Caffeine releases a glucose-primed endoplasmic reticulum Ca^{2+} pool in the insulin secreting cell line INS-1

Alessandra Gamberucci, Rosella Fulceri, William Pralong, Gabor Bánhegyi, Paola Marcolongo, Sharlene L. Watkins, Angelo Benedetti*

Istituto di Patologia Generale, Università di Siena, 53100 Siena, Italy

Received 27 January 1999

Abstract Caffeine mobilized an intracellular Ca^{2+} pool in intact fura-2-loaded INS-1 cells in suspension exposed to high (16 mM) [glucose], while a minor effect was observed with low (2 mM) [glucose]. Cells were kept in a medium containing diaxozide or no Ca^{2+} to prevent the influx of extracellular Ca^{2+}. The caffeine-sensitive intracellular Ca^{2+} pool was within the endoplasmic reticulum since it was depleted by the inhibitor of the reticular Ca^{2+} pumps thapsigargin and the $InsP_3$-dependent agonist carbachol. No effect of caffeine was observed in the parent glucose-insensitive RINmF5 cells. In microsomes from INS-1 but not RINmF5 cells, the type 2 ryanodine receptor was present as revealed by Western blotting. It was concluded that the endoplasmic reticulum of INS-1 cells possesses caffeine-sensitive type 2 ryanodine receptors Ca^{2+} channels.

Key words: Endoplasmic reticulum Ca^{2+} store; Insulin-secreting cell; Ryanodine receptor

1. Introduction

A key event in the insulin secretion process is the elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In insulin secreting cells two major mechanisms underlie (oscillatory) increases in $[Ca^{2+}]_i$, i.e. extracellular Ca^{2+} inflow through voltage-operated channels and mobilization of Ca^{2+} ions stored in the endoplasmic reticulum (ER) [1,2]. Increased glucose fueling ultimately results in the elevation of the cell ATP/ADP ratio which causes plasma membrane depolarization, and in turn activation of voltage-operated Ca^{2+} channels [3]. Mobilization of intracellular ER Ca^{2+} stores can result from the opening of ER Ca^{2+} channels, such as the $InsP_3$ receptors and the ryanodine receptors Ca^{2+}-sensitive channels (RyRs) [4]. While it is well assessed that $InsP_3$-induced mobilization of Ca^{2+} is operative in insulin secreting cells [5], the presence and the role of RyRs in β-cells and insulin secreting cell lines remains controversial ([5,6] and references therein). Early suggestions that β-cells might possess RyRs arose from the fact that caffeine, the classic pharmacological agonist of RyRs, causes the increase in $[Ca^{2+}]_i$ [7,8]. Caffeine, however, was subsequently shown to be capable of elevating $[Ca^{2+}]_i$ independently of ER Ca^{2+} mobilization, i.e. by depolarizing β-cells and hence activating the Ca^{2+} inflow through voltage operated channels [9]. Other studies (reviewed in [5]) showed that the putative physiological agonist of (type 2) RyR cyclic ADP ribose can or cannot mobilize ER Ca^{2+} in insulin secreting cells. Two recent reports on single β-cells from ob/ob mice also gave controversial results. Islam et al. [10] showed that caffeine can release ER Ca^{2+}, which was evident upon stimulation of cAMP-dependent phosphorylation. On the other hand, Tengholm et al. [11] observed no decrease in ER Ca^{2+} levels, i.e. no ER Ca^{2+} mobilization upon caffeine or ryanodine treatment of digitonin-permeabilized single β-cells.

Here we have characterized a caffeine-releasable ER Ca^{2+} pool in the glucose-sensitive INS-1 cells in suspension. This pool was evident in INS-1 cells maintained in the presence of stimulatory concentrations of glucose, and virtually absent in the glucose-insensitive parent line RINmF5. The caffeine effect was found to be associated with an evident type 2 RyR expression in the INS-1 line, whilst little expression of RyRs was found in RINmF5 cells.

