Original Article

Inositol (1,4,5)-Trisphosphate Dynamics and Intracellular Calcium Oscillations in Pancreatic β-Cells

Natalia A. Tamarina,[1] Andrey Kuznetsov,[1] Christopher J. Rhodes,[2] Vytautas P. Bindokas,[1] and Louis H. Philipson[1]

Glucose-stimulated insulin secretion is associated with transients of intracellular calcium concentration ($[Ca^{2+}]_i$) in the pancreatic β-cell. We tested the hypothesis that inositol (1,4,5)-trisphosphate [$Ins(1,4,5)P_3$] $[Ca^{2+}]_i$ release is incorporated in glucose-induced $[Ca^{2+}]_i$ oscillations in mouse islets and MIN6 cells. We found that depletion of intracellular Ca^{2+} stores with thapsigargin increased the oscillation frequency by twofold and inhibited the slow recovery phase of $[Ca^{2+}]_i$ oscillations. We employed a pleckstrin homology domain–containing fluorescent biosensor, phospholipase Cδ pleckstrin homology domain–enhanced green fluorescent protein, to visualize $Ins(1,4,5)P_3$ dynamics in insulin-secreting MIN6 cells and mouse islets in real time using a video-rate confocal system. In both types of cells, stimulation with carbamoylcholine (CCh) and depolarization with KCl results in an increase in $Ins(1,4,5)P_3$ accumulation in the cytoplasm. When stimulated with glucose, the $Ins(1,4,5)P_3$ concentration in the cytoplasm oscillates in parallel with oscillations of $[Ca^{2+}]_i$. Maximal accumulation of $Ins(1,4,5)P_3$ in these oscillations coincides with the peak of $[Ca^{2+}]_i$ and tracks changes in frequencies induced by the voltage-gated K^+ channel blockade. We show that $Ins(1,4,5)P_3$ release in insulin-secreting cells can be stimulated by depolarization-induced Ca^{2+} flux. We conclude that $Ins(1,4,5)P_3$ concentration oscillates in parallel with $[Ca^{2+}]_i$ in response to glucose stimulation, but it is not the driving force for $[Ca^{2+}]_i$ oscillations. *Diabetes* 54:3073–3081, 2005

In the pancreatic β-cell, glucose stimulation is associated with increased intracellular calcium ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ increase and subsequent $[Ca^{2+}]_i$ oscillations are initiated by glucose metabolism through an increase in the adenosine 5′-triphosphate (ATP) level, a decrease in adenosine 5′-diphosphate level, and the subsequent closure of ATP-sensitive K^+ channels (1). A number of reports have suggested that the endoplasmic reticulum plays a role in the regulation of the electrical activity and $[Ca^{2+}]_i$ responses in the pancreatic β-cell (2–5). Calcium stores in the endoplasmic reticulum provide a regulated source of Ca^{2+}, which can be quickly mobilized upon hormonal stimulation in insulin-secreting cells (6). The endoplasmic reticulum also provides a source of Ca^{2+} that contributes to $[Ca^{2+}]_i$ increase in the cytoplasm during glucose stimulation (3,5,7), along with the extracellular Ca^{2+} influx through L-type Ca^{2+} channels. It has been suggested that the endoplasmic reticulum is replete with Ca^{2+} when cells are depolarized and Ca^{2+} is released upon repolarization (8,9).

Although some studies have suggested that the endoplasmic reticulum Ca^{2+} stores are mainly discharged by a passive leak of Ca^{2+} into the cytoplasm (8), others have indicated that gated efflux of endoplasmic reticulum Ca^{2+} is an important signaling mechanism in the glucose-stimulated β-cell (10,11). β-Cells have been shown to contain inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] receptors (12), ryanodine receptors (4,10), and Ca^{2+} channels gated by cyclic ADP-ribose (13) or nicotinic acid adenine dinucleotide phosphate (14,15). $Ins(1,4,5)P_3$-dependent mobilization of endoplasmic reticulum Ca^{2+} has been well described in β-cells as part of the response to various agonists, such as acetylcholine, purine nucleotides, or histamines. However, it is still unclear whether $Ins(1,4,5)P_3$ signaling is important in glucose-dependent β-cell activation, particularly for $[Ca^{2+}]_i$ oscillations. Our simulations of β-cell calcium responses, taking into account ATP and Na^+ homeostasis as well as uptake and release of Ca^{2+} by the endoplasmic reticulum, indicate that Ca^{2+} in the endoplasmic reticulum is one of the main variables driving $[Ca^{2+}]_i$ oscillations (16). Our model suggested that the effect of regulated Ca^{2+} efflux from the intracellular stores could shape the pattern of slow calcium oscillations. Using a biosensor capable of reporting $Ins(1,4,5)P_3$ (17) and confocal microscopy, we have demonstrated dynamics of $Ins(1,4,5)P_3$ in mouse primary β-cells and in MIN6 insulinoma cells upon stimulation with glucose and carbamoylcholine (CCh), as well as depolarization with KCl. We show that $Ins(1,4,5)P_3$ concentration oscillates in parallel with $[Ca^{2+}]_i$ in response to glucose stimulation, but it is not the driving force for $[Ca^{2+}]_i$ oscillations.

RESEARCH DESIGN AND METHODS

Isolation and culturing of mouse islets of Langerhans and islet cells. Islets were isolated from the pancreata of 1- to 5-month-old C57BL/J6 mice (The Jackson Laboratories) as described previously (7). To dissociate islets into fragments, they were incubated 5–20 min in dilute trypsin/EDTA solution (Sigma, St. Louis, MO), titurated to dissociate the cells, and plated on glass coverslips in RPMI 1640 medium supplemented with 10% FCS, 11.6 mmol/l

From the [1]Department of Medicine, University of Chicago, Chicago, Illinois; and the [2]Pacific Northwest Research Institute, Seattle, Washington.

Address correspondence and reprint requests to Louis H. Philipson, Department of Medicine, MC 1027, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637. E-mail: l-philipson@uchicago.edu.

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ATP, adenosine 5′-triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; CCh, carbamoylcholine; EGFP, enhanced green fluorescent protein; $Ins(1,4,5)P_3$, inositol (1,4,5)-trisphosphate; KRBB, Krebs-Ringer bicarbonate buffer; LSCM, laser scanning confocal microscope; PHD, pleckstrin homology domain; PIP_2, phosphatidylinositol(4,5)-bisphosphate; PLC, phospholipase C; TEA, tetraethylammonium.

glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin on glass coverslips for 1–4 days. We did not observe any significant association of $[Ca^{2+}]_i$ oscillation frequency with time in culture.

