrane depolarization and influx of Ca^{2+} through voltage-sensitive L-type Ca^{2+} channels. The elevated ATP/ADP ratio seen in the presence of high levels of glucose has been postulated to mediate the glucose-induced closure of the K_{ATP} channels and rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), or alternatively to be a consequence of activation of mitochondrial dehydrogenases by the increase in $[Ca^{2+}]_i$. To distinguish between these two possibilities, the time course of the change in the ATP/ADP ratio was determined in comparison with that of $[Ca^{2+}]_i$. We here show that a severalfold rise in the ATP/ADP ratio occurs rapidly on stimulation of suspensions of mouse pancreatic β-cells with glucose. The change in the ATP/ADP ratio is an early event that begins within 20–40 s and precedes the rise in $[Ca^{2+}]_i$. The temporal relationship indicates that the adenine nucleotide changes cannot be a consequence of the $[Ca^{2+}]_i$ changes and may indeed be the connecting link between glucose metabolism and $[Ca^{2+}]_i$ changes. When the cells were sequentially treated with high glucose concentration, clonidine and finally high extracellular Ca^{2+} concentration to induce synchronized oscillations in $[Ca^{2+}]_i$ in the cell suspension, corresponding oscillations in the ATP/ADP ratio were observed. Glucose 6-phosphate levels oscillated out of phase with the ATP/ADP ratio. These results support the hypothesis that the Ca^{2+} oscillations previously observed in glucose-stimulated single islets or β-cells may reflect oscillations in the ATP/ADP ratio that accompany oscillatory glycolysis.

INTRODUCTION

A rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), owing to influx through voltage-gated L-type Ca^{2+} channels in the plasma membrane, is a central component of the stimulus–secretion coupling mechanism leading to insulin release by the pancreatic β-cell [1]. Glucose stimulation of secretion requires metabolism of the sugar [2], but the connecting linkage between its metabolism and the rise in $[Ca^{2+}]_i$ is not established. The mechanism seems to involve closure of ATP-sensitive K^+ (K_{ATP}) channels [3–8], to cause membrane depolarization. In excised inside-out patches, these K_{ATP} channels are closed by subphysiological levels of ATP; however, counteracting effects of ADP and the requirement for closure of nearly all channels should allow regulation by ATP and ADP at their physiological concentrations [7–9]. The ATP/ADP ratio is elevated in isolated islets incubated with stimulatory glucose concentrations (reviewed extensively in [10]). However, there have been few time-course data, especially in comparison with changes in $[Ca^{2+}]_i$. An alternative proposal has been made that the increase in the ATP/ADP ratio could be a consequence, rather than a cause, of the Ca^{2+} rise, owing to activation of mitochondrial dehydrogenases by Ca^{2+} and enhanced oxidative phosphorylation [11–13].

We recently developed a highly sensitive bioluminescent assay for ADP suitable for high ATP/ADP ratios, in which endogenous ATP is first removed by reaction with ATP sulphurylase [14]. We have now applied this method to timed samples from suspensions of mouse pancreatic β-cells, in which $[Ca^{2+}]_i$ was also monitored by fura-2 fluorescence. Our results show that the glucose-induced rise in the ATP/ADP ratio precedes, and therefore cannot be a consequence of, the rise in $[Ca^{2+}]_i$; thus it may indeed be a link in the stimulus–secretion coupling mechanism. Furthermore when the suspension was subsequently treated with clonidine and high Ca^{2+} concentration to induce synchronized oscillations in $[Ca^{2+}]_i$ [15,16], oscillations in the ATP/ADP ratio and glucose 6-phosphate level were observed. This is the first demonstration of such metabolite oscillations in intact pancreatic β-cells. The data support the hypothesis that oscillations of glycolysis and the ATP/ADP ratio may underlie previously observed oscillations in $[Ca^{2+}]_i$ in single β-cells and islets, and oscillations in insulin secretion *in vivo* and *in vitro*.

