SPONTANEOUS CALCIUM OSCILLATIONS IN
CLONAL ENDOCRINE PANCREATIC GLUCAGON-SECRETING CELLS

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Summary: Measurements of the cytosolic calcium concentration in single cells of the clonal endocrine pancreatic glucagon-secreting cell line INR1 G9 revealed the existence of spontaneous calcium oscillations in 20 - 70 % of these cells. Inhibition of these spontaneous oscillations by thapsigargin as well as the phospholipase C inhibitor U 73122 demonstrated involvement of calcium release from intracellular stores, probably mediated by a high basal activity of phospholipase C. Removal of extracellular calcium but not the L-type calcium channel antagonists verapamil or nifedipine terminated the spontaneous oscillations, suggesting that calcium influx by a pathway distinct from L-type channels contributed to the oscillations. Similar spontaneous calcium oscillations could be the pacemaker of pulsatile glucagon release in endocrine pancreatic A-cells. © 1994 Academic Press, Inc.

INR1 G9 is a clonal glucagon-secreting cell line derived from hamster endocrine pancreas (1). Glucagon production and secretion by INR1 G9 cells shares the basic characteristics of *in vivo* production and secretion of this hormone (2), for example stimulation of secretion by arginine, theophylline, and forskolin, and inhibition of secretion by somatostatin (3). INR1 G9 cells are therefore used as a model for endocrine pancreatic A-cells (4, 5, 6), which can be isolated only in low number, and with potentially altered properties.

We report the occurence of spontaneous oscillations of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in G9 cells. This finding is of interest with regard to the mechanism of pulsatile islet glucagon secretion *in vivo*.

Material and Methods

Chemicals
Fura-2 AM and fura-2 free acid were purchased from Molecular Probes (Eugene, Oregon, USA); thapsigargin was from L. C. Services (Boston, Massachusetts, USA); and U 73122 was a gift

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Abbreviations: $[Ca^{2+}]cyt$, cytosolic free calcium ion concentration; PLC,phospholipase C; PKC, protein kinase C; TPA 12-O-tetradecanoylphorbol 13-acetate.

from Dr. J. Bleasdale, Upjohn. Cell culture reagents were from Gibco. All other chemicals were obtained from Sigma.

Growth and preparation of cells

INR1 G9 cells were grown in RPMI 1640 medium supplemented with 10 % (by vol.) fetal calf serum, 100 IU penicillin/ml, and 100 μg streptomycin/ml. For measurements of the cytosolic calcium concentration the cells were seeded onto uncoated glass coverslips and allowed to grow in the described medium for three days. The measurements were performed with subconfluent cell cultures.

Measurement of the cytosolic calcium concentration

For loading of cells with the fluorescent calcium indicator fura-2, G9 cells adherent to glass coverslips were incubated with 5 μM fura-2 AM for 45 min, at 37° C, in the same medium used for growing the cells. For measurement of $[Ca^{2+}]_{cyt}$, G9 cell-carrying coverslips were transferred to a closed incubation chamber, the coverslip representing the center part of the chamber bottom. The chamber was mounted onto the stage of a Zeiss Axiovert inverted microscope, fitting tightly into the aperture of a thermostatted metal block. The cells in the incubation chamber were continuously superfused at 1 ml/min. Solution changes were rapidly accomplished by means of a valve attached to an 8-chambered superfusion reservoir. The reservoir and the metal block on the microscope stage were thermostatted to 37° C. The standard superfusion buffer contained (mM): 137 NaCl, 4.7 KCl, 0.56 $MgCl_2$, 1.28 $CaCl_2$, 1.0 Na_2HPO_4, 10 D-glucose, 10 HEPES. pH was adjusted to 7.4 with NaOH. Any use of superfusion buffers with a different composition is indicated in the text. Prior to experiments, the superfusion solutions were equilibrated with 100 % O_2.

Measurement of $[Ca^{2+}]_{cyt}$ was performed using an Attofluor digital imaging system (Rockville, Maryland, USA), with alternating excitation of cells at 334 and 380 nm, monitoring of the resultant emission at 510 nm by an intensified CCD camera and subsequent digitizing, as described previously (7). The traces shown in the figures represent the mean $[Ca^{2+}]$ values in user-defined regions of interest (ROI), one ROI completely covering a single cell. Usually, between 15 and 50 cells were monitored during one experiment.

Results

Upon superfusion with standard superfusion buffer without any additions, G9 cells displayed rythmic fluctuations of $[Ca^{2+}]_{cyt}$ (Fig. 1 a) in 20 to 70 % of the cells examined during a single experiment. During these spontaneous oscillations, $[Ca^{2+}]_{cyt}$ oscillated between a trough level of 129 +/- 10 nM (mean +/- standard deviation, determined from 10 experiments and 10 cells in each) and a peak level of 214 +/- 55 nM, with a peak-to-peak interval of 93 +/- 38 s. In comparison, non-oscillating cells displayed a basal $[Ca^{2+}]_{cyt}$ of 128 +/- 18 nM. In most cases the spontaneous oscillations were already in progress at the beginning of a measurement. Spontaneous oscillations tended to cease during prolonged measurements but typically lasted for longer than 45 min. The oscillations were not affected by variations of the ambient glucose concentration between 5 and 15 mM.