Recombinant adenoviruses. Recombinant adenoviruses were generated to express phospholipase C∂ pleckstrin homology domain–enhanced green fluorescent protein (PLC∂PHD-EGFP) driven by the cytomegalovirus early promoter as previously described (18,19) using a bacterial recombination method in BJ5183 *E. coli* cells (20). Adenovirus-expressing green fluorescent protein was used as a control virus. The amplification and purification of these adenoviruses and their infection protocol were as previously described (18,19). Viral transduction of mouse islets was performed after they were cultured for 2 days on glass coverslips with 5×10^8 pfu/ml for 3 h, followed by imaging 1–2 days posttransduction. In MIN6 cells, the intracellular localization of PLC∂PHD-EGFP was the same with either plasmid DNA transfection or adenovirus delivery (not shown). The expression of PLC∂PHD-EGFP did not affect $[Ca^{2+}]_i$ responses to glucose or CCh in mouse islets (not shown).

Cell culture. Insulin-secreting MIN6 cells were maintained in a humidified incubator with 95% air: 5% CO_2 at 37°C in medium (Dulbecco's modified Eagle's medium, high-glucose medium supplemented with 10% FCS, 25 mmol/l glucose, 100 IU/ml penicillin, and 100 μg/ml streptomycin). MIN6 cells on coverslips were transfected with plasmid-expressing PLC∂PHD-EGFP driven by the cytomegalovirus early promoter (gift of Dr. Tobias Meyer, Stanford University, Stanford, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 1–3 days before the experiment.

Measurement of $[Ca^{2+}]_i$ with fura-2. Mouse or rat pancreatic islets and MIN6 mouse insulinoma cells were loaded with fura-2 for 30 min to 1 h at 37°C in the corresponding growth medium supplemented with 5 μmol/l acetoxymethyl ester of fura-2 (Molecular Probes, Eugene, OR). Fluorescence imaging was performed using a charged coupled device–based imaging system and MetaFluor software (Universal Imaging). During imaging experiments, cells or islets were kept at 37°C and constantly perifused with appropriate Krebs-Ringer bicarbonate buffer (KRBB) containing (in mmol/l) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 25 $NaHCO_3$, with varying concentrations of glucose as needed and appropriate inhibitors at a flow rate of 2.5 ml/min. $[Ca^{2+}]_i$ was expressed as the ratio of fluorescence intensity (at 535/30 nm) after illumination at 340 nm and 380 nm. Calibration of fura-2 was performed using the Fura-2 Calcium Imaging Calibration Kit (Molecular Probes). At least three independent preparations of islets were used for each experimental protocol.

Fluorescent measurements of EGFP and $[Ca^{2+}]_i$ in MIN6 and islets. Between 1 and 2 h before the experiment, cells were loaded with 10 μmol/l acetoxymethyl ester of Fura Red in growth medium (10 μl of 1 mmol/l Fura Red-AM in DMSO was first mixed with 10 μl of 20% Pluronic F127 and then diluted with 1 ml medium). At least three independent preparations of transfected cells or islets were used for each experimental protocol.

Imaging experiments were performed in real time using the Leica SP2 AOBS spectral laser scanning confocal microscope (LSCM) with the Leica DMIRE2 inverted microscope (Leica Microsystems, Heidelberg, Germany). Photoexcitation of both fluorophores was achieved by illumination at 488 nm (Ar laser), and emitted light was acquired at 520–540 nm (EGFP) and at 630–690 (Fura Red). Data acquisition and analysis were performed with Leica Confocal software (Leica Microsystems). Alternatively, a Nipkow dual rotating-disk confocal scanner (CSU10; Yokogawa Electric, Tokyo, Japan) and inverted microscope (Olympus, Tokyo, Japan) were used for confocal imaging. Photoexcitation was achieved by illumination with the 488 nm line of the Kr-Ar mixed-gas laser (series 43; Melles-Griot, Carlsbad, CA). Emitted light was filtered through the 530/30 nm (for EGFP signal) or 660/50 nm (for Fura Red signal) emission filter (Omega Filters, Brattleboro, VT). Data acquisition and analysis were performed with MetaFluor or MetaMorph software (Universal Imaging, Downingtown, PA).

Statistical analysis was performed using Student's t test. Statistical significance was set at $P = 0.05$; results were presented as means ± SE.

RESULTS

Mouse islets at elevated glucose (10–18 mmol/l) often display $[Ca^{2+}]_i$ oscillations (16). To study the role of the intracellular Ca^{2+} stores in the generation of these oscillations, endoplasmic reticulum stores were emptied following treatment with thapsigargin, a specific inhibitor of the sarcoplasmic or endoplasmic reticulum ATPase pump (21). Thapsigargin (1 μmol/l) was added to islets incubated in 14 mmol/l glucose; the emptying of the stores was verified by the absence of the $[Ca^{2+}]_i$ discharge peak that occurs in untreated islets after the addition of 0.25 mmol/l CCh. Two groups of islets were selected based on their oscillation frequency (22): "slow," with oscillations 0.1–1.0/min (39 islets, average 0.34 ± 0.06/min) and "fast," with oscillations faster than 2.0/min (19 islets, average 3.2 ± 0.8/min).

In the first group, thapsigargin produced a sustained elevation of $[Ca^{2+}]_i$ (2) in ~10% of islets (4/39 islets), whereas in another subset (6/39 islets), oscillations occurred irregularly (Fig. 1*B*). The majority of islets (29/39 islets) continued to oscillate after the addition of thapsigargin (Fig. 1*A*). In these islets, thapsigargin brought about a change in the oscillation waveform. The descending phase (downstroke) of each oscillation was accelerated and lacked the prolonged recovery "tail" characteristic of the slow-type oscillations (Fig. 1*A* and *C*). Shortening the lag time between two consecutive oscillations contributed to an increase in the oscillation frequency by 2.11 ± 0.17–fold ($P < 0.01$, $n = 29$). observed with thapsigargin treatment (Fig. 1*C*).

In the group of islets with fast oscillations, there were also a few islets (3/19) that displayed a sustained increase in $[Ca^{2+}]_i$ after the addition of thapsigargin. In the majority of islets (16/19), $[Ca^{2+}]_i$ oscillations persisted after thapsigargin addition. In these islets, thapsigargin produced an increase in the frequency of $[Ca^{2+}]_i$ oscillations by 1.72 ± 0.56–fold ($P < 0.01$, $n = 16$) and a change in the waveform, similar to the islets with slower oscillations (Fig. 1*D*).

