Biochemical and Biophysical Research Communications **275,** 103–107 (2000)
doi:10.1006/bbrc.2000.3228, available online at http://www.idealibrary.com on IDEAL®

Glucose-6-Phosphatase in the Insulin Secreting Cell Line INS-1

Rosella Fulceri, Tamás Kardon, Gábor Bánhegyi, William-F. Pralong, Alessandra Gamberucci, Paola Marcolongo, and Angelo Benedetti[1]
Department of Pathophysiology and Experimental Medicine, University of Siena, 53100 Siena, Italy

Received June 30, 2000

The glucose-6-phosphatase system of the glucose sensitive insulin secreting rat insulinoma cells (INS-1) was investigated. INS-1 cells contain easily detectable levels of glucose-6-phosphatase enzyme protein (assessed by Western blotting) and have a very significant enzymatic activity. The features of the enzyme (Km and Vmax values, sensitivity to acidic pH, partial latency, and double immunoreactive band) are similar to those of the hepatic form. On the other hand, hardly detectable levels of glucose-6-phosphatase activity and protein were present in the parent glucose insensitive RINm5F cell line. The mRNA of the glucose-6-phosphate transporter was also more abundant in the INS-1 cells. The results support the view that the glucose-6-phosphatase system of the β-cell is associated with the regulation of insulin secretion. © 2000 Academic Press

Glucose-6-phosphatase (G6Pase; EC 3.1.3.9.) catalyses the terminal reaction of gluconeogenesis and glycogenolysis, i.e. the conversion of glucose-6-phosphate to glucose [1, 2]. Classic liver G6Pase is intimately associated with the endoplasmic reticulum (ER) and appears to be a multicomponent system [3] which comprises: i) the enzyme protein whose active site is located within the ER lumen [4–7], ii) a transport protein (G6PT) which mediates the entry of the substrate glucose-6-phosphate (G6P) into the ER lumen [6–7] and iii) at least two transport systems which allow the exit to the cytosol of the hydrolysis products Pi and glucose [8]. The enzyme protein and its gene have been identified in rats and humans [9]. A cDNA encoding a putative ER G6P transporter (G6PT) has been cloned [10]. The human G6PT gene spans a genomic region of approximately 4kb, consists of nine exons and maps to human chromosome 11q23.3 [11]. G6Pase has also been unequivocally demonstrated to be present in kidney [2, 9, 12]. Lower levels of the enzyme protein have also been found in other organs, e.g. in intestinal and gall bladder mucosa, in the human fetal adrenal gland and in some (but not all) brain astrocytes [see 13 for refs].

The extent of a glucose futile cycling due to the simultaneous action of glucokinase and G6Pase affects the flow through glycolysis and consequently alters the intracellular ATP levels. In diabetic animals, the increased activity of this cycle is supposed to impair the secretion of insulin [14]. Moreover, the intraluminal hydrolysis of glucose-6-phosphate is known to promote calcium storage in the hepatic ER [5]. A recent report indicates that the transport of glucose-6-phosphate into the lumen of the ER results in enhanced calcium sequestration in various cell types, even in the absence of intraluminal G6Pase activity [15]. Both the alteration of the ATP level and calcium sequestration in the ER may regulate the glucose stimulated insulin secretion and these events can be influenced by the components of the G6Pase system. Therefore, it is an important question whether the existence of G6Pase is significant in normal and diabetic β-cells. Relatively low [16–19], negligible [20] or high [21] G6Pase activity has been detected in normal mammalian islets. G6Pase activity has been also shown to be several fold higher in islets from diabetic [22, 23] and from obese hyperglycemic [14, 24] than from normal animals and an increased expression of the G6Pase enzyme mRNA was found during the development of diabetes in Zucker rats [25].

The glucose sensitive insulin secreting INS-1 rat insulinoma β-cell line [26] is widely used as a satisfactory model system for studying the regulation of insulin secretion. However, its G6Pase system has not been described unequivocally; even the absence of G6Pase protein and activity has been reported in these cells [27], which would mean that the glucose futile cycling

Abbreviations used: ER, endoplasmic reticulum; G6Pase, glucose-6-phosphatase; G6PT, glucose-6-phosphate transporter.