2. Materials and methods

2.1. Materials

Carbachol, thapsigargin, diazoxide, dibutyryl cyclic AMP and caffeine were obtained from Sigma (St. Louis, MO, USA). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, USA). All other chemicals were of analytical grade.

2.2. Cells

INS-1 cells, between passages 35 and 80, were grown in monolayer culture [12] in RPMI 1640 medium containing 11 mM glucose supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (5% CO_2, 95% air), until they reached approximately 80% confluence (6–7 days). Subsequently, the cells were washed twice with PBS (phosphate buffered saline) at 37°C and cultured for an additional 24–36 h in the culture medium as above but containing 4 mM glucose. RINmF5 cells were cultured as above except that mercaptoethanol and sodium pyruvate were omitted from the medium. Cells were detached by incubating in PBS containing 0.0125% trypsin and 0.2 mM EGTA for 3 min at 37°C, washed twice with cold RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, and resuspended (approximately 2.5×10^6 cells/ml) in Krebs medium (136 mM NaCl, 3.6 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM KH_2PO_4, 5 mM $NaHCO_3$ 10 mM HEPES, pH 7.4) containing 16 or 2 mM glucose, as indicated in the individual experiments.

The glucose sensitivity of INS-1 cells was assessed with stimulating glucose concentrations which resulted in biphasic changes in $[Ca^{2+}]_i$ and this effect could be reverted by diazoxide [12]. Accordingly, high glucose caused a progressive depolarization of the cells, which could also be reversed by diazoxide. Insulin secretion assessed in parallel (by standard RIA procedure) under the same conditions resulted in a 5-fold increase over basal (2 mM) at 16 mM glucose. This indicated that the INS-1 cell line employed here was a relevant model to study the glucose-induced events involved in insulin secretion.

*Corresponding author. Fax: (39) (577) 227 009.
E-mail: benedetti@unisi.it

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; ER, endoplasmic reticulum; RyR(s), ryanodine receptor; fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

PII: S0014-5793(99)00220-3

2.3. $[Ca^{2+}]_i$ measurements

Cells were loaded with 3 μM fura-2 acetoxymethyl ester according to [13]. After loading, the cells were kept at 0–4°C in Krebs buffer until used. Just before the experiment, a 1 ml aliquot of the cell suspensions was rapidly centrifuged, and resuspended in fresh medium to give a final concentration of 1.25×10^6 cells/ml. Fluorescence was measured with a Perkin-Elmer LS3B fluorometer (excitation and emission wavelengths, 340 and 509 nm, respectively) equipped with magnetic stirring and temperature control. To minimize leakage of trapped fura-2, 200 μM sulfinpyrazone was included in the medium [14]. The results were not affected by sulfinpyrazone. At the end of each incubation, digitonin (50 μg/ml) and EGTA (20 mM) were added in order to measure maximal (F_{max}) and minimal (F_{min}) fluorescence values, respectively. Fluorescence mV output signals were acquired at 0.25 s intervals, using MacLab hardware (AD Instruments), equipped with a Chart v3.2.5. software. The K_d for the Ca^{2+}-fura-2 complex was assumed to be 224 nM at 37°C [15]. Values of $[Ca^{2+}]_i$ were calculated by using CA Cricket Graph III software according to the formula $[Ca^{2+}]_i = K_d[(F-F_{min})/(F_{max}-F)]$.

2.4. Western blot analysis of ryanodine receptors

Microsomal membrane fractions were obtained from INS-1 and RINmF5 cells, rabbit fast-twitch skeletal muscles and rat hippocampus as reported elsewhere [16]. Microsomal proteins were separated on 5% SDS-PAGE and transferred on PVDF membranes following standard procedures. Rabbit polyclonal antibodies against the three known isoforms of the ryanodine receptor (types 1, 2, and 3) prepared as reported [16] were kindly supplied by Dr. V. Sorrentino (Department of Biomedical Sciences, Siena, Italy). For Western blotting, PVDF filters were incubated overnight at room temperature with 1/3000 dilution of the specific antisera [16]. An amplified alkaline phosphatase immunoblot assay was used for detection with a BCIP-NBT substrate system.