There were other distinctions between the two groups of islets. Treatment of fast-oscillating islets with thapsigargin usually brought an immediate increase of $[Ca^{2+}]_i$, most likely originating from the emptying of intracellular stores. The absence of this increase in slowly oscillating islets might indicate a lower filling state of the Ca^{2+} stores. Another difference involved a thapsigargin-stimulated increase in amplitude of oscillations by 2.3 ± 0.6–fold ($P < 0.05$, $n = 16$) in fast-oscillating islets, whereas in the group of slowly oscillating islets, this parameter did not change.

Thus, it appears that the control of $[Ca^{2+}]_i$ oscillation frequency and the generation of the waveform are modulated by endoplasmic reticulum $[Ca^{2+}]$ stores. We therefore further tested the hypothesis that in β-cells, $[Ca^{2+}]_i$ oscillations are accompanied by simultaneous cyclical activation of thapsigargin-sensitive Ca^{2+}stores.

Thapsigargin-sensitive calcium stores in many cell types can be discharged by phospholipase C (PLC)-dependent generation of Ins(1,4,5)P_3. To test the hypothesis that Ins(1,4,5)P_3 is involved in the regulation of $[Ca^{2+}]_i$, changes in mouse islets, and MIN6 insulinoma cells, we employed an Ins(1,4,5)P_3 biosensor, which has been previously used to monitor Ins(1,4,5)P_3 metabolism in madindarbycanine kidney epithelial cells (17), in Chinese hamster ovary cells (23), in Purkinje neurons (24), and in INS-1 cells (25). The fusion protein PLC∂PHD-EGFP combines the pleckstrin homology domain (PHD) of PLC∂ with the EGFP. The PHD of PLCδ is highly specific for phosphate residues in positions 4 and 5 of inositol phosphates and therefore, it binds phosphatidylinositol (4,5)-bisphosphate (PIP_2) and Ins(1,4,5)P_3 (26).

In Fig. 2, the stimulation of MIN6 cells with the muscarinic receptor agonist CCh is illustrated. In nonstimulated cells expressing PLC∂PHD-EGFP, the fluorescent EGFP labeling was located mostly in the plasma membrane (Fig. 2*A* and *B*), which reflects the binding of the PLC∂PHD to PIP_2. After stimulation with CCh, accompanied by the generation of Ins(1,4,5)P_3, the PLC∂PHD-EGFP fluorescent label was translocated into the cytoplasm (Fig. 2*C* and *D*),

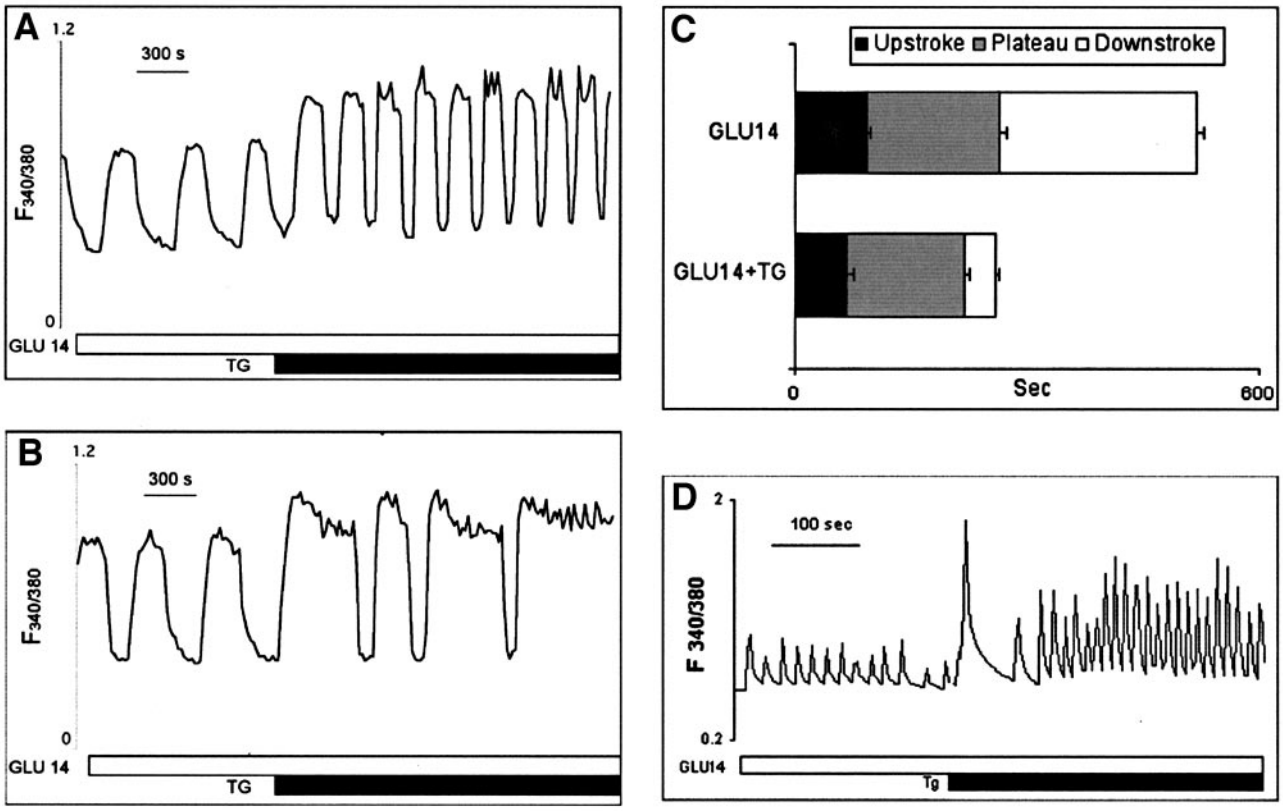

FIG. 1. Islet $[Ca^{2+}]_i$ after sarcoplasmic or endoplasmic reticulum ATPase inhibition. $[Ca^{2+}]_i$ recorded with fura-2 (expressed as F340 nm/380 nm). Islets were incubated in KRBB 14 mmol/l glucose, with 1 μmol/l thapsigargin (Tg) added as indicated. Data are representative of 29 (*A*) and 6 (*B*) of 39 slow-oscillating and 16 (*C*) of 19 fast-oscillating islets.

where it bound soluble $Ins(1,4,5)P_3$. Line-scan graphs (Fig. 2*B* and *D*) show the decrease of the EGFP fluorescence intensity in the plasma membrane of the cell and the gain of intensity in the cytoplasmic region of the cell. By measuring fluorescence in a cytoplasmic region, we were able to monitor the dynamics of $Ins(1,4,5)P_3$ generation.