EXPERIMENTAL

Pancreatic islet cells (over 90 % β-cells) were prepared from adult non-inbred obese hyperglycaemic mice (*ob/ob*) as described previously [17] and cultured overnight in RPMI 1640 culture medium supplemented with 10 % (v/v) fetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 60 μg/ml gentamycin. The cells were loaded with Ca^{2+} indicator by incubation with 2 μM fura-2/AM for 45 min in the culture medium (which contained 11 mM glucose). They were then washed in medium containing 140 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM Hepes, pH 7.4, and 1 mg/ml BSA. The cells (about 4×10^6) were suspended in 2.5 ml of fresh washing medium in a stirred cuvette placed in a Perkin-Elmer LS5 spectrofluorometer or in a SPEX Fluorolog-2 system for monitoring $[Ca^{2+}]_i$ changes by 340 or 340/380 nm excitation ratio [18] respectively at 37 °C. Intracellular Ca^{2+} levels could not be directly calibrated, and the $[Ca^{2+}]_i$ changes presented are relative changes in fluorescence. After a stable baseline was reached, glucose (20 mM) was added. The time that the cells spent in the absence of glucose, that is, the time needed for washing, suspending and establishment of baseline, was 10–15 min. Each experiment used a separate preparation of islets.

For assay of ATP and ADP, 50 μl samples were taken from the cell suspension being monitored for $[Ca^{2+}]_i$ changes, at

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; K_{ATP} channel, ATP-sensitive K^+ channel.

‡ To whom correspondence should be addressed.

various times before and after the glucose addition, and added immediately to chilled microcentrifuge tubes containing 10 μl of 10% (w/v) trichloroacetic acid and vortexed. After centrifugation, the supernatants were neutralized by ether extraction, freeze-dried (SpeedVac) and stored at -80 °C until they were redissolved in 0.5 ml water for assay. ATP and ADP were assayed by bioluminescent methods as described previously [14]. In brief, aliquots of each deproteinized sample were assayed for ATP directly with luciferase. Other aliquots were treated with ATP sulphurylase in the presence of molybdate, to hydrolyse the endogenous ATP to AMP and PP_i. After inactivation of the sulphurylase, ADP was converted to ATP with pyruvate kinase and phosphoenolpyruvate for measurement with luciferase. The removal of endogenous ATP greatly increases the precision of the ADP measurement; duplicate measurements generally agree within 10% [14]. Data are presented as the ATP/ADP ratio, which is unaffected by loss of sample volume in the ether extraction or by the numbers of cells. Glucose 6-phosphate was measured by enzyme cycling [19]; because of a significant reaction of the glucose (present at vastly higher concentrations) with glucose 6-phosphate dehydrogenase under these conditions, samples were also assayed for glucose spectrophotometrically [19], and samples and standards in the glucose 6-phosphate assay were run with the same concentration of glucose. A systematic correction of 1.8 was applied to compensate for apparent inhibition of cycling rate by the 25% volume of processed samples generally used, as determined with added glucose 6-phosphate. Glucose 6-phosphate values given are normalized to (ATP + ADP) content to remove effects of variability of numbers of cells in sample aliquots or loss of material in processing.

RESULTS

When glucose was added to suspensions of β-cells, the ATP/ADP ratio increased severalfold, as illustrated in Figure 1 (left panels). The rise was detectable between 20 and 40 s after glucose addition and usually reached a maximum by 60–90 s (68 ± 11 s; mean $\pm$ S.E.M., $n = 7$). Measurements of $[Ca^{2+}]_i$ in the same suspensions showed a rise that occurred later (Figure 1, right panels), beginning at 68 ± 5 s and reaching near-maximum at 128 ± 10 s. The beginning of the rise in the ATP/ADP ratio preceded the rise in $[Ca^{2+}]_i$ in every case ($P < 0.0001$); in most (five out of seven) experiments the ATP/ADP ratio reached maximum by the time the $[Ca^{2+}]_i$ rise began.