3. Results

To evidence a caffeine-sensitive ER Ca^{2+} pool of INS-1 cells we adopted an experimental protocol suitable to keep the ER Ca^{2+} stores maximally full and to prevent extracellular Ca^{2+} inflow through voltage operated channels. To this end, fura-2-loaded INS-1 cells were incubated in the presence of high (16 mM) [glucose] and diazoxide. Several previous reports (e.g. [17–19]) showed, in fact, that glucose has a priming effect on ER Ca^{2+} stores of insulin secreting cells, i.e. the fueling of insulin secreting cells with high glucose concentrations can enlarge the size of ER Ca^{2+} stores mobilized by carbachol [19] and thapsigargin (Fulceri et al., unpublished data). Diazoxide hyperpolarizes cells, thus preventing glucose-induced depolarization and subsequent activation of voltage sensitive Ca^{2+} channels. A similar protocol was already used to study the role of glucose on carbachol-induced cytosolic Ca^{2+} oscillations in insulin secreting cells [20]. Under these experimental conditions, caffeine addition resulted in an evident increase of $[Ca^{2+}]_i$ in INS-1 cells (Figs. 1a and 3a). Instead, caffeine stimulation of INS-1 cells in the presence of a low (2 mM) [glucose] caused barely detectable $[Ca^{2+}]_i$ rises (Fig. 1b). In the parent glucose-insensitive RINmF5 cells, caffeine poorly affected $[Ca^{2+}]_i$ both in the presence of high (Fig. 1c) and low glucose concentrations (Fig. 1d). The addition of the SERCA type ER Ca^{2+} pump inhibitor thapsigargin [21] caused rises in $[Ca^{2+}]_i$ both in INS-1 and RINmF5 cells previously treated with caffeine (Fig. 1). The $[Ca^{2+}]_i$ rises induced by thapsigargin, however, were higher in INS-1 cells challenged with high [glucose] (Fig. 1b), as compared to those maintained in the presence of low [glucose] (Fig. 1a). This indicated that glucose enlarged not only the caffeine ER Ca^{2+} pool, but also the (caffeine-insensitive) thapsigargin-sensitive one. $[Ca^{2+}]_i$ elevation (nM) caused caffeine was 9 ± 6 in control cells and 46 ± 10 in high glucose-treated cells ($n=3$, means ± S.D.). $[Ca^{2+}]_i$ elevations (nM) caused by the sequential addition of caffeine and thapsigargin were 9 ± 6 and 60 ± 8 in control cells and 46 ± 10 and 107 ± 19 in high glucose-treated cells ($n=3$, means ± S.D.). Stimulation of cAMP-dependent phosphorylation did not appear to be involved in the caffeine effect. The membrane permeant analogue of cAMP dibutyryl cAMP, in fact, did not modify the caffeine-induced $[Ca^{2+}]_i$ rise. In fact, $[Ca^{2+}]_i$ elevations (nM) caused by caffeine were 10 ± 8 in control cells and 41 ± 11 in high glucose-treated