[1] To whom correspondence should be addressed at Dipartimento di Fisiopatologia e Medicina Sperimentale, Viale A. Moro n°1, 53100-Siena, Italy. E-mail: benedetti@unisi.it.

0006-291X/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

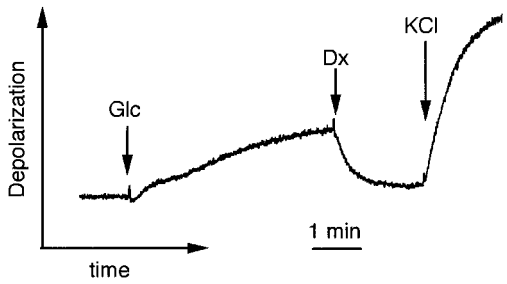

FIG. 1. Changes in plasma membrane potential induced by glucose in the INS-1 cells. Plasma membrane potential was monitored with the fluorescent probe bis-oxonol as detailed under Materials and Methods. Additions as indicated were: 14 mM glucose (Glc), 300 μM diazoxide (Dz), and 30 mM KCl.

does not play an indispensable role in the regulation of insulin secretion. In the present study, we characterize the G6Pase system in the glucose-sensitive insulin secreting cell line INS-1. We provide evidence that INS-1 cells possess components (phosphohydrolase and glucose-6-phosphate transporter) of the G6Pase system similarly to the liver. Moreover, the G6Pase system is virtually absent in the glucose-insensitive parent line RINm5F, which indicates that its expression can follow the cell differentiation transition from the glucose-insensitive to the glucose-sensitive status.

MATERIALS AND METHODS

Materials. RPMI 1640 was purchased from Gibco and all of the other supplements of the culture media were from Sigma Chemical (St. Louis, MO). Glucose-6-phosphate (dipotassium salt), digitonin, and alamethicin were obtained from Sigma Chemical (St. Louis, MO). Bis-oxonol was from Molecular Probes (Eugene, OR). Monospecific polyclonal antiserum (IgG fraction) to the G6Pase enzyme was kindly supplied by Dr. Ann Burchell, Dundee, Scotland. D-[^{14}C(U)]glucose-6-phosphate (300 mCi/mMol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). All other chemicals were of analytical grade.

Cells. INS-1 cells, between passages n° 35–80, were grown in monolayer cultures [26] in RPMI 1640 medium containing 11 mM glucose supplemented with 10% heat-inactivated fetal calf serum (HFCS), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM mercaptoethanol, 100 UI/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (5% CO_2, 95% air). RINm5F cells were cultured as above except that mercaptoethanol and sodium pyruvate were omitted.

Because of the possible loss of the glucose responsiveness in INS-1 cells during culturing, the effect of stimulatory concentrations of glucose on plasma membrane potential [26] was routinely verified in the INS-1 cells employed in the present study. Plasma membrane potential was measured with the fluorescent probe bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (bis-oxonol) [26, 28]. INS-1 cells resuspended in a medium containing 2 mM glucose presented with an evident decrease in plasma membrane potential upon addition of 14 mM glucose, and the depolarization was reverted by the hyperpolarizing drug diaxozide (Fig. 1). These responses were consistent with the original report on the INS-1 cell line [26].

