Abstract We have investigated intracellular Ca\(^{2+}\) mobilization in oscillations of cytoplasmic Ca\(^{2+}\) in response to glucagon-like peptide 1 (GLP-1) and glucose in clonal HIT insulinoma cells with a confocal laser-scanning microscope (CLSM). We also used electron probe X-ray microanalysis to determine the GLP-1- and glucose-induced changes in electrolyte levels in the cytoplasm and insulin granules of the cells. GLP-1 produced 10- to 35-s duration oscillations in cytoplasmic Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)), both with and without Ca\(^{2+}\) in the extracellular solution, suggesting that Ca\(^{2+}\) is mobilized from intracellular Ca\(^{2+}\) stores, namely secretory granules. Glucose caused 1- to 3-min duration oscillatory increases in \([\text{Ca}^{2+}]_i\) when the extracellular solution contained Ca\(^{2+}\). When the cells were cultured without Ca\(^{2+}\) (no Ca\(^{2+}\) added, 1 mM EGTA), an oscillatory \([\text{Ca}^{2+}]_i\) increase of amplitude and short duration (12–35 s) was produced by 11 mM glucose, and the oscillation was inhibited by ruthenium red. X-ray microanalysis showed that stimulation with glucose increased the total Ca concentration in the cytoplasm and decreased it in the insulin granules with and without Ca\(^{2+}\) in the extracellular solution. The application of glucose significantly decreased K, and increased Na and Cl in the cytoplasm when the extracellular solution contained Ca\(^{2+}\). Our result also suggests that the \([\text{Ca}^{2+}]_i\) oscillation induced by glucose is involved in the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores through the ryanodine receptor, which is blocked by ruthenium red, and/or through the inositol trisphosphate receptor that may be present in the membrane of insulin granules.

Key words Ca\(^{2+}\) oscillation · Confocal laser scanning microscopy · GLP-1 · Glucose · Insulin granule · Intracellular Ca\(^{2+}\) store · Pancreatic \(\beta\) cell · X-ray microanalysis

Introduction

Dean and Matthews were the first to show that applying extracellular glucose to pancreatic islet cells evokes a cyclical depolarization of the transmembrane potential [11, 12, 34]. These action potentials correspond to insulin secretion. Moreover, stimulation–secretion coupling in pancreatic \(\beta\) cells has been proposed, and the insulin secretion oscillates in synchrony with the increase in intracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)) in the \(\beta\) cells of islets. Therefore, \([\text{Ca}^{2+}]_i\) is an important determinant of insulin secretion [3, 4, 17, 18, 25, 35, 40]. The clonal pancreatic \(\beta\) cell line HIT secretes insulin in an oscillatory manner [9, 10, 29]. It has been reported that the glucose-induced secretion of insulin by pancreatic \(\beta\) cells causes the following sequence of events: the closing of ATP-sensitive K\(^+\) channels in the plasma membrane, membrane depolarization, the opening of voltage-dependent Ca\(^{2+}\) channels, Ca\(^{2+}\) influx, an increase in the concentration of cytoplasmic Ca\(^{2+}\) and serial exocytosis of secretory granules in the apical cytoplasm [2, 35, 40, 41]. However, it has been shown that glucose can stimulate insulin secretion independently from its action on ATP-sensitive K\(^+\) channels and Ca\(^{2+}\) influx [1, 14].

The temporal and spatial regulation of intracellular Ca\(^{2+}\) in response to external stimuli has recently been investigated in many cells. Cytosolic Ca\(^{2+}\) oscillations induced by an agonist are primarily due to the release of Ca\(^{2+}\) from intracellular stores through inositol 1,4,5-trisphosphate (InsP\(_3\)) or ryanodine receptors, or both [5, 31, 39]. Studies have suggested that the endoplasmic reticulum and/or the secretory granules may respond to the intracellular Ca\(^{2+}\) stores in pancreatic \(\beta\) cells [6, 8]. However, there is little information about the temporal and spatial mobilization of Ca\(^{2+}\) following the stimula-
tion of insulin secretion from pancreatic β cells. It is reported that glucagon-like peptide-1-(7–36) amide (GLP-1) is produced from proglucagon, a precursor of glucagon. It stimulates insulin secretion from pancreatic β cells, and GLP-1 activates adenylate cyclase, leading to an increase in intracellular cAMP and protein kinase A activation [13, 22].