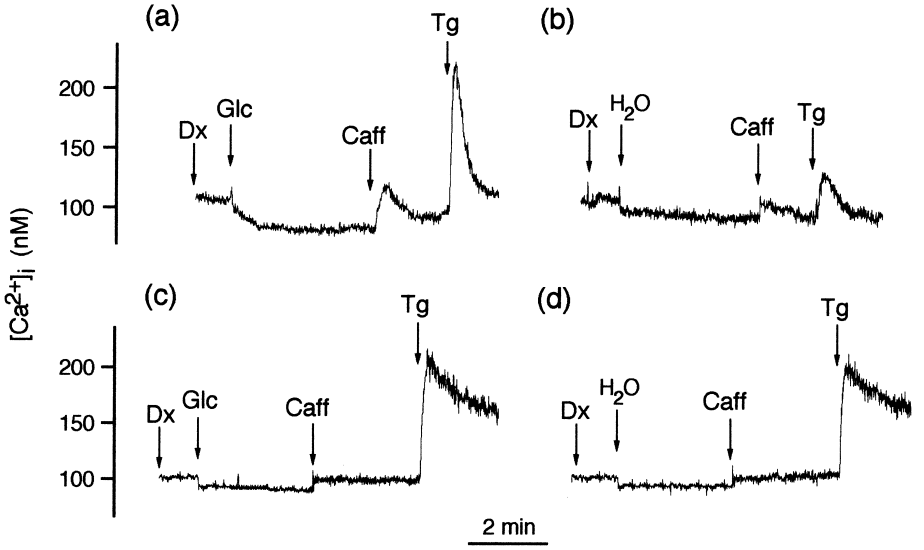

Fig. 1. Effect of caffeine on $[Ca^{2+}]_i$ of INS-1 and RINmF5 cells exposed to 'high' and 'low' glucose. INS-1 cells (a and b) and RINmF5 (c and d) cells were loaded with fura-2 and resuspended in a medium containing low (2 mM) glucose to give 1.25×10^6 cells/ml as indicated in Section 2. Additions as indicated were: glucose 16 mM (Glc), 32 μl of H_2O, 300 μM diaxozide (Dx), 10 mM caffeine (Caff), and 0.1 μM thapsigargin (Tg). The calibrated $[Ca^{2+}]_i$ values are presented on the left side of each trace. Here and elsewhere in this work, the results are representative of at least three different experiments.

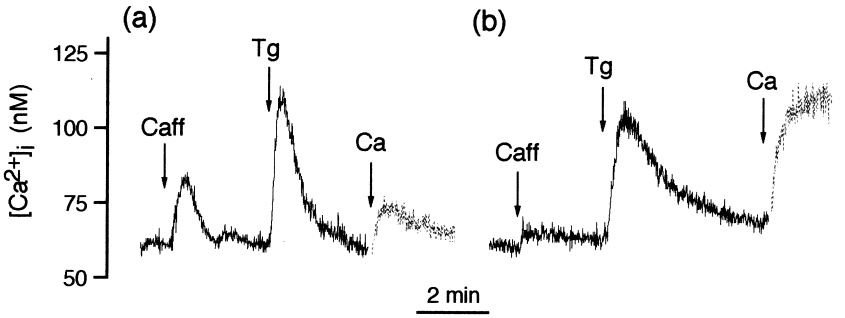

Fig. 2. Effect of caffeine on $[Ca^{2+}]_i$ of INS-1 and RINmF5 cells exposed to 'high' glucose in the absence of extracellular Ca^{2+}. INS-1 cells (trace a) and RINmF5 (trace b) cells were incubated as reported in Fig. 1, except that the medium contained no added Ca^{2+}, 100 μM EGTA and 16 mM glucose. Additions as indicated were: 10 mM caffeine (Caff), 0.1 μM thapsigargin (Tg), and 1.5 mM $CaCl_2$ (Ca).

cells that have been previously exposed to 200 μM dibutyryl cyclic AMP for 3 min ($n = 3$, means ± S.D.). Dibutyryl cyclic AMP itself did not cause a measurable change of $[Ca^{2+}]_i$.