Assay of glucose-6-phosphatase activity. G6Pase activity was measured in cells whose plasma membrane was permeabilized with digitonin (1 μmol/10^6 cells, 5 min., at 37°C) on the basis of D-[^{14}C(U)]glucose production from D-[^{14}C(U)]glucose-6-phosphate according to [7]. At substrate concentrations higher than 2 mM, the enzyme activity was also evaluated by measuring glucose production with a Glucose (Trinder) kit (Sigma, St. Louis, MO). To permeabilize ER membranes, alamethicin (25 μg/mg cell protein) was added to the G6Pase assay medium, as described previously [29]. Where indicated, G6Pase was inactivated by preincubation of (digitonin-permeabilized) cells in 20 mM sodium acetate buffer, pH 5, for 10 min., at 37°C [30]. The G6Pase assay system consisted of: 0.6–1 mg of cell protein per ml in 100 mM KCl, 20 mM NaCl, 3.5 mM $MgCl_2$, 20 mM Mops (pH 7.2), and G-6-Pi (0.2, 0.5, 1, 2, 5, or 20 mM); 30 min of incubation, at 37°C.

Western blot analysis of the G6Pase enzyme. Microsomal membranes were prepared as reported [7]. Proteins were separated by electrophoresis on SDS polyacrylamide gels (15%), and electrophoretically transferred to nitro-cellulose [13]. Western blots were immunoreacted with a sheep IgG previously shown to be monospecific for the G6Pase enzyme [31]. The immunoreactive band was revealed by a biotin-streptavidin horseradish peroxidase linked detection system with 4-chloro-1-napththol as the substrate.

Northern blot analysis of the G6PT. Total RNA was extracted from INS-1 and RINm5F cells by the acid guanidinium thiocyanate method [32]. Total RNA (30 μg/sample) was separated on a 1.3% formaldehyde-agarose gel and transferred to Hybond-N Nylon membrane (Amersham Pharmacia Biotech) as detailed earlier. The membrane was hybridized with α-^{32}P dCTP labelled probe (human liver cDNA mutated in glycogen storage disease type 1b [10, 11]) as detailed elsewhere [33].

RESULTS

Previous work [21] indicates that G6Pase in β-cells is very unstable displaying a time-dependent loss in enzyme activity during subcellular fractionation. To avoid this difficulty, we measured G6Pase activity directly in the ER *in situ*, i.e. in cells whose plasma membrane has been permeabilized by digitonin [34, 35]. Classic hepatic microsomal G6Pase activity is (partially) latent; it increases after disruption of the ER membrane barrier, because the transport of glucose-6-phosphate into vesicles is the rate-limiting step for the intralumenal phosphohydrolase activity [7]. Therefore, enzyme latency was assessed by the subsequent permeabilization of ER (endo)membranes with the pore-forming peptide alamethicin [36], as previously performed in isolated hepatocytes [37]. The activity of the hepatic microsomal G6Pase is known to be inactivated by acidic pH pretreatment, while that of nonspecific phosphatases is not [2, 13, 30]. The enzyme activity was therefore assayed in (permeabilized) INS-1 cells preincubated at acidic pH.

The concentration dependence of the hydrolytic activity of glucose-6-phosphatase in digitonin-permeabilized INS-1 cells is shown in Fig. 2a. A portion of the total hydrolytic activity activity was still present following the preincubation of cells at acidic pH (Fig. 2a, dotted lines). By subtracting the acidic pH insensitive portion from the total hydrolytic activity, the acidic pH-sensitive portion of glucose-6-phosphate hydrolysis was obtained, referred to here as G6Pase activity (Fig. 2b). G6Pase activity was partially latent as it increased