To elucidate the possible role of intracellular Ca\(^{2+}\) mobilization in the oscillation of [Ca\(^{2+}\)], produced by GLP-1 or glucose, we imaged [Ca\(^{2+}\)], in clonal HIT insulinoma cells using a laser-scanning confocal microscope (CLSM). In addition, we used electron probe X-ray microanalysis to determine changes in electrolyte levels, such as Na, K, Cl and Ca, in the cytoplasm and insulin granules of the cells induced by exposure to GLP-1 and glucose in the presence or absence of Ca\(^{2+}\) in the perfusate. Microanalysis was carried out with freshly frozen ultrathin sections of cultured HIT cells.

**Materials and methods**

**HIT cell cultures**

The original culture from which the material for these studies was obtained was provided by American Type Culture Collection (Rockville, Md., USA). All studies were performed with culture passages 65–78. HIT cells were grown in 5% CO\(_2\) /95% O\(_2\) at 37°C and maintained in Ham’s F12K medium containing 10% fetal bovine serum and 100 U/ml penicillin.

Ca\(^{2+}\) imaging with a confocal microscope

The cells were cultured in a glass-bottomed culture dishes with Ham’s F12K medium and were washed with a physiological salt solution (PSS) containing (in mM): 140 NaCl, 4.7 KCl, 1.2 CaCl\(_2\), 1.13 MgCl\(_2\), 3 glucose and 10 HEPES, pH 7.4. After rinsing with PSS, the cells were loaded with 3 µM Fluo 3-acetoxymethyl ester (AM) (Molecular Probes, Eugene, Ore., USA) in a PSS for 30 min in the dark at room temperature. The cells in the dish were continuously superfused with the PSS at a flow rate of 1 ml/min by a peristaltic pump and maintained at 37°C in an electronic incubator (DIA Med, Tokyo, Japan), which was placed on the stage of an inverted microscope (Leica Fluovert, Heidelberg, Germany). To measure fuel- or hormone-evoked Ca\(^{2+}\) signals, 11 mM glucose was added to the perfusate, and glucagon-like peptide-1-(7–36) amide (GLP-1) was used at a concentration of 30 mM in the bath solution. Ruthenium red was used at a concentration of 100 µM in the bath solution. Confocal images of [Ca\(^{2+}\)], were acquired with a confocal image analysis system (CLSM-DIAPLAN, Leica Lasertechnik, Heidelberg, Germany). The cells were irradiated through a 40× oil-immersion objective (Leitz Wetzlar, Heidelberg, Germany) with an excitation beam (488 nm) produced by an argon ion laser tube. Fluorescence images of 256×256 pixels with a resolution of 0.25 µm/pixel were recorded every 4–15 s.

We used the in vivo calibration method proposed by Kao et al. [27]. The cells were superfused with ionomycin, and when fluorescence reached the maximum, they were superfused with a medium containing 5 mM Mn\(^{2+}\), replacing Ca\(^{2+}\), until fluorescence reached a new stable level. At that point, the cells were treated with digitonin. [Ca\(^{2+}\)], was estimated according to the following equations:

\[
F_{\text{max}} = (F_{\text{Mn}} - F_{\text{dig}})/0.2 + F_{\text{dig}}
\]

\[
F_{\text{min}} = (F_{\text{max}} - F_{\text{dig}})/40 + F_{\text{dig}}
\]

\[
[Ca^{2+}] = K_d (F - F_{\text{min}})/(F_{\text{max}} - F)
\]

where \(F_{\text{min}}\) is the minimum fluorescence, \(F_{\text{max}}\) is the maximum fluorescence, \(F_{\text{Mn}}\) is the fluorescence in the presence of Mn, \(F_{\text{dig}}\) is that in the presence of digitonin and \(K_d\) the dissociation constant, was 400 nM for Fluo-3 at vertebrate ionic strength [27].

