THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 19, Issue of May 7, pp. 19396–19400, 2004
Printed in U.S.A.

Oscillations of Phospholipase C Activity Triggered by Depolarization and Ca^{2+} Influx in Insulin-secreting Cells*

Received for publication, February 26, 2004, and in revised form, March 23, 2004
Published, JBC Papers in Press, March 25, 2004, DOI 10.1074/jbc.C400088200

Sophia Thore, Oleg Dyachok, and Anders Tengholm‡

From the Department of Medical Cell Biology, Uppsala University, Biomedical Centre, Box 571, SE-75123 Uppsala, Sweden

Phospholipase C (PLC) is a ubiquitous enzyme involved in the regulation of a variety of cellular processes. Its dependence on Ca^{2+} is well recognized, but it is not known how PLC activity is affected by physiological variations of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). Here, we applied evanescent wave microscopy to monitor PLC activity in parallel with $[Ca^{2+}]_i$ in individual insulin-secreting INS-1 cells using the phosphatidylinositol 4,5-bisphosphate- and inositol 1,4,5-trisphosphate-binding pleckstrin homology domain from PLCδ_1 fused to green fluorescent protein ($PH_{PLC\delta1}$-GFP) and the Ca^{2+} indicator fura red. In resting cells, $PH_{PLC\delta1}$-GFP was located predominantly at the plasma membrane. Activation of PLC by muscarinic or purinergic receptor stimulation resulted in $PH_{PLC\delta1}$-GFP translocation from the plasma membrane to the cytoplasm, detected as a decrease in evanescent wave-excited $PH_{PLC\delta1}$-GFP fluorescence. Using this translocation as a measure of PLC activity, we found that depolarization by raising extracellular $[K^+]$ triggered activation of the enzyme. This effect could be attributed both to a rise of $[Ca^{2+}]_i$ and to depolarization *per se*, because some translocation persisted during depolarization in a Ca^{2+}-deficient medium containing the Ca^{2+} chelator EGTA. Moreover, oscillations of $[Ca^{2+}]_i$ resulting from depolarization with Ca^{2+} influx evoked concentration-dependent periodic activation of PLC. We conclude that PLC activity is under tight dynamic control of $[Ca^{2+}]_i$. In insulin-secreting β-cells, this mechanism provides a link between Ca^{2+} influx and release from intracellular stores that may be important in the regulation of insulin secretion.

Phospholipase C (PLC)[1] is a ubiquitous enzyme that catalyzes hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in formation of the Ca^{2+}-mobilizing messenger inositol 1,4,5-trisphosphate (IP_3) and the protein kinase C (PKC) activator diacylglycerol (DAG) (1). PLC signaling is involved in the regulation of a variety of cellular processes, including the secretion of insulin from pancreatic β-cells (2, 3). Exocytosis of insulin secretory granules in response to glucose stimulation is the final step in a chain of events starting with the uptake and metabolism of the sugar, which leads to an increase in the cytoplasmic ATP/ADP ratio, closure of K_{ATP} channels with membrane depolarization, and Ca^{2+} influx through voltage-dependent channels (4). The resulting rise in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers exocytosis of the insulin-containing granules. The elevation of $[Ca^{2+}]_i$ is usually manifested as complex patterns of oscillations, involving periodic depolarization (5) as well as intracellular mobilization (6), but the underlying mechanisms are incompletely understood. PLC participates in the regulation of insulin secretion by mediating signals from G protein-coupled receptors for various hormones and neurotransmitters such as acetylcholine (7). It has also been demonstrated that the second phase of glucose-induced insulin secretion correlates with activation of PLC (3). This enhancement of secretion may be mediated, at least in part, by DAG-induced PKC activation (3).

To date, 11 isoforms of PLC have been characterized, all of them requiring Ca^{2+} for activity (8). Early studies (9–11) of PLC in islets of Langerhans showed that stimulation with depolarizing concentrations of KCl resulted in a significant rise in inositol phosphate generation, indicating that elevation of $[Ca^{2+}]_i$ might be sufficient to activate PLC. However, little is known about the kinetics of PLC activation in insulin-secreting cells. In particular, it is unknown whether the depolarization-induced oscillations of $[Ca^{2+}]_i$ are associated with periodic activation of PLC with resulting generation of IP_3 and release of Ca^{2+} from internal stores.