In MIN6 cells, we found that $Ins(1,4,5)P_3$ generation is also activated by treatment of cells with 30 mmol/l KCl

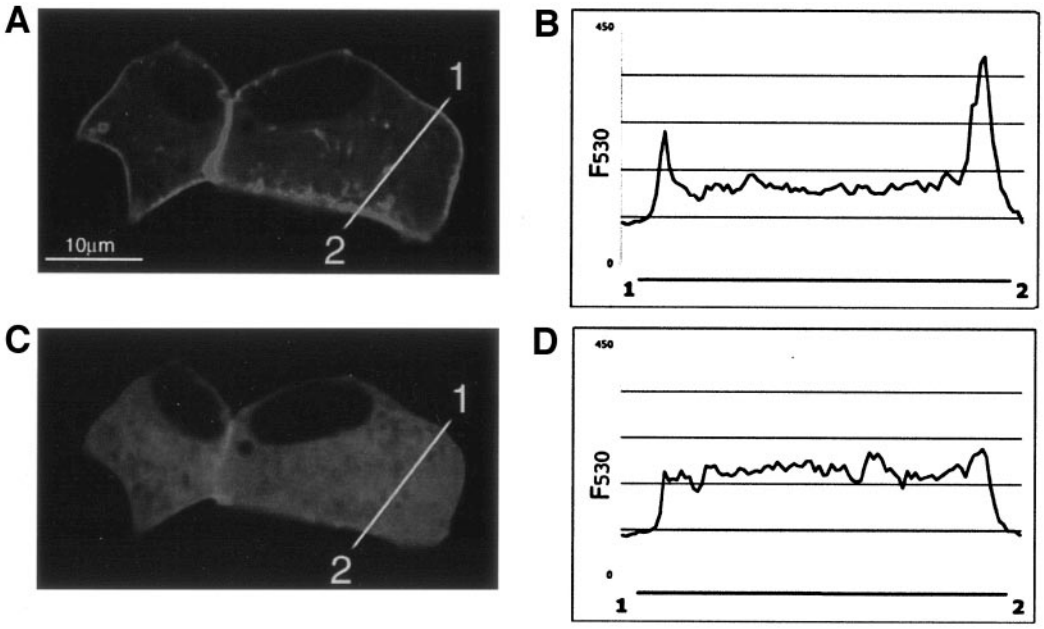

FIG. 2. PLC activation leads to the translocation of PLC∂PHD-EGFP to the cytoplasm. MIN6 cell expressing pPLC∂PHD-EGFP in KRBB 2 mmol/l glucose (*A*) and after the addition of 0.25 mmol/l CCh (*C*). Line-scan graphs (*B* and *D*) reflect the distribution of PLC∂PHD-EGFP across the cell in *A* and *C*, respectively.