**X-ray microanalysis**

HIT cells were harvested from dishes using trypsin (0.05%) and EDTA (0.02%). They were spun in the centrifuge at 100 g, and the pellet was resuspended in PSS. For the stimulation, 30 nM GLP-1 or 20 mM glucose was added to the cells suspended in PSS for 1–10 min. After removal from the centrifuge, the pellet was quickly frozen in liquid nitrogen by being pressed against the wall of a metal block cooled to liquid nitrogen temperature. Cryosections (0.1–0.2 µm thick) were cut at −130°C (MT-7000/CR21, RMC, Arizona, USA) and mounted on gold grids, then freeze-dried at −130°C overnight and carbon coated (PTS Systems, Stone Ridge, N.Y., USA). The X-ray microanalysis was done with a Hitachi H-7100 electron microscope interfaced with a Kevex Si (Li) detector and an EMAX-3770 X-ray microanalyzer system (Horiba, Kyoto, Japan). The microanalyzer was operated at 75 kV. A probe current of 10–9–10–10 A was used, and the analysis was usually carried out for 100–200 s. To estimate of local dry-mass fractions, we analyzed the frozen hydrated sections. The grids with frozen sections were set in the cooling specimen holder of the electron microscope (GATAN 626-DH, Institute, Warrendale, Pa., USA), and the X-ray microanalysis was immediately carried out [36]. The X-ray energy spectra and further data processing (to obtain the final concentration values) were performed by an on-line computer system. The special utility programs included some statistical analyses. Details of these procedures are reported elsewhere [32, 36]. With frozen hydrated and dehydrated sections of the glands, the dry-mass fractions in the cytoplasm were estimated to be: 23% for normal tissue, 23% for GLP-1-stimulated tissue, 22% for glucose-stimulated tissue, and 23% for glucose stimulation in the absence of Ca\(^{2+}\). In the insulin granules the dry mass fractions were as follows: 34% for normal tissue, 32% for GLP-1- and glucose-stimulated tissues, and 33% for glucose stimulation in the absence of Ca\(^{2+}\).

**Results**

Effect of glucose or GLP-1 on [Ca\(^{2+}\)], determined by CLSM

Figure 1 shows the synchronous increases in Ca\(^{2+}\) in the nucleus and cytoplasm of an isolated single β cell after stimulation with 11 mM glucose cultured in an external solution containing 3 mM sugar and Ca\(^{2+}\). The pseudocolor images (Fig. 1A, a) were taken at the times shown. After the experiment, acridine orange was added to locate the nucleus. Ca\(^{2+}\) signals were detected within the cell enclosed in the squares. The increase in intranuclear Ca\(^{2+}\) was greater in amplitude and longer in duration than that of intracellular Ca\(^{2+}\) (Fig. 1B). The increase in [Ca\(^{2+}\)] does not reveal typical oscillation, which may be due to a lack of communication with cells [28]. The perinuclear space (the lumen of the nuclear envelope) is continuous with the lumen of the endoplasmic reticulum, and it stores intracellular Ca\(^{2+}\). The difference between Ca\(^{2+}\) increases in the cytoplasm and in the nucleus may be the result of the nucleus generating its own Ca\(^{2+}\) signals. Specifically, the nucleus has its own InsP\(_3\)-sensitive Ca\(^{2+}\) store, and Ca\(^{2+}\) signals in the nucleus and cytoplasm can be uncoupled [15]. Figure 2A shows the cytoplasmic...