In some experiments the ATP/ADP ratio rose monotonically to a fairly constant plateau after glucose stimulation (e.g. Figure 1C). In other experiments the ATP/ADP ratio appeared to oscillate (e.g. Figures 1B and 1D). However, under these conditions oscillations in $[Ca^{2+}]_i$ were not visible.

Subsequent treatment of the β-cell suspension with clonidine, followed by addition of high Ca^{2+} concentration to the buffer, led to damped oscillations in $[Ca^{2+}]_i$ with a period of several minutes (Figure 2), as reported previously [15,16]. Measurement of the ATP/ADP ratio showed oscillations of similar period, with rises in the ATP/ADP ratio roughly correlating with rises in $[Ca^{2+}]_i$. The rise in the ATP/ADP ratio seems to begin slightly before the rise in $[Ca^{2+}]_i$, but without the time difference of 30–60 s seen on the initial addition of glucose (in this experiment, too; results not shown but included in the means above). As discussed below, this could be due to activation of protein kinase C. The glucose 6-phosphate level also appeared to oscillate, but roughly in opposite phase to the ATP/ADP ratio, as would be expected from previous studies of glycolytic oscillations in muscle extracts. Although they could still be seen because of the continuous nature of the Ca^{2+} trace, the Ca^{2+} oscillations in Figure 2 rapidly lost amplitude, by a factor of 3 or 4, presumably owing to loss of synchrony in the cell suspension. Because of the smaller number of points and greater error in the metabolite assays, oscillations in these parameters were no longer clearly visible after the first one or two oscillations.

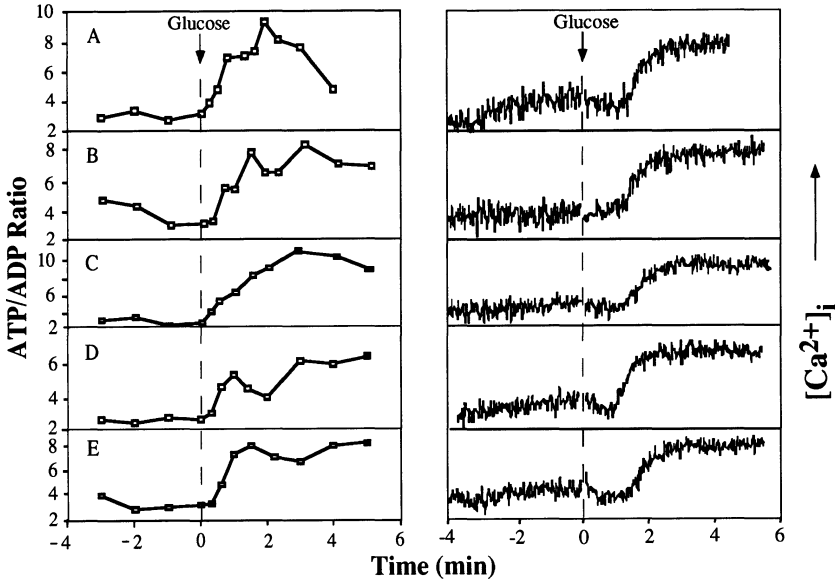

Figure 1 Glucose-stimulated rises in the ATP/ADP ratio and $[Ca^{2+}]_i$ in suspensions of mouse β-cells

Results from five separate experiments are shown. Glucose (20 mM) was added at zero time. $[Ca^{2+}]_i$ was monitored continuously with fura-2 (right panels). Samples were taken at the times indicated by the experimental points and assayed for ATP and ADP (left panels).