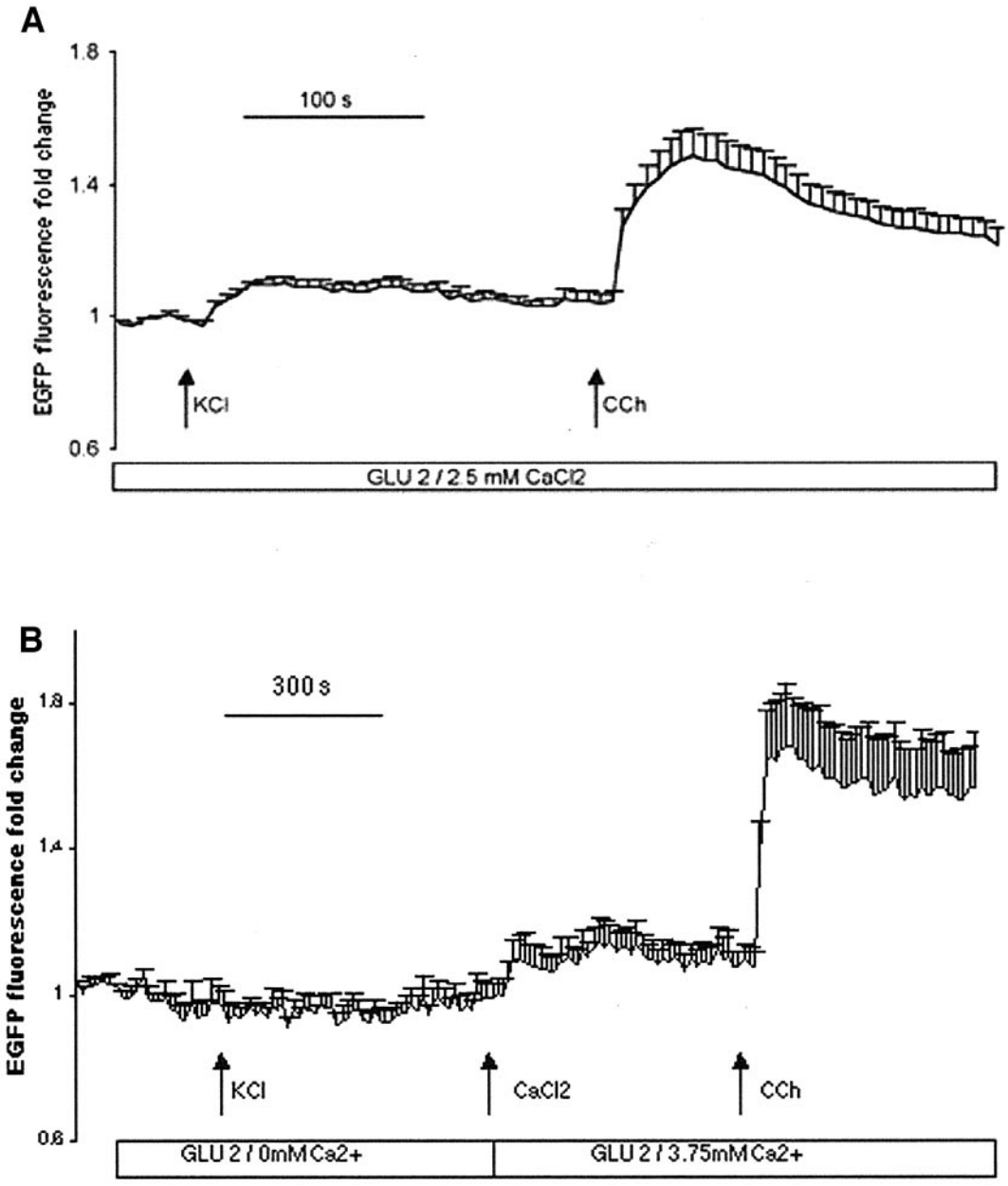

FIG. 3. KCl, CCh, and Ca^{2+} induce PLC activation in MIN6 cells. PLC∂PHD-EGFP–expressing cells were incubated in KRBB 2 mmol/l glucose (*A*), and Ca^{2+}-free KRBB 2 mmol/l glucose (*B*). KCl (30 mmol/l), $CaCl_2$ (3.75 mmol/l), or CCh (0.25 mmol/l) were added as indicated. Traces represent cytoplasmic PLC∂PHD-EGFP fluorescence, an average of 15 (*A*) and 12 (*B*) cells.

(Fig. 3*A*). Stimulation with KCl caused a 10.0 ± 2.4% increase in PLC∂PHD-EGFP translocation, which was significantly less than that observed following CCh treatment (48.4 ± 7.5%, $P < 0.05$, $n = 65$). Simultaneous detection of $[Ca^{2+}]_i$ with Fura Red confirmed that both KCl treatment and subsequent stimulation with CCh are accompanied by increases in $[Ca^{2+}]_i$ (not shown). To determine the role of extracellular Ca^{2+} in depolarization-stimulated PLC activation, stimulation with KCl (30 mmol/l) (in Ca^{2+}-free KRBB containing 0.1 mmol/l EGTA) produced no detectable increase in Ins(1,4,5)P_3 (Fig. 3*B*). Adding Ca^{2+} back to the incubation medium immediately stimulated Ins(1,4,5)P_3 accumulation in KCl-depolarized cells.

Mouse insulinoma cells can display $[Ca^{2+}]_i$ oscillations resembling those in pancreatic islets (16,27). Stimulation of MIN6 cells with high glucose (14 mmol/l) results in a modest nonoscillatory increase in $[Ca^{2+}]_i$ level. However, subsequent addition of 10–20 mmol/l tetraethylammonium (TEA), a potassium channel blocker, reveals an oscillatory response (Fig. 4*A*). TEA-induced $[Ca^{2+}]_i$ oscillations in MIN6 cells are glucose-dependent, since TEA does not stimulate oscillations in low glucose (Fig. 4*B*). We sought to determine whether $[Ca^{2+}]_i$ oscillations in MIN6 cells are associated with PLC activity and generation of Ins(1,4,5)P_3.

Next, we studied simultaneous changes of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ in MIN6 cells. TEA prompted clusters of 5–10 cells (Fig. 4*C*) to start synchronous $[Ca^{2+}]_i$ oscillations (indicated by decreases in the Fura Red emission in Fig. 4*D–F*) with a frequency of 0.3 to 1/min. In PLC∂PHD-EGFP–expressing cells, TEA invoked periodic increases in cytoplasmic EGFP fluorescence (536 nm emission), with a frequency identical to $[Ca^{2+}]_i$ oscillations (0.3 to