One of the most widely used methods for measuring PLC activity is labeling cells with *myo*-[^{3}H]inositol. However, this approach typically provides poor time resolution and no information about the response kinetics in individual cells. It has recently become possible to measure PLC activity in real time in individual living cells using the pleckstrin homology (PH) domain from PLCδ_1 fused to green fluorescent protein ($PH_{PLC\delta1}$-GFP) (12, 13). This PH domain binds specifically to 4,5-phosphorylated PIP_2 and IP_3 but only weakly to other phospholipids or inositol polyphosphates (14). $PH_{PLC\delta1}$-GFP was introduced to monitor plasma membrane PIP_2 concentration during PLC activation with receptor agonists or Ca^{2+} ionophores (12, 13), but was later employed it as a probe for the cytoplasmic IP_3 concentration (15, 16). Upon activation of PLC, $PH_{PLC\delta1}$-GFP dissociates from the plasma membrane into the cytoplasm, a translocation that can be detected with confocal or fluorescence resonance energy transfer (FRET) microscopy (17).

In the present study, we imaged the plasma membrane concentration of $PH_{PLC\delta1}$-GFP by applying evanescent wave microscopy, also known as total internal reflection fluorescence

* This study was supported by Grant 14643 from the Swedish Research Council and by grants from the European Foundation for the Study of Diabetes/Novo Nordisk and the Novo Nordisk Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A "Wenner Gren fellow" supported by the Wenner-Gren Foundations. To whom correspondence should be addressed. Tel.: 46-18-4714481; Fax: 46-18-4714059; E-mail: anders.tengholm@medcellbiol.uu.se.

[1] The abbreviations used are: PLC, phospholipase C; PH, pleckstrin homology; GFP, green fluorescent protein; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; DAG, diacylglycerol; ER, endoplasmic reticulum; PM, palmitoylated and myristoylated.

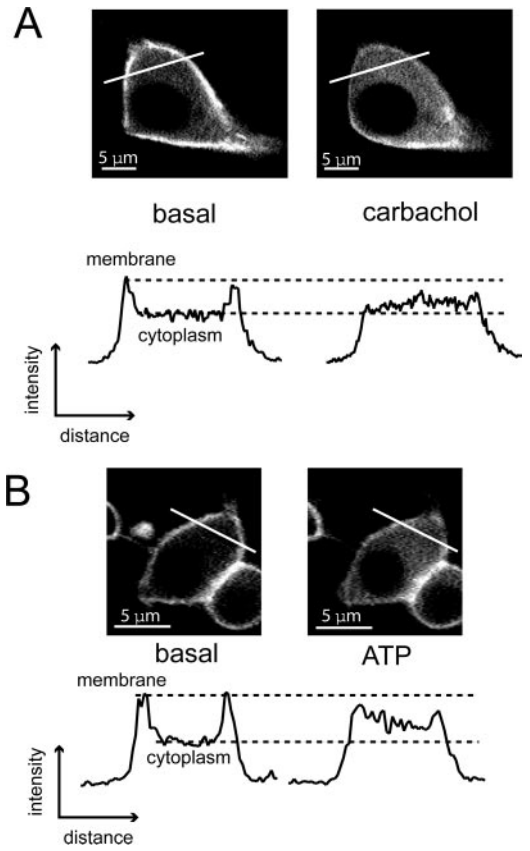

FIG. 1. **Visualization of phospholipase C activation in individual insulin-secreting cells.** INS-1 cells were transiently transfected with $PH_{PLC\delta1}$-GFP and visualized 24 h later with confocal microscopy under basal conditions and at 1 min after stimulation with 100 μM carbachol (*A*) or ATP (*B*). Translocation of the biosensor occurred from the plasma membrane to the cytoplasm. The graphs show the fluorescence intensity along the *lines* indicated in the images. Data are representative of 6–10 independent experiments.