To eliminate any contribution to the observed caffeine-induced $[Ca^{2+}]_i$ rises by Ca^{2+} inflow from extracellular fluid, the protocol adopted for the experiments in Fig. 1b was repeated in the absence of extracellular Ca^{2+}. As shown in Fig. 2, in the presence of high [glucose] an evident caffeine-induced $[Ca^{2+}]_i$ rise was present in INS-1 but not in RINmF5 cells. In the presence of low [glucose] barely detectable caffeine-induced and minor thapsigargin-induced $[Ca^{2+}]_i$ rises were observed (not shown). It should be noted that Ca^{2+} readmission caused a lower $[Ca^{2+}]_i$ overshoot in INS-1 cells as compared to RINmF5 cells (compare Fig. 2a,b) which is consistent with recent data by others [22]. Assuming that Ca^{2+} readmission to thapsigargin-treated cells maintained in a Ca^{2+} free medium reflects capacitative Ca^{2+} influx (e.g. [13]), this observation indicated a minor representation of the capacitative Ca^{2+} influx pathway in INS-1 as compared to RINmF5 cells.

To further characterize the glucose-primed caffeine-sensitive intracellular Ca^{2+} pool of INS-1 cells, fura-2-loaded cells were exposed to different consecutive combined treatments with caffeine, carbachol, and thapsigargin. Carbachol and thapsigargin were used at their supramaximal concentrations (1 μM and 300 μM, respectively). In these (and in the above shown) experiments, 10 mM caffeine was used, since this concentration did not substantially interfere with the fura-2 assay (as verified in parallel experiments). As illustrated in Fig. 3a, after a caffeine-induced $[Ca^{2+}]_i$ rise was accomplished, carbachol resulted in little $[Ca^{2+}]_i$ rises. Previous mobilization of ER Ca^{2+} by carbachol largely reduced the caffeine-induced $[Ca^{2+}]_i$ rise (Fig. 3b). These results suggest that the pools containing $InsP_3$ gated channels and RyRs are largely overlapping. Discharging of ER Ca^{2+} stores by thapsigargin (Fig. 3c) resulted in no further $[Ca^{2+}]_i$ rises by caffeine (and carbachol), which indicated that the caffeine-sensitive Ca^{2+} pool was comprised within that possessing the SERCA type Ca^{2+} pumps, i.e. the ER compartment [23].

Consistent with the Ca^{2+} mobilizing effect of caffeine, western blot analysis of INS-1 microsomal proteins revealed a positive reaction for the RyR type 2 isoform (Fig. 4, lanes 2 and 4). The degree of expression, however, was at least one order of magnitude lower than in brain microsomes used as a positive control for the type 2 RyR expression (Fig. 4, lane 3). A comparable intensity of reaction was observed in fact, with 60 μg and 5 μg of membrane proteins in INS-1 and brain microsomes, respectively (compare lanes 2 and 3 of Fig. 4). A barely detectable reaction was present in RINmF5 microsomes (Fig. 4, lanes 1 and 5). No apparent reaction was present in liver microsomes (as a negative control; Fig. 4, lane 6). The other known isoforms of RyR, i.e. types 1 and 3, were both virtually absent in both INS-1 and RINmF5 cells, as revealed by Western blotting with the proper antibody [23] (not shown).

4. Discussion

The present data indicate that the RyR agonist caffeine does release (a part of) ER Ca^{2+} and that type 2 RyRs are expressed in the insulin-secreting glucose-sensitive INS-1 cell