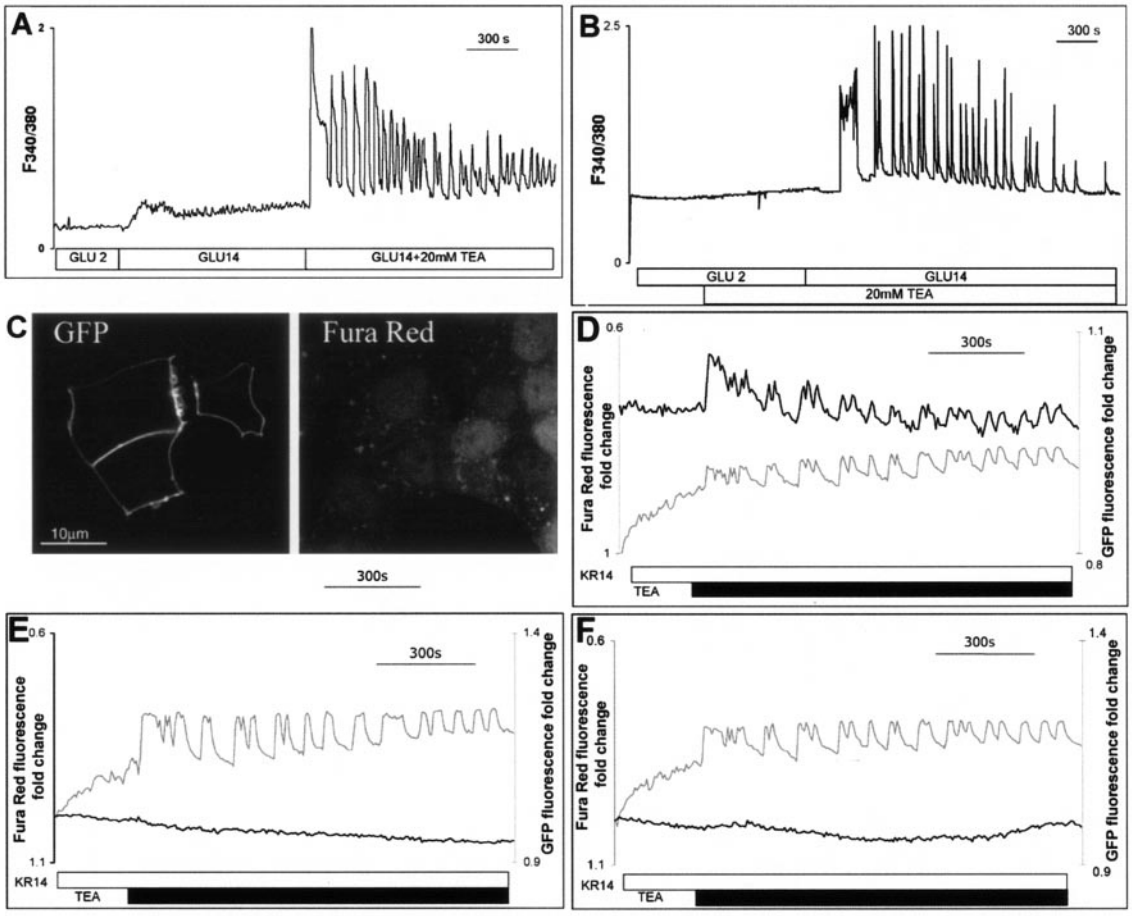

FIG. 4. PLC activation and $[Ca^{2+}]_i$ in MIN6 cells. MIN6 cells were loaded with fura-2 AM (*A* and *B*), Fura Red (*C-F*), along with pPLC∂PHD-EGFP plasmid. Cells were incubated in KRBB 2 mmol/l or 14 mmol/l glucose and TEA as indicated. Traces represent recordings of $[Ca^{2+}]_i$ with fura-2 (as 340-to-380 ratio) in individual whole cells (*A* and *B*), parallel confocal microscopy recordings of relative $[Ca^{2+}]_i$ change with Fura Red (gray trace, inverted scale) and PLC activity (cytoplasmic PLC∂PHD-EGFP, black trace) in cytoplasm of a PLC∂PHD-EGFP–positive cell (*D*), cytoplasm of an PLC∂PHD-EGFP–negative cell (*E*), and nucleus of an PLC∂PHD-EGFP–positive cell (*F*). Data are representative of 30 (*A*), 42 (*B*), 20 (*D* and *F*), or 15 (*E*) cells.

1/min) (Fig. 4*D*). In the nuclei of the same cells (Fig. 4*E*), there were no oscillations of EGFP fluorescence, which correlated with the fact that PLC∂PHD-EGFP is not directed to the nucleus. Similarly, there were no oscillations of EGFP fluorescence in the cytoplasm of control cells not expressing PLC∂PHD-EGFP (Fig. 4*F*). Therefore, the oscillations of EGFP fluorescence in the cytoplasm are directly related to the expression of PLC∂PHD-EGFP and are not the result of the Fura Red emissions. In the majority of experiments (five out of six), the dynamics of PLC∂PHD-EGFP fluorescence in the cytoplasm correlated with increases in $[Ca^{2+}]_i$. These findings indicate that periodic translocations of PLC∂PHD-EGFP [generation of $Ins(1,4,5)P_3$ and decrease in PIP_2] were coincident with peaks of $[Ca^{2+}]_i$ in MIN6 cells stimulated with glucose/TEA.

In mouse islets transduced with adenoviral vector–expressing PLC∂PHD-EGFP, 5–20 cells per islet on the surface area expressed PLC∂PHD-EGFP ("green" cells). Double immunofluorescence staining with anti-insulin and anti-GFP antibodies revealed that most of the EGFP-

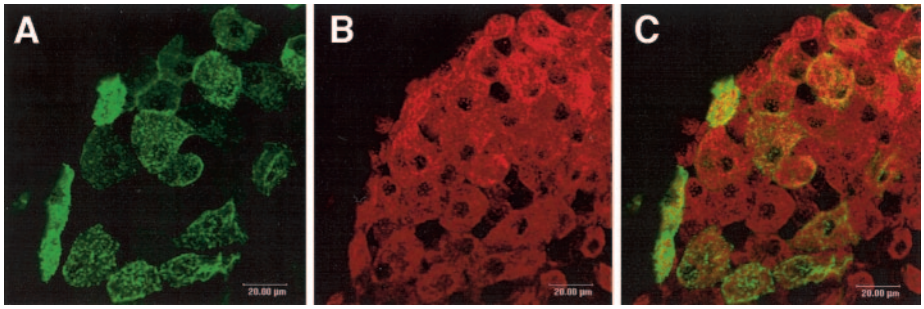

FIG. 5. Mouse islet transduced with AdPLC∂PHD-EGFP adenovirus. Mouse islets were transduced with AdPLC∂PHD-EGFP and immunostained with anti-EGFP antibody (*A*), anti-insulin antibody (*B*), and overlay (*C*). Note that all the EGFP-positive cells in this representative field for were also positive for insulin.