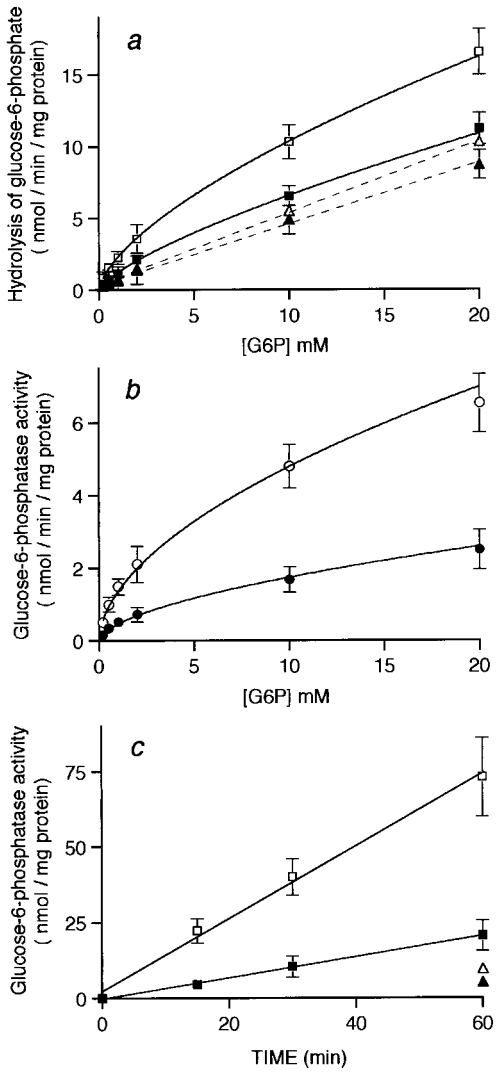

FIG. 2. G6Pase activity in digitonin-permeabilized INS-1 cells. In panel (a) the hydrolysis of G-6-Pi was measured in digitonin-permeabilized INS-1 cells before (continuous lines) and after (dotted lines) preincubation at acidic pH as detailed under Materials and Methods. In panel (b) the acidic pH-sensitive glucose-6-phosphatase activity is calculated from data of panel (a). Empty symbols indicate cells whose ER membranes were further permeabilized with alamethicin. In panel (c) the time course of acidic pH-sensitive G6Pase activity in the presence of 1 mM glucose-6-phosphate is shown in digitonin (dark squares) and digitonin plus alamethicin (empty squares) -treated INS-1 cells. In panel (c) the acidic pH-sensitive glucose-6-phosphatase activity at 60 min. of incubation in digitonin (dark triangles) and digitonin plus alamethicin (empty triangles) -treated RINm5F cells is also shown for comparison. Data are means ± SD of 3 to 5 experiments.

after ER membrane permeabilization at all the concentrations of the substrate assayed. The time course of the enzyme activity in the presence of 1 mM substrate is shown in Fig. 2c; G6Pase activity was constant up to 1h incubation. These features of the catalytic activity are largely similar to those seen with the classic hepatic microsomal G6Pase [1, 2, 38].

In digitonin-permeabilized RINm5F cells, an acidic pH-sensitive G6Pase activity was barely detectable (see Fig. 2c, triangles). A hydrolytic activity towards glucose-6-phosphate resistant to acidic-pH treatment, and without latency (almost the same in the presence and the absence of alamethicin) was however detectable in RINm5F cells (data not shown).

Apparent Km values of G6Pase activity (estimated by dual reciprocal plotting of data in Fig. 2b) were 1,92 and 1,63 mM in INS-1 cells with intact and permeabilized ER membranes, respectively. Km values in the millimolar range have been repeatedly reported for the hepatic microsomal enzyme [1, 2, 38]. The Vmax of the INS-1 cell G6Pase was 1,65 and 4,33 nmol per min per mg of cell protein, in cells with intact and alamethicin-permeabilized ER membranes, respectively.

The G6Pase enzyme protein was present in INS-1 cells, as revealed by Western blot analysis (Fig. 2). The immunoreactivity seen with INS-1 microsomes appeared, however, to be lower than that observed in rat liver microsomes. A comparable intensity of immunoreactivity was present in the band(s) representing the G6Pase enzyme protein with 5 μg of liver and 30 μg of INS-1 microsomal protein (Fig. 3, cf. lane 1 and 2). The immunoreactive G6Pase enzyme of INS-1 microsomes displayed a double band similar to the classic liver enzyme ([30, 31]; see also Fig. 3, lane 1). In RINm5F microsomes, an immunoreactive band could also be observed, but its intensity was much lower than that of INS-1. A comparable immunoreactivity was present with 5 and 30 μg of protein in INS-1 and RINm5F microsomes, respectively (Fig. 3; compare lane 3 with 4). With RINm5F microsomes, the Western blot analysis did not reveal a double band even at higher protein concentrations (up to 60 μg; data not shown).