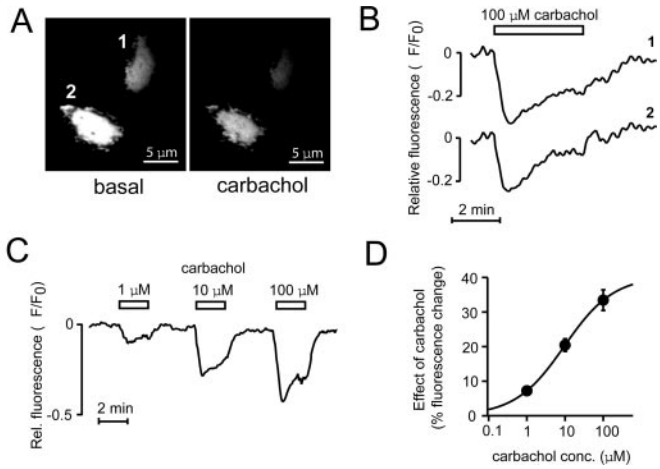

FIG. 2. **Evanescent wave microscopy imaging of phospholipase C activity in individual insulin-secreting cells.** *A*, INS-1 cells were transfected with $PH_{PLC\delta1}$-GFP and 24 h later were imaged before and 1 min after stimulation with 100 μM carbachol. *B*, time course of evanescent wave-excited fluorescence averaged in regions corresponding to the coverslip contact area of the cells shown in *A*. Data are representative of 55 cells from 14 independent experiments. *C*, recording of evanescent wave-excited fluorescence from an individual INS-1 cell expressing $PH_{PLC\delta1}$-GFP during stimulation with increasing concentrations of carbachol. *D*, dose dependence of carbachol-induced $PH_{PLC\delta1}$-GFP translocation. Data points are means ± S.E. (n = 11). The *line* is a fit of the 33 data points to a Hill equation.

microscopy. In this technique, fluorescent molecules are excited with an evanescent wave traveling in a thin layer of ~100 nm above the interface between a coverslip and the aqueous solution bathing the adherent cells. Hence, fluorescent molecules closely adjacent to the plasma membrane are selectively excited, whereas those in the cell interior are not (18, 19). Imaging can therefore be made with improved resolution, lower background, and minimal phototoxicity compared with confocal microscopy. We applied this novel approach to monitor PLC activation in individual insulin-secreting INS-1 cells. Simultaneous measurements of PLC activation and $[Ca^{2+}]_i$ enabled us to demonstrate that oscillations of $[Ca^{2+}]_i$ resulting from periodic influx of the ion are associated with cyclic activation of PLC.

EXPERIMENTAL PROCEDURES

Materials—Reagents of analytical grade and deionized water were used. Thapsigargin and the acetoxymethyl esters of the Ca^{2+} indicators fura-2 and fura red were from Molecular Probes. HEPES was obtained from Roche Applied Science, and Invitrogen provided RPMI 1640 medium and fetal calf serum. All other chemicals were from Sigma. Plasmids encoding the fusion construct between the PH domain of $PLC\delta_1$ and GFP (12) as well as GFP targeted to the plasma membrane via covalent lipid modifications (20) were kindly provided by Dr. Tobias Meyer, Stanford University.

Cell Culture and Transfection—Insulin-secreting INS-1 cells (passage 90–120 (21)) were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium containing 5.5 mM glucose and supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. Following plating onto 25-mm coverslips at a density of 1.5×10^5/ml, the cells were transiently transfected with 2 μg of plasmid DNA in a ratio of 1:2.5 with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and further cultured for 12–24 h.

Fluorescence Microscopy—Before experiments were done, the cells were transferred to a buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 25 mM HEPES with pH adjusted to 7.40 with NaOH. Where indicated, transfected cells were loaded with the Ca^{2+} indicator fura-2 or fura red by a 30- or 40-min incubation at 37 °C with their acetoxymethyl esters at 1 or 10 μM, respectively. The coverslips were used as exchangeable bottoms of a 50-μl open chamber and superfused with buffer at a rate of 0.3 ml/min. All experiments were performed at 37 °C. $[Ca^{2+}]_i$ imaging of fura-2 loaded cells was performed using an epifluorescence imaging system as described previously (22).