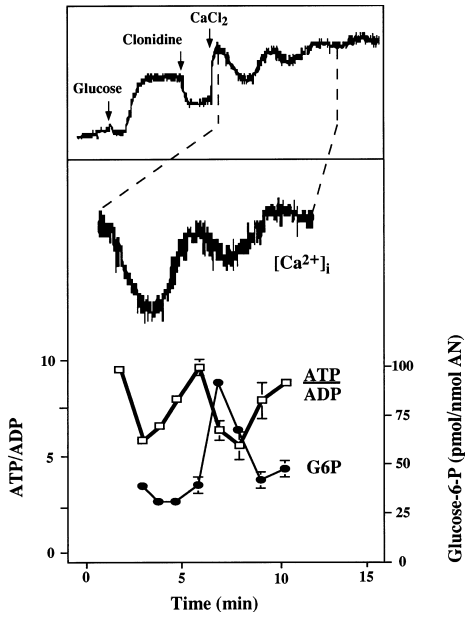

Figure 2 Oscillations in ATP/ADP ratio, glucose 6-phosphate and $[Ca^{2+}]_i$ induced by clonidine/high Ca^{2+} treatment

Glucose (20 mM), clonidine (10 nM) and an additional 5 mM $CaCl_2$ were added, thereby inducing Ca^{2+} oscillations as shown in the upper panel. The first oscillations are shown in expanded scale in the lower panel. Samples were taken at the times indicated by the experimental points and assayed for ATP, ADP and glucose 6-phosphate (G6P). Glucose 6-phosphate values are normalized to adenine nucleotide content (AN = ATP + ADP). Data for the ATP/ADP ratio are means and ranges of duplicate assays. Data for glucose 6-phosphate are means ± S.E.M. for three to six replicate measurements.

DISCUSSION

The time courses presented here, showing a substantial rise in the ATP/ADP ratio of pancreatic β-cells beginning shortly after glucose stimulation and preceding the rise in $[Ca^{2+}]_i$, clearly establish that the change in the ATP/ADP ratio is not a consequence of Ca^{2+} activation of mitochondrial dehydrogenase enzymes, as proposed previously [11–13]. This is in agreement with the observation by M. Ohta and M. Erecinska (unpublished work, cited in [10]) that the glucose-stimulated rise in the ATP/ADP ratio is not prevented by Ca^{2+} channel blockers that prevent the rise in $[Ca^{2+}]_i$. The time-course data are consistent with a role of ATP and ADP as the connecting linkage between increased glucose metabolism and the ionic events that lead to release of insulin. According to that model, enhanced glycolysis raises ATP and lowers ADP, thus closing K_{ATP} channels. This in turn causes membrane depolarization, opening of voltage-sensitive L-type Ca^{2+} channels and influx of Ca^{2+}. This sequence of events is in agreement with observations of an increase in endogenous pyridine nucleotide fluorescence (a metabolic parameter) preceding membrane depolarization and the increase in $[Ca^{2+}]_i$ [20–22]. These data are also consistant with observations of Duchen et al. [22] that closure of K_{ATP} channels occurs earlier than the $[Ca^{2+}]_i$ rise. The actual parameter sensed by the K_{ATP} channel under physiological conditions might be the ADP concentration (in the presence of inhibitory ATP), rather than the ATP/ADP ratio *per se* [9].

The basal and glucose-stimulated values of the ATP/ADP ratio seen here in suspensions of β-cells from *ob/ob* mice are similar to values reported for rat islets (reviewed in [10]). It should, however, be noted that the actual change in the *cytoplasmic* ATP/ADP ratio may be greater, because the analysed levels include significant amounts of ATP and ADP in secretory granules and other compartments [10].

The large increase in the ATP/ADP ratio on glucose stimulation appears to be an unusual characteristic of the pancreatic β-cell that is not seen in most other respiring cells that can use other fuels. (In the absence of glucose, islets use endogenous fuels, principally fatty acids, and can maintain a constant rate of oxygen consumption over 2 h [2]. In the presence of respiratory inhibitors, energy production in all cells is totally dependent on glycolysis and the ATP/ADP ratio generally decreases in the absence of glucose.) Liver is the other tissue that has high levels of glucokinase and thus responds metabolically to changes in glucose concentration; however, glucose causes little if any change in the ATP/ADP ratio in liver *in vivo* [23], the perfused liver [24] or isolated hepatocytes [25], despite large increases in glycolytic intermediates indicative of accelerated glycolysis. Similarly, little increase and in some cases a decrease in the ATP/ADP ratio with elevated glucose has been seen in resting skeletal muscle [26], subcutaneous tumours [27], ascites tumour cells [28–30] and yeast [31]. The severalfold change in the ATP/ADP ratio in glucose-stimulated β-cells may reflect the particular signalling role of the adenine nucleotides in the stimulus–secretion coupling via the K_{ATP} channel, rather than their ubiquitous role in energy coupling for biosynthetic, transport or contractile processes.