Northern blot analysis was used to evaluate G6PT mRNA levels in INS-1 and RINm5F cells. The mRNA was hardly detectable in the latter cell type, while in INS-1 cells it could be demonstrated (Fig. 4).

DISCUSSION

The data presented in this study show that the components of the glucose-6-phosphatase system (the phosphohydrolase activity and the glucose-6-phos-

FIG. 3. Western blot analysis of the G6Pase enzyme protein in microsomes from rat liver, INS-1 and RINm5F cells. Microsomal membranes from INS-1 and RINm5F cells, and rat liver were subjected to SDS/PAGE and immunoblotted with an anti-glucose-6-phosphatase antibody as detailed under Materials and Methods. Lane 1, rat liver (5 μg of protein); lanes 2 and 3, INS-1 cells (30 and 5 μg of protein, respectively); lane 5, RINm5F cells (30 μg of protein).

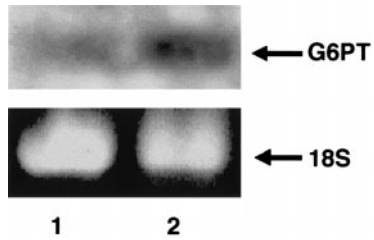

FIG. 4. Northern blot analysis of G6PT mRNA in the RINm5F and INS-1 cell lines. Total RNA (30 μg/sample) from INS-1 and RINm5F cells was separated by formaldehyde-containing agarose gel electrophoresis and blotted to a GeneScreen Plus Nylon membrane as described under Materials and Methods. The membrane was hybridized with α-^{32}P dCTP labelled probe (human liver cDNA mutated in glycogen storage disease type 1b [10,11]) and autoradiography was used to visualize G6PT mRNA. Ethidium bromide staining indicated the equal amount of loaded RNA. Lane 1, RNA from RINm5F; lane 2, from INS-1 cells.

phate transporter G6PT) are expressed in the glucose-sensitive insulin secreting cell line INS-1 and they are almost completely absent in the glucose-insensitive line RINm5F. Activity measurements and Western blot data (Figs. 2 and 3) indicate that INS-1, but not RINm5F, cells possess clearly detectable levels of the G6Pase enzyme. Several properties of the G6Pase enzyme in INS-1 cells are very similar to those of the well-characterized classic hepatic enzyme. The enzyme activity of INS-1 cells is partially latent, can be inhibited by acidic pH preincubation and has a Km in the mM range. The enzyme protein has the same apparent molecular weight and, similarly to the hepatic form, exhibits a double immunoreactive band on the Western blot. Assuming ER proteins to be at least 5% of cell proteins [39], Vmax values of 33 and 87 nmol per min per mg of ER protein could be calculated (intact and permeabilized ER membrane, respectively). These values are comparable with the G6Pase activity measured in rat liver microsomes under identical incubation conditions (i.e., in a cytosol-like medium at pH 7.2): Vmax of 153 and 233 nmol/min/mg protein in intact and disrupted microsomes, respectively [38]. The lower enzyme activity in INS-1 cells is consistent with the relative representation of the protein in INS-1 cells and in rat liver. Moreover, Northern blot analysis of the two insulinoma lines revealed a several fold higher expression of the mRNA for G6PT in INS-1 cells (Fig. 4).

Our results show that the return of the insulin storing and secreting ability of the cells is accompanied by the appearance of the lost glucose cycling capacity. The parent RINm5F cell line exhibits very low constitutive insulin secretion [26] together with practically absent glucokinase [40] and G6Pase activities and moderate G6PT expression. In contrast, the glucose sensitive insulin secreting INS-1 cells are equipped with remarkable glucokinase [41, 42] and G6Pase activities, and G6PT expression. Although glucokinase can be regarded as the principal glucose sensor of the β-cell, glucose cycling might also affect the glucose-dependent secretion of insulin. Experimental evidence shows that changed glucose cycling due to altered glucokinase or G6Pase activities results in the impairment of insulin secretion [41–43]. The representation of G6Pase and G6PT proteins can also affect calcium sequestration in the ER [5]; in agreement with this assumption higher calcium accumulation was observed upon glucose-6-phosphatase addition in the ER of permeabilized INS-1 cells compared to RIN5mF cells (Fulceri, Banhegyi, Pralong and Benedetti, unpublished results).