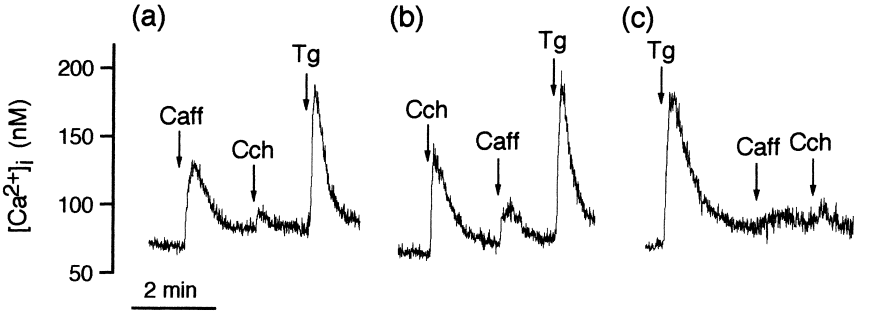

Fig. 3. Effects of the sequential combined treatments with caffeine, carbachol and thapsigargin on $[Ca^{2+}]_i$ of INS-1 cells exposed to 'high' glucose. INS-1 cells were incubated as reported in Fig. 1 in a medium containing 16 mM glucose, and treated with 300 μM diaxozide prior to adding the different compounds. Additions as indicated were: 10 mM caffeine (Caff), 300 μM carbachol (Cch), and 0.1 μM thapsigargin (Tg).

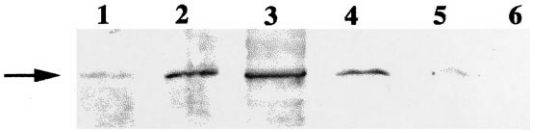

Fig. 4. Western blot analysis of RyR isoform 2 protein in microsomal membranes of INS-1 and RINmF5 cells. Microsomal membranes from INS-1 and RINmF5 cells, rat liver, and rat brain were subjected to SDS/PAGE and immunoblotted with anti-RyR2 antibodies as detailed in Section 3. INS-1 cells, 60 and 40 μg of protein: lanes 2 and 4 respectively. RINmF5 cells, 60 and 40 μg of protein: lanes 1 and 5 respectively. Rat brain microsomes, 5 μg of protein: lane 3. Rat liver microsomes, 20 μg of protein: lane 6.

line. To evidence the activity of caffeine, we took advantage of the priming effects of glucose on ER Ca^{2+} stores (e.g. [17–19]). This effect of glucose is not specific for the caffeine-sensitive ER Ca^{2+} pool as it has been observed also for the $InsP_3$-[17–19] and the thapsigargin-releasable (Fulceri et al., unpublished data, and present work) ER Ca^{2+} stores in different insulin secreting cells. The mechanism through which glucose enlarges the ER Ca^{2+} stores is not the object of the present paper. Whatever the mechanism however, glucose can have a multiple effect on Ca^{2+} signaling in insulin secreting cells, the sugar not only activates Ca^{2+} inflow via increasing ATP/ADP ratio, but can also prime the intracellular Ca^{2+} stores mobilized by $InsP_3$ or eventually Ca^{2+} itself. The latter case might be relevant in the generation of oscillations in $[Ca^{2+}]_i$ and would logically involve ER Ca^{2+}-sensitive Ca^{2+} channels such as type 2 RyR.

In intact single β-cells from ob/ob mice caffeine was found to be capable of releasing ER Ca^{2+} upon stimulation of cAMP-dependent phosphorylation [10]. In INS-1 cells, stimulation of cAMP-dependent phosphorylation does not appear to be required for the caffeine activity (see Section 3). It is possible that the (cAMP-dependent) phosphorylation status of INS-1 cells is constitutively higher as compared to ob/ob mouse β-cells. Alternatively, type 2 RyrR activity might not require cAMP-dependent phosphorylation in INS-1 cells. In another recent report [11], caffeine failed to lower the ER Ca^{2+} levels in digitonin-permeabilized ob/ob mouse β-cells. As a possible explanation for this discrepancy with the present data and those of [10], the lack of caffeine effect in permeabilized ob/ob mouse β-cells might be due to the loss of phosphorylating components during the permeabilization procedure. Consistent with this speculation, no Ca^{2+}-releasing activity of caffeine has been previously observed in digitonin-permeabilized INS-1 cells [24].

Functional (caffeine-sensitive) type 2 RyRs appear to be present in the glucose-responsive insulin-secreting INS-1 cells, but not in the parent glucose-insensitive RINmF5 cells. It is tempting therefore, to speculate that the expression of RyRs can follow the cell differentiation transition from the glucose-insensitive to the glucose-sensitive status, similarly to other components of the glucose-responsive machinery (e.g. glucokinase and plasma membrane glucose transporters GLUT2).