The oscillations ceased immediately when the standard incubation buffer was replaced with a solution containing no added calcium but 1 mM EGTA (Ethyleneglycol-bis (ß-aminoethylether) N, N, N', N'-tetraacetic acid) (Fig. 1 b), and readdition of calcium-containing buffer caused prompt reappearance of oscillations. Addition of agents that block L-type voltage-dependent calcium channels, 20 μM verapamil (Fig. 1 c) or 5 μM nifedipine (not shown), had no effect on the spontaneous calcium oscillations. Calcium entry pathways other than L-type channels, for
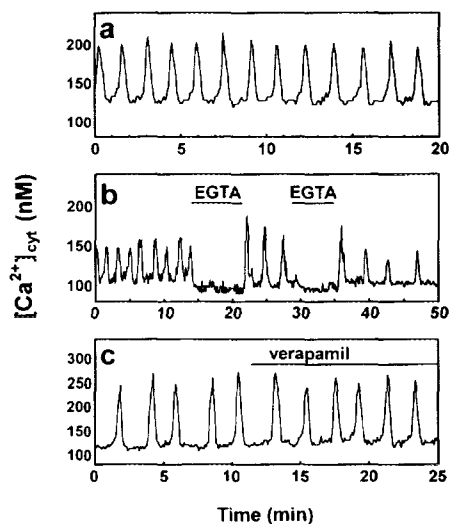
Fig. 1.

Spontaneous oscillations of $[Ca^{2+}]_{cyt}$ in INR1 G9 glucagon-secreting cells and the involvement of calcium influx in this phenomenon. 1 a, typical example of spontaneous oscillations. Fura-2-loaded cells attached to glass coverslips were superfused with standard superfusion buffer, 37^{0} C. 1 b, intermittent superfusion with medium containing 1 mM EGTA and no added calcium is associated with rapid termination and immediate reappearance of oscillations. 1 c, 20 μM of the L-type calcium channel antagonist verapamil does not inhibit the spontaneous oscillations.

example receptor- and second messenger-operated calcium channels as well as the store depletion-regulated pathway, can be inhibited by $LaCl_3$, or by higher concentrations of $NiCl_2$ (1-2 mM) (8). However, the use of these agents was unfeasible with G9 cells. Greater than 300 μM $NiCl_2$ or greater than 100 μM $LaCl_3$ caused a slow but steady and irreversible rise of the cytosolic calcium concentration. This suggested that the cells were rendered leaky by this treatment.

Thapsigargin, a specific inhibitor of intracellular calcium pumps, depletes intracellular calcium stores (9). Application of thapsigargin (Fig. 2 a) terminated the spontaneous calcium oscillations in G9 cells. U 73122 (1 μM), an inhibitor of phospholipase C (PLC) (7), also inhibited the spontaneous oscillations (Fig.2 b), while it did not affect $[Ca^{2+}]_{cyt}$ in non-oscillating cells (not shown). The spontaneous oscillations were also terminated by superfusion with 0.5 μM of the protein kinase C (PKC) inhibitor staurosporine (Fig. 2 c), followed by an elevation of $[Ca^{2+}]_{cyt}$ to levels intermediate between peak and trough values of the oscillations. The PKC-stimulating phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) completely terminated the oscillations (Fig. 2 d), in accordance with the kown feedback inhibition of PLC by PKC (10).

The membrane-permeable cyclic nucleotide analogues 8-bromo cAMP (500 μM) and 8-bromo cGMP (100 μM) had no influence on the spontaneous calcium oscillations, and, moreover, showed no influence on cytosolic calcium in non-oscillating G9 cells.

In non-oscillating G9 cells, oscillations similar in shape and frequency to the spontaneous oscillations could be induced by superfusion with the muscarinic receptor agonist carbachol in concentrations from 10^{-4} to 10^{-6} M (Fig. 3 a). These oscillations were affected in the same way as the spontaneous oscillations by Ca^{2+} removal, verapamil, nifedipine, thapsigargin, U 73122,
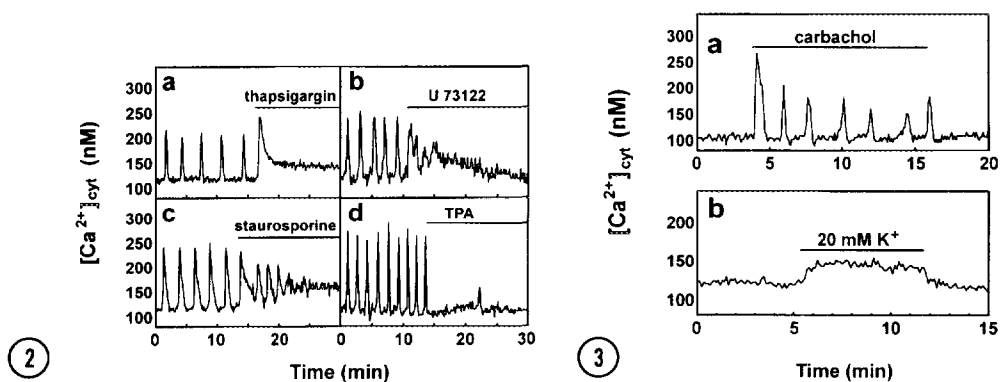
Fig. 2.
Dependence of spontaneous $[Ca^{2+}]_{cyt}$ oscillations in G9 cells on intracellular calcium stores and the phospholipase C/protein kinase C system. 2 a, superfusion with medium containing 1 μM thapsigargin, an inhibitor of intracellular calcium pumps, terminates the spontaneous oscillations and generates a sustained elevation of $[Ca^{2+}]_{cyt}$. 2 b, 1 μM U73122 causes a termination of spontaneous oscillations, $[Ca^{2+}]_{cyt}$ returning to a basal level. 2 c, 0.5 μM staurosporine also terminates the spontaneous oscillations, but generates a sustained $[Ca^{2+}]_{cyt}$ elevation, intermediate between peak and trough values of the oscillations. 2 d, superfusion with 10 nM TPA (12-O-tetradecanoylphorbol 13-acetate) mediates a complete suppression of spontaneous oscillations.