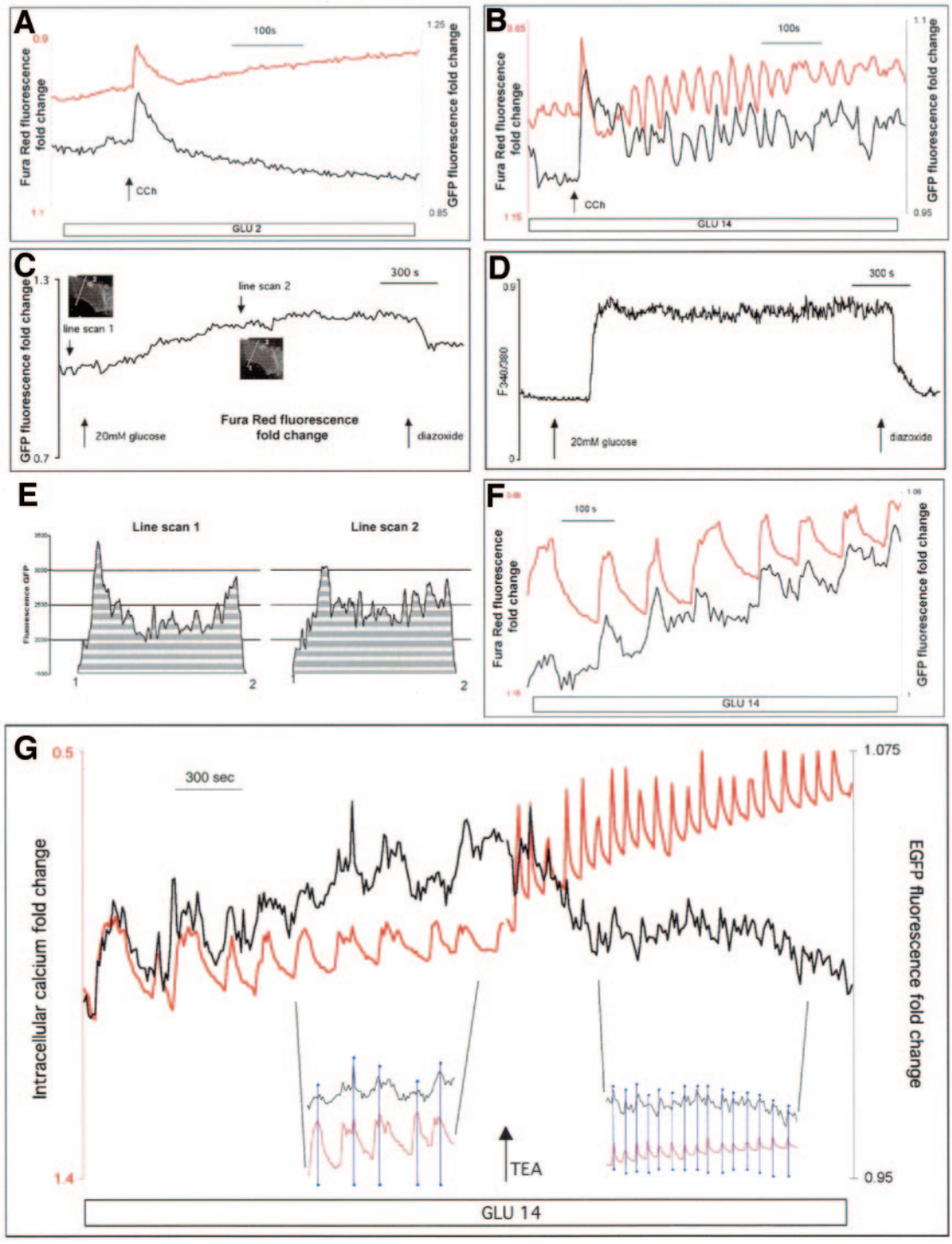

FIG. 6. Simultaneous measures of $[Ca^{2+}]_i$ and PLC activity in intact mouse islets. Mouse islets were transduced with AdPLC∂PHD-EGFP, loaded with Fura Red, and analyzed using simultaneous confocal microscopy recordings. Graphs indicate combined relative $[Ca^{2+}]_i$ change with Fura Red (red trace, inverted scale) and PLC activity (cytoplasmic PLC∂PHD-EGFP, black trace) (*A*, *B*, *F*, and *G*), PLC activity alone (cytoplasmic PLC∂PHD-EGFP) (*C*), $[Ca^{2+}]_i$ (fura-2, expressed as F340 nm/380 nm) (*D*); fluorescence intensity across the cell in trace (*C*) at the time indicated by arrows (line scan 1 and 2) (*E*). Cell image at time points 1 and 2 is illustrated by inset. Cells were incubated in KRBB 2 mmol/l, 14 mmol/l, 20 mmol/l glucose, and 0.25 mmol/l CCh, 200 μmol/l diazoxide, or 20 mmol/l TEA as indicated. Inset in *G* correlates the peaks of PLC activity with $[Ca^{2+}]_i$ oscillations. Each trace represents 15 (*A*), 7 (*B*), 14 (*C*), 20 (*F*), or 7 (*G*) cells.

expressing cells were also positive for insulin (Fig. 5). One to three cells from each islet in close proximity to each other and in the same focal plane were studied in individual experiments.

CCh (0.25 mmol/l) treatment of islets in low glucose induced a translocation of PLC∂PHD-EGFP from its membrane-bound location into the cytoplasm. This translocation was accompanied by an increase in $[Ca^{2+}]_i$ (Fig. 6*A*). CCh treatment in 14 mmol/l glucose led to oscillations in both PLC activity and $[Ca^{2+}]_i$ (after an initial increase) (Fig. 6*B*) in about half of the islets observed. Treatment of islets with KCl (30 mmol/l) induced a translocation of PLC∂PHD-EGFP from plasma membrane to the cytoplasm along with an increase in $[Ca^{2+}]_i$ (not shown).

We also sought to determine whether we could visualize if Ins(1,4,5)P_3 generation in islets is stimulated by an increase in glucose concentration alone. Islets expressing PLC∂PHD-EGFP were first incubated in low glucose (2 mmol/l) for 2–3 h, followed by high glucose (14 mmol/l or 20 mmol/l) for 15–30 min (shorter incubations in low glucose gave a less robust response on translocation without affecting $[Ca^{2+}]_i$ responses [not shown]). Each experiment was concluded with the addition of CCh (0.25 mmol/l) to ensure that the Ins(1,4,5)P_3 signaling remained functional.

Islets responded to the step increase in glucose concentration (from 2 to 20 mmol/l) with a sustained increase in $[Ca^{2+}]_i$ (Fig. 6*D*), and an increase in cytoplasmic PLC∂PHD-EGFP fluorescence was observed in ~70% of experiments (Fig. 6*C*). This increase was accompanied by a decrease in membrane-bound fluorescence, which indicates a translocation of PLC∂PHD-EGFP from the plasma membrane region into the cytoplasm (Fig. 6*E*). Addition of diazoxide (200 μmol/l), an activator of ATP-sensitive K^+ channels that hyperpolarizes the β-cell and stops voltage-dependent $[Ca^{2+}]_i$ influx (Fig. 6*D*), also decreased the cytoplasmic PLC∂PHD-EGFP fluorescence (Fig. 6*C*). This shows that PLC activity in β-cells is sensitive to changes in glucose concentration and is likely mediated by $[Ca^{2+}]_i$.

Mouse islets stimulated with 14 mmol/l glucose typically exhibit $[Ca^{2+}]_i$ oscillations. In these conditions, we observed repeated PLC∂PHD-EGFP translocations, which coincided with $[Ca^{2+}]_i$ oscillations (Fig. 6*F*). The frequency of oscillations varied from 0.25 to 2.26 per min, which shows that both slow and faster oscillations were accompanied by PLC activation. Simultaneous recording of Fura Red and PLC∂PHD-EGFP fluorescence showed that the decrements in Fura Red fluorescence ($[Ca^{2+}]_i$ increase) were almost simultaneous with peaks of cytoplasmic PLC∂PHD-EGFP fluorescence [Ins(1,4,5)P_3 increase]. Islet fragments consisting of groups of 5–50 cells also experienced glucose-dependent synchronous oscillations of Ins(1,4,5)P_3 (not shown).

Treatment of mouse islet β-cells with a blocker of delayed rectifier K^+ channels, TEA, resulted in an increase in frequency and amplitude of $[Ca^{2+}]_i$ oscillations (16,27,28). Treatment of glucose-stimulated islets with TEA (20 mmol/l) changed the frequency of PLC∂PHD-EGFP translocations [Ins(1,4,5)P_3 accumulation] exactly in step with the change in $[Ca^{2+}]_i$ oscillation frequency (Fig. 6*G*). This indicates that the $[Ca^{2+}]_i$ changes in the β-cell could be the activating mechanism for oscillations in PLC activity.

DISCUSSION

The regulation of intracellular Ca^{2+} stores in insulin-secreting cells is complex (29). There are several Ca^{2+} storage pools that can be discharged independently or semi-independently via various agonists. We sought to determine whether in addition to well-known pathways leading to insulin secretion via membrane depolarization following ATP-sensitive K^+ channel blockade, glucose is also capable of discharging intracellular Ca^{2+} storage pools.