In summary, both ER proteins, G6PT and G6Pase, necessary for effective glucose cycling and calcium sequestration can be demonstrated in INS-1 cells. The expression of these proteins coincides with the existence of the glucose-sensitive insulin secretion indicating a possible connection between the two phenomena.

ACKNOWLEDGMENTS

This work was supported in part by an Italian Telethon grant (n° E.638) to A. Benedetti, a grant from EC (Biotechnology Project PL97 0286), Siena University grants (MURST 60%) to R. Fulceri, and by the Hungarian-Italian Intergovernmental S&T Co-operation Program. A. Benedetti and G. Bánhegyi were recipients of a NATO Linkage Grant.

REFERENCES

1. Nordlie, R. C., and Sukalski, K. A. (1985) *in* The Enzymes of Biological Membranes (Martonosi, A. N., Ed.), pp. 349–398, Plenum Press, New York.
2. Chen, Y. T., and Burchell, A. (1995) *in* The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.), pp. 935–965, McGraw-Hill.
3. Arion, W. J., Lange, A. J., Walls, E. H., and Ballas, L. M. (1980) *J. Biol. Chem.* **255,** 10396–10406.
4. Leskes, A., Siekevitz, P., and Palade, G. E. (1971) *J. Cell Biol.* **49,** 264–287.
5. Wolf, B. A., Colca, J. R., Comens, P. G., Turk, J., and McDaniel, M. L. (1986) *J. Biol. Chem.* **261,** 16284–16287.
6. Fulceri, R., Bellomo, G., Gamberucci, A., Scott, H. M., Burchell, A., and Benedetti, A. (1992) *Biochem. J.* **286,** 813–817.
7. Bánhegyi, G., Marcolongo, P., Fulceri, R., Hinds, C., Burchell, A., and Benedetti, A. (1997) *J. Biol. Chem.* **272,** 13584–13590.
8. Bánhegyi, G., Marcolongo, P., Burchell, A., and Benedetti, A. (1998) *Arch. Biochem. Biophys.* **359,** 133–138.
9. Chou, Y. J., and Mansfield, B. C. (1999) *Trends Endocrinol. Metab.* **10,** 104–113.
10. Gerin, I., Veiga-da-Cunha, M., Achouri, Y., Collet, J. F., and Van Shaftingen, E. (1997) *FEBS Lett.* **419,** 235–238.
11. Marcolongo, P., Barone, V., Priori, G., Pirola, B., Giglio, S., Biasucci, G., Zammarchi, E., Parenti, G., Burchell, A., Benedetti, A., and Sorrentino, V. (1998) *FEBS Lett.* **436,** 247–250.
12. Colilla, W., Jorgenson, R. A., and Nordlie, R. C. (1975) *Biochim. Biophys. Acta* **377,** 117–125.
13. Gamberucci, A., Marcolongo, P., Fulceri, R., Giunti, R., Watkins, S. L., Waddell, I.-D., Burchell, A., and Benedetti, A. (1996) *Molec. Membr. Biology* **13,** 103–108.