Oscillations in $[Ca^{2+}]_i$ have been observed previously in single islets and single β-cells on stimulation with glucose [13,21,32–36], as well as in a variety of cells stimulated with hormones or neurotransmitters [37,38]. The problem in observing such oscillations in a suspension of cells may be largely a matter of inducing synchrony in the population. The effectiveness of the clonidine/high Ca^{2+} treatment used here may be due to the repolarization caused by the α_2-adrenergic agonist; similar effects can also be obtained with galanin or somatostatin [15]. Whether the treatment with high Ca^{2+} concentration itself has a role in inducing synchrony, or merely increases the magnitude of the Ca^{2+} fluxes and thus the amplitude and visibility of the oscillations, remains to be determined. Clonidine addition has also been shown to induce regular oscillations in single mouse islets [36].

A number of practical rationales have been given for oscillations in $[Ca^{2+}]_i$, including signal-to-noise advantages of pulsed signals and avoidance of deleterious effects of high steady-state $[Ca^{2+}]_i$ [37,38]. In the pancreatic β-cell the oscillations in $[Ca^{2+}]_i$ may be of particular physiological importance. Insulin secretion itself is pulsatile, in man and animals *in vivo*, from the perfused pancreas and even from groups of perifused islets [33,39–42]. Recent studies have shown synchrony of $[Ca^{2+}]_i$ and insulin secretion oscillations in single islets [35,36]. The oscillatory nature of insulin secretion is perturbed in patients with Type II diabetes and their near relatives [43,44]. It has been demonstrated that insulin infused in an oscillatory manner is more effective than a steady infusion in regulating the blood glucose concentration, perhaps by avoiding down-regulation of insulin receptors [39,40].

We have proposed that oscillatory glycolysis and associated oscillations in the ATP/ADP ratio may be responsible for the observed oscillations in $[Ca^{2+}]_i$ and insulin secretion in glucose-stimulated pancreatic islets [33,45]. This proposal is based on our studies of spontaneous oscillations of glycolysis and the ATP/ADP ratio in skeletal muscle extracts [46–50] and the ability of such metabolic oscillations to cause oscillations in Ca^{2+} handling [45] and O_2 consumption in permeabilized β-cells (V. N. Civelek, J. T. Deeney, G. E. Fusonie, B. E. Corkey and K. Tornheim, unpublished work). Glycolytic oscillations have also been shown

to occur in other extracts (heart, yeast) and cell suspensions (yeast, ascites cells) [51–57]. The observations of oscillations in the ATP/ADP ratio and glucose 6-phosphate level shown here are the first direct evidence of oscillations in adenine nucleotides and glycolytic intermediates in intact glucose-stimulated β-cells. They are consistent with the oscillations in O_2 consumption previously shown by us in perifused islets [33], with oscillations in lactate release [58], and with oscillations in NAD(P)H fluorescence seen in single β-cells [13,21]. Interestingly, in the oscillations in Figure 2, the rise in the ATP/ADP ratio seems to begin only slightly before the rise in $[Ca^{2+}]_i$; there is clearly not the degree of temporal separation seen on the initial addition of glucose. The decreased lag may be due to the prior and continuing glucose stimulation, perhaps because of activation of protein kinase C that occurs on glucose stimulation [59]. It has previously been shown that down-regulation of protein kinase C substantially delays the glucose-induced rise in $[Ca^{2+}]_i$ by an effect on the Ca^{2+} channel, whereas acute stimulation of protein kinase C seemed to shorten the lag [60].