Thapsigargin-sensitive calcium stores in many cell types can be activated by PLC-dependent generation of Ins(1,4,5)P_3. Secretagogue stimulation, as well as depolarization of the membrane potential can activate PLC and lead to Ins(1,4,5)P_3 production in RINm5F cells (30), murine islets (31,32), and βTC3 cells (33). Stimulation of Ins(1,4,5)P_3 production by secretagogues was partially attributed to the effect of $[Ca^{2+}]_i$ increase in RINmf cells (30).

However, none of the studies correlated the generation of Ins(1,4,5)P_3 with glucose-stimulated $[Ca^{2+}]_i$ oscillations. To test whether Ins(1,4,5)P_3 is directly involved in the regulation of $[Ca^{2+}]_i$ changes in mouse islets and MIN6 insulinoma cells, we used the Ins(1,4,5)P_3/PIP_2 biosensor PLC∂PHD-EGFP. Intracellular translocation of this biosensor is thought to be dependent upon both the decrease of PIP_2 in the membrane and the increase of Ins(1,4,5)P_3 in the cytoplasm (17,34,35). Although this system is semi-quantitative, it is uniquely suited to real-time imaging and correlation of PLC activation with other cellular processes. We found that both video-rate spinning disk confocal microscopy and LSCM could report translocation, and each method had certain advantages. The dynamics of the processes induced by muscarinic stimulation and glucose/TEA in MIN6 cells could be analyzed by video-rate confocal microscopy (Figs. 2 and 4), but the smaller changes induced by glucose in islets were best observed with the somewhat increased sensitivity and enhanced band separation of our LSCM system (Fig. 6*G*).

We found that in primary mouse islets, stimulation with glucose can lead to oscillatory Ins(1,4,5)P_3 generation. Cytoplasmic Ins(1,4,5)P_3 increased with every $[Ca^{2+}]_i$ increase so that the two processes were tightly coordinated. A change in frequency of $[Ca^{2+}]_i$ oscillations in islets, induced by K_V channel blockade, was immediately reflected in the frequency of Ins(1,4,5)P_3 biosensor translocation oscillations. To our knowledge, this is the first report of dynamic visualization of glucose-induced Ins(1,4,5)P_3 release in primary β-cells. In MIN6 cells, glucose-dependent oscillations of $[Ca^{2+}]_i$ in the presence of TEA were also accompanied by coordinated oscillations of Ins(1,4,5)P_3 similar to those in islets.

It was tempting to argue that $[Ca^{2+}]_i$ changes precede oscillatory changes of PLC activity, which can be seen in some of the graphs depicting simultaneous measurements. This conclusion seems logical based on the $[Ca^{2+}]_i$-dependence of PLC activation during depolarization with KCl and its sensitivity to diazoxide and TEA. However, experimental factors such as the differences in the diffusion times of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 might complicate the measurement and make correlation less certain. In addition, since Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ can influence each other, we can expect that at different phases of the oscillations, one or the other might assume the "leading" role.

We recently suggested a role for Ins(1,4,5)P_3 signaling in our mathematical model of slow $[Ca^{2+}]_i$ oscillations in β-cells (16). According to our model, Ca^{2+} mobilization from intracellular stores plays an important role in $[Ca^{2+}]_i$ oscillations in pancreatic β-cells. We proposed that endoplasmic reticulum calcium stores are filled, and PLC is activated at the upstroke of each $[Ca^{2+}]_i$ oscillation. Both reach maximum shortly after $[Ca^{2+}]_i$ is maximal, thus providing a source of Ins(1,4,5)P_3 and a means for emptying the endoplasmic reticulum Ca^{2+} stores. Our data support this hypothesis since the increases in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 coincide.

The powerful signaling capacity of Ins(1,4,5)P_3 makes it a potentially important factor in regulating glucose responses of the β-cell, including $[Ca^{2+}]_i$ oscillations. Ins(1,4,5)P_3-mediated $[Ca^{2+}]_{endoplasmic\ reticulum}$ release

could potentially affect membrane potential by activating Ca^{2+}-activated K^+ channels in the β-cell (36,37), or store-operated channels (16,38). In addition, cyclical activation of PLC suggests that diacylglycerol and PKC pathways are also likely to be glucose activated (39), which increases the potential importance of oscillations in β-cell physiology.

In Chinese hamster ovary cells expressing the α_{1B}-adrenergic receptor, $[Ca^{2+}]_i$ oscillations are capable of stimulating transient increases in Ins(1,4,5)P_3 via a positive feedback effect on PLC (40). Ca^{2+}-dependent activation has been reported for PLC∂ (41) and PLC-β (30). Both of these isoforms are expressed in pancreatic β-cells (31). Barker et al. (42) observed coordinate oscillations of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 in suspensions of mouse β-cells that were stimulated to undergo synchronous oscillations by sequential applications of glucose (20 mmol/l), the α_2-adrenergic agonist clonidine, and increased extracellular $[Ca^{2+}]$ (5 mmol/l) (43). Although these experiments did not represent normal glucose-induced oscillations and did not measure $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 simultaneously in individual cells, they also suggest the presence of feedback mechanisms that connect $[Ca^{2+}]_i$ and PLC activity in β-cells. The PLC∂PHD-EGFP indicator was also used recently to simultaneously measure $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 in INS-1 cells (25). This study established a positive correlation between changes of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 in INS-1 insulinoma cells, although $[Ca^{2+}]_i$ oscillations in INS-1 cells studied by these authors were not glucose dependent.

Mechanisms that could be responsible for cyclic increases of Ins(1,4,5)P_3 include direct activation of PLC by $[Ca^{2+}]_i$ increase (40), indirect effect through $[Ca^{2+}]_i$-sensitive factors, or by cyclical stimulation of G-protein coupled receptors (42). It is also conceivable that glucose-stimulated Ins(1,4,5)P_3 generation, no matter what its origin, is amplified by both membrane depolarization and Ca^{2+} influx. Additional factors resulting from secretagogue exposure or receptor stimulation are able to modify the sensitivity of PLC to Ca^{2+} in insulin secreting cells and also lead to partial desensitization to glucose stimulation, which might occur in hyperglycemic states (44). Further studies of PLC activity in insulin secreting cells are likely to contribute to the understanding and treatment of insulin secretion defects in type 2 diabetes.

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