14. Khan, A., Chandramouli, V., Östenson, C.-G., Ahrén, B., Schumann, W. C., Löw, H., Landau, B. R., and Efendic, S. (1989) *J. Biol. Chem.* **264,** 9732–9733.
15. Chen, P. Y., Csutora, P., Veyna-Burke, N. A., and Marchese, R. B. (1998) *Diabetes* **47,** 874–881.
16. Ashcroft, S. J. H., and Randle, P. J. (1968) *Nature* **219,** 857–858.
17. Matschinsky, F. M., and Ellerman, J. E. (1968) *J. Biol. Chem.* **243,** 2370–2376.
18. Khan, A., Hong-Lie, C., Landau, B. R. (1995) *Endocrinology* **49,** 219–225.
19. Sweet, I. R., Najafi, H., Li, G., Grodberg, J., and Matschinsky, F. M. (1997) *Am. J. Physiol.* **272,** E696–E711.
20. Giroix, M. H., Sener, A., and Malaisse, W. J. (1987) *Mol. Cell. Endocrinol.* **49,** 219–222.
21. Waddell, I. D., and Burchell, A. (1988) *Biochem. J.* **255,** 471–476.
22. Khan, A., Chandramouli, V., Östenson, C.-G., Löw, H., Landau, B. R., and Efendic, S. (1990) *Diabetes* **39,** 456–459.
23. Cao, H.-L. (1995) *Diabetologia* **38,** A–143.
24. Khan, A., Chandramouli, V., Östenson, C.-G., Berggren, P.-O., Löw, H., Landau, B. R., and Efendic, S. (1990) *Endocrinology* **126,** 2413–2416.
25. Tokuyama, Y., Sturis, J., DePaoli, A. M., Takeda, J., Stoffel, M., Tang, J., Sun, X., Polonsky, K. S., and Bell, G. I. (1995) *Diabetes* **44,** 1447–1475.
26. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A., and Wollheim, C. B. (1992) *Endocrinology* **130,** 167–178.
27. Trinh, K., Minassian, C., Lange, A. J., O'Doherty, R. M., and Newgard, C. B. (1997) *J. Biol. Chem.* **272,** 24837–24842.
28. Gamberucci, A., Innocenti, B., Fulceri, R., Bánhegyi, G., Giunti, R., Pozzan, T., and Benedetti, A. (1994) *J. Biol. Chem.* **269,** 23597–23602.
29. Bánhegyi, G., Garzó, T., Fulceri, R., Benedetti, A., and Mandl, J. (1993) *FEBS Lett.* **328,** 149–152.
30. Burchell, A., and Waddell, I.-D. (1991) *Biochim. Biophys. Acta* **1092,** 129–137.
31. Burchell, A., Waddell, I.-D., Countaway, J.-L., Arion, W. J., and Hume, R. (1988) *FEBS Lett.* **242,** 153–156.
32. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
33. Middleditch, C., Clottes, E., and Burchell, A. (1998) *FEBS Lett.* **433,** 33–36.
34. Jorgenson, R. A., and Nordlie, R. C. (1980) *J. Biol. Chem.* **255,** 5907–5915.
35. Benedetti, A., Fulceri, R., Romani, A., and Comporti, M. (1987) *Biochim. Biophys. Acta* **928,** 282–286.
36. Ojcius, D. M., and Young, J. D.-E. (1991) *Trends Biochem. Sci.* **16,** 225–229.
37. Bànhegyi, G., Garzò, T., Fulceri, R., Benedetti, A., and Mandl, J. (1993) *FEBS Lett.* **328,** 149–152.
38. Marcolongo, P., Bànhegyi, G., Benedetti, A., Hinds, C. J., and Burchell, A. (1998) *J. Clin. Endocrinol. Metab.* **83,** 224–229.
39. Bygrave, F. L., and Benedetti, A. (1996) *Cell Calcium* **19,** 547–551.
40. Halban, P. A., Praz, G. A., and Wollheim, C. B. (1983) *Biochem. J.* **212,** 439–443.
41. Wang, H., and Iynedjian, P. B. (1997) *J. Biol. Chem.* **272,** 25731–25736.
42. Wang, H., and Iynedjian, P. B. (1997) *Proc. Natl. Acad. Sci.* **94,** 4372–4377.
43. Iizuka, K., Nakajima, H., Ono, A., Okita, K., Miyazaki, J., Miyagawa, J., Namba, M., Hanafusa, T., and Matsuzawa, Y. (2000) *J. Endocrinol.* **164,** 307–314.