We thank Ms. Maggie Phan for her assistance in preparing the figures. This work was supported by United States Public Health Service Grants DK-31559, DK-35914 and DK-46200 and grants from the Swedish Medical Research Council (03X-09890, 19X-00034), the Juvenile Diabetes Foundation International and the Bank of Sweden Tercentenary Foundation.

REFERENCES

1 Prentki, M. and Matschinsky, F. M. (1987) Physiol. Rev. **67**, 1185–1248
2 Meglasson, M. D. and Matschinsky, F. M. (1986) Diabetes Metab. Rev. **2**, 163–214
3 Cook, D. L. and Hales, C. N. (1984) Nature (London) **311**, 271–273
4 Ashcroft, F. M., Harrison, D. E. and Ashcroft, S. J. H. (1984) Nature (London) **312**, 446–448
5 Misler, S., Falke, L. C., Gillis, K. and McDaniel, M. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7119–7123
6 Ashcroft, F. M. (1988) Annu. Rev. Neurosci. **11**, 97–118
7 Dunne, M. J. and Peterson, O. H. (1991) Biochim. Biophys. Acta **1071**, 67–82
8 Ashcroft, F. and Rorsman, P. (1990) Biochem. Soc. Trans. **18**, 109–111
9 Hopkins, W. F., Fatherazi, S., Peter-Riesch, B., Corkey, B. E. and Cook, D. (1992) J. Membr. Biol. **129**, 287–296
10 Erecinska, M., Bryla, J., Michalik, M., Meglasson, M. D. and Nelson, D. (1992) Biochim. Biophys. Acta **1101**, 273–295
11 Denton, R. M. and McCormack, J. G. (1985) Am. J. Physiol. **249**, E543–E554
12 McCormack, J. G., Longo, E. and Corkey, B. E. (1990) Biochem. J. **267**, 527–530
13 Pralong, W.-F., Spät, A. and Wollheim, C. B. (1994) J. Biol. Chem. **269**, 27310–27314
14 Schultz, V., Sussman, I., Bokvist, K. and Tornheim, K. (1993) Anal. Biochem. **215**, 302–304
15 Nilsson, T., Arkhammar, P., Rorsman, P. and Berggren, P.-O. (1989) J. Biol. Chem. **264**, 973–980
16 Barker, C. J., Nilsson, T., Kirk, C. J., Michell, R. H. and Berggren, P.-O. (1994) Biochem. J. **297**, 265–268
17 Juntti-Berggren, L., Arkhammar, P., Nilsson, T., Rorsman, P. and Berggren, P.-O. (1991) J. Biol. Chem. **266**, 23537–23541
18 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
19 Lowry, O. H. and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York
20 Gilon, P. and Henquin, J.-C. (1992) J. Biol. Chem. **267**, 20713–20720
21 Pralong, W. F., Bartley, C. and Wollheim, C. B. (1990) EMBO J. **9**, 53–60
22 Duchen, M. R., Smith, P. A. and Ashcroft, F. M. (1993) Biochem. J. **294**, 35–42
23 Van Schaftingen, E., Hue, L. and Hers, H.-G. (1980) Biochem. J. **192**, 263–271
24 Brunengraber, H., Boutry, M. and Lowenstein, J. M. (1973) J. Biol. Chem. **248**, 2656–2669
25 Hue, L. (1982) Biochem. J. **206**, 359–365
26 Thomsen, C., Jensen, K. E., Astrup, A., Bulow, J. and Henriksen, O. (1989) Acta Physiol. Scand. **137**, 335–339
27 Schaefer, C., Mayer, W. K., Kruger, W. and Vaupel, P. (1993) J. Cancer Res. Clin. Oncol. **119**, 599–608
28 Maitra, P. K. and Chance, B. (1965) in Control of Energy Metabolism (Chance, B., Estabrook, R. W. and Williamson, J. R., eds.), pp. 157–175, Academic Press, New York