Acknowledgements: This work was supported by a Grant from EC (Biotechnology Project PL97 0286), and by Siena University grants (MURST 60%) to R. Fulceri. G. Bánhegyi was the recipient of a Research Fellowship in Siena from the European Science Foundation. S.L. Watkins was the recipient of a Research Fellowship from the Siena Medical Faculty.

References

[1] Prentky, M. and Matschinsky, F.M. (1987) Physiol. Rev. 67, 1185–1248.
[2] Hermans, M.P. and Henquin, J.C. (1989) Diabetes 38, 198–204.
[3] Matschinsky, F.M. (1996) Diabetes 45, 223–241.
[4] Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) Physiol. Rev. 74, 595–636.
[5] Islam, M.S. and Berggren, P.O. (1997) Diabetologia 40, 1480–1484.
[6] Islam, M.S., Kindmark, H., Larsson, O. and Berggren, P.O. (1997) Biochem. J. 321, 347–354.
[7] Herchuelz, A. and Lebrun, P. (1993) Biochem. Pharmacol. 45, 7–11.
[8] Roe, M.W., Lancaster, M.E., Mertz, R.J., Worley III, J.F. and Dukes, I.D. (1993) J. Biol. Chem. 268, 9953–9956.
[9] Islam, M.S., Larsson, O., Nilsson, T. and Berggren, P.O. (1995) Biochem. J. 306, 679–686.
[10] Islam, M.S., Leibiger, I., Leibiger, B., Rossi, D., Sorrentino, V., Ekstrom, T.J., Westerblad, H., Andrade, F.H. and Berggren, P.O. (1998) Proc. Natl. Acad. Sci. USA 95, 6145–6150.
[11] Tengholm, A., Hagman, C., Gylfe, E. and Hellman, B. (1998) Diabetes 47, 1224–1230.
[12] Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P.A. and Wollheim, C.B. (1992) Endocrinology 130, 167–178.
[13] Gamberucci, A., Innocenti, B., Fulceri, R., Bànhegyi, G., Giunti, R., Pozzan, T. and Benedetti, A. (1994) J. Biol. Chem. 269, 23597–23602.
[14] Li, G., Milani, D., Dunne, M.J., Pralong, W.F., Theler, J.M., Petersen, O.H. and Wollheim, C.B. (1991) J. Biol. Chem. 266, 3449–3457.
[15] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3445.
[16] Giannini, G., Conti, A., Mammarella, S., Scrobogna, M. and Sorrentino, V. (1995) J. Cell Biol. 128, 893–904.
[17] Sato, Y., Nenquin, M. and Henquin, J.C. (1987) FEBS Lett. 421, 115–119.
[18] Chow, R.H., Lund, P.E., Loser, S., Panten, U. and Gylfe, E. (1995) J. Physiol. 485, 607–617.
[19] Theler, J.M., Mollard, P., Guerineau, N., Vacher, P., Pralong, W.F., Sehlegel, W. and Wollheim, C.B. (1992) J. Biol. Chem. 267, 18110–18117.
[20] Gylfe, E. (1991) Pflugers Arch. Eur. J. Physiol. 43, 419–639.
[21] Thansrup, O., Cullen, P.J., Drobak, B.J., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA 87, 2466–2470.
[22] Liu, Y.J. and Gylfe, E. (1997) Cell Calcium 22, 277–286.
[23] Gamberucci, A., Fulceri, R., Tarroni, P., Giunti, R., Marcolongo, P., Sorrentino, V. and Benedetti, A. (1995) Cell Calcium 17, 431–441.
[24] Rutter, G.A., Theler, J.-M. and Wollheim, C.B. (1994) Cell Calcium 16, 71–80.