Fig. 3.
In not spontaneously oscillating cells, $[Ca^{2+}]_{cyt}$ oscillations are generated by the phospholipase C-activating muscarinic agonist carbachol but not by elevation of the extracellular potassium concentration. 3 a, typical oscillations generated by superfusion with 10^{-5} M carbachol. 2 b, the bar indicates a change of the extracellular potassium concentration from 4.7 mM to 20 mM.

staurosporine, and TPA (not shown). In contrast, elevation of extracellular potassium to depolarize the cells raised $[Ca^{2+}]_{cyt}$, but never caused oscillations (Fig. 3 b).

Discussion

Our results suggest that in G9 cells spontaneous calcium oscillations are generated by repetitive calcium release from intracellular stores, induced by a high basal activity of phospholipase C, and concomitant calcium influx by a pathway distinct from L-type voltage-dependent calcium channels. Regarding the effect of the protein kinase C inhibitor staurosporine, protein kinase C appears to transform the elevation of $[Ca^{2+}]_{cyt}$ caused by PLC-induced release of stored calcium into oscillations. This activity of PKC may be intermittent or of an intermediate degree, since TPA, generally causing a strong and permanent PKC activation (11), completely terminated the spontaneous oscillations. According to one model for the generation of $[Ca^{2+}]_{cyt}$ oscillations (12), PKC might act by feedback inhibition of PLC.

Carbachol, known to elevate $[Ca^{2+}]_{cyt}$ via activation of PLC, induced calcium oscillations similar to those that occured spontaneously. In contrast, potassium-induced $[Ca^{2+}]_{cyt}$ elevation did not generate oscillations at all. This emphasizes the importance of PLC and intracellular calcium stores for $[Ca^{2+}]_{cyt}$ oscillations in G9 cells.

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Only a small number of $[Ca^{2+}]_{cyt}$ measurements has been performed with isolated A-cells (13, 14). No $[Ca^{2+}]_{cyt}$ oscillations were found in these studies. However, $[Ca^{2+}]_{cyt}$ in these cells also was not elevated by high concentrations of carbachol (13), despite the known stimulation of glucagon secretion by this agent (15) and the typical activation of phospholipase C by carbachol. The phospholipase C signal transduction pathway may have been impaired in the isolated A-cells. $[Ca^{2+}]_{cyt}$ oscillations in G9 cells are similar to spontaneous $[Ca^{2+}]_{cyt}$ oscillations in adrenal chromaffin cells (16) but profoundly distinct from $[Ca^{2+}]_{cyt}$ oscillations in endocrine pancreatic B-cells (17) or cell lines derived thereof (18), the dominant type of $[Ca^{2+}]_{cyt}$ oscillations in the latter cell type being dependent on cell depolarization and calcium influx via L-type channels (19). The type of $[Ca^{2+}]_{cyt}$ oscillations in G9 cells fulfils general requirements for any $[Ca^{2+}]_{cyt}$ oscillation pattern in endocrine pancreatic A-cells. For example, it is compatible with pronounced basal glucagon secretion, since the $[Ca^{2+}]_{cyt}$ oscillations in G9 cells occur spontaneously. It is also compatible with the reported pulsatility of glucagon release, approximately 1 pulse per 4 min in isolated islets (20), since the frequency of oscillations in G9 cells is close to this value. Furthermore, a calcium store- and phospholipase C-based oscillation mechanism in A-cells would be in accordance with the inverse relationship between glucagon and insulin secretion (21) despite the connection of these cell types via gap junctions (22), since this oscillation mechanism would allow $[Ca^{2+}]_{cyt}$ in A-cells to rise independently of B-cell depolarization. In summary, the $[Ca^{2+}]_{cyt}$ oscillations described here could have a function in glucagon secretion by endocrine pancreatic A-cells.

Acknowledgments

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