29 Wu, R. (1965) in Control of Energy Metabolism (Chance, B., Estabrook, R. W. and Williamson, J. R., eds.), pp. 187–192, Academic Press, New York
30 Nelson, B. D., Kabir, F. and Muchiri, P. (1984) Biochem. J. **219**, 159–164
31 Theobald, U., Mailinger, W., Reuss, M. and Rizzi, M. (1993) Anal. Biochem. **214**, 31–37
32 Valdeolmillos, M., Santos, R. M., Contreras, D., Soria, B. and Rosario, L. M. (1989) FEBS Lett. **259**, 19–23
33 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
34 Grapengiesser, E., Gylfe, E. and Hellman, B. (1988) Biochem. Biophys. Res. Commun. **151**, 1299–1304
35 Gilon, P., Shepherd, R. M. and Henquin, J.-C. (1993) J. Biol. Chem. **268**, 22265–22268
36 Bergsten, P., Grapengiesser, E., Gylfe, E., Tengholm, A. and Hellman, B. (1994) J. Biol. Chem. **269**, 8749–8753
37 Berridge, M. J. and Galione, A. (1988) FASEB J. **2**, 3074–3082
38 Tsien, R. W. and Tsien, R. Y. (1990) Annu. Rev. Cell Biol. **6**, 715–760
39 Weigle, D. S. (1987) Diabetes **36**, 764–775
40 Lefèbvre, P. J., Paolisso, G., Scheen, A. J. and Henquin, J. C. (1987) Diabetologia **30**, 443–452
41 Chou, H.-F. and Ipp, E. (1990) Diabetes **39**, 112–117
42 Bergstrom, R. W., Fujimoto, W. Y., Teller, D. C. and de Haen, C. (1989) Am. J. Physiol. **257**, E479–E485
43 Matthews, D. R., Lang, D. A., Burnett, M. A. and Turner, R. C. (1983) Diabetologia **24**, 231–237
44 O'Rahilly, S., Turner, R. C. and Matthews, D. R. (1988) New Engl. J. Med. **318**, 1225–1230
45 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
46 Tornheim, K. and Lowenstein, J. M. (1974) J. Biol. Chem. **249**, 3241–3247
47 Tornheim, K. and Lowenstein, J. M. (1975) J. Biol. Chem. **250**, 6304–6314
48 Tornheim, K. (1988) J. Biol. Chem. **263**, 2619–2624
49 Andrés, V., Schultz, V. and Tornheim, K. (1990) J. Biol. Chem. **265**, 21441–21447
50 Tornheim, K., Andrés, V. and Schultz, V. (1991) J. Biol. Chem. **266**, 15575–15578
51 Frenkel, R. (1968) Arch. Biochem. Biophys. **125**, 157–165
52 Chance, B., Schoener, B. and Elsaesser, S. (1965) J. Biol. Chem. **240**, 3170–3181
53 Betz, A. and Chance, B. (1965) Arch. Biochem. Biophys. **109**, 585–594
54 Hess, B., Boiteux, A. and Kruger, J. (1969) Adv. Enz. Regul. **7**, 149–167
55 Hess, B. and Boiteux, A. (1971) Annu. Rev. Biochem. **40**, 237–258
56 Ibsen, K. H. and Schiller, K. W. (1967) Biochim. Biophys. Acta **131**, 407–411
57 Das, J. and Busse, H. G. (1991) Biophys. J. **60**, 369–379
58 Chou, H.-F., Berman, N. and Ipp, E. (1992) Am. J. Physiol. **262**, E800–E805
59 Calle, R. C., Ganesan, S., Smallwood, J. I. and Rasmussen, H. (1992) J. Biol. Chem. **267**, 18723–18727
60 Arkhammar, P., Juntti-Berggren, L., Larsson, O., Welsh, M., Nanberg, E., Sjoholm, A., Kohler, M. and Berggren, P.-O. (1994) J. Biol. Chem. **269**, 2743–2749

Received 24 July 1995/4 October 1995; accepted 10 October 1995