The subcellular distribution of the $PH_{PLC\delta1}$-GFP construct was analyzed using a laser-scanning confocal microscope (Odyssey XL, Noran Instruments) equipped with a 60 × 1.40-NA objective (Nikon). Plasma membrane concentrations of the GFP constructs were measured using an evanescent wave microscopy setup built around an Eclipse TE2000 microscope (Nikon). The 488-nm beam of an argon ion laser (Creative Laser Production, Munich, Germany) was homogenized and expanded by a rotating light shaping diffuser (Physical Optics Corp.) and refocused onto the back focal plane of a 60 × 1.45-NA objective (Nikon). The laser beam was moved toward the periphery of the objective aperture until the critical angle was exceeded and total internal reflection occurred. The fluorescence excited by the evanescent field was detected using an Orca-ER camera (Hamamatsu) with a fire-wire interface and MetaMorph software (Universal Imaging Corp.). Selection of emission wavelength was made with interference (525 nm, 25 nm half bandwidth for GFP) and long pass (>630 nm for fura red) filters (Chroma Technology Corp.) mounted in a Lambda 10–2 filter wheel (Sutter Instruments) capable of changing positions within 60 ms. Images (or image pairs) were acquired every 5 s. To minimize exposure of the cells to the potentially harmful laser light, the beam was blocked by an electronic shutter (Sutter Instruments) between image captures.

Data Analysis—Image analysis was made with MetaMorph, MetaFluor (Universal Imaging), or ImageJ (W. S. Rasband, National Institutes of Health, rsb.info.nih.gov/ij) software. GFP and fura red fluorescence intensities are expressed as changes relative to initial fluorescence ($\Delta F/F_o$) after subtraction of background. Curve fitting was made with IgorPro software (Wavemetrics, Lake Oswego, OR). All data are presented as mean values ± S.E.

RESULTS

Confocal and Evanescent Wave Microscopy Imaging of PLC Activity—Confocal microscopy of INS-1 cells expressing

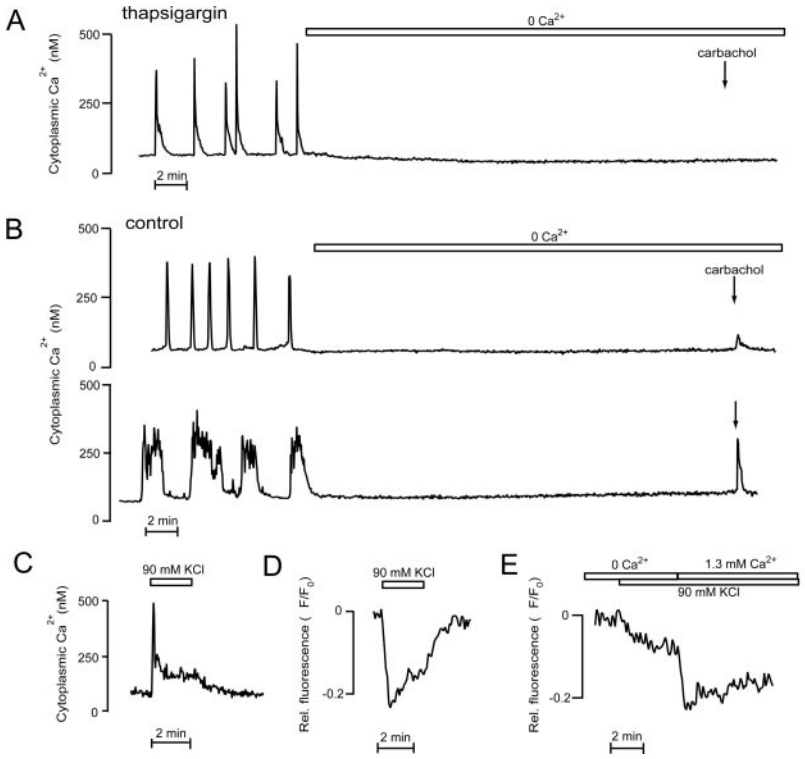

FIG. 3. **Depolarization and Ca^{2+} influx trigger phospholipase C activation in individual insulin-secreting cells.** *A*, INS-1 cells were loaded with the Ca^{2+} indicator fura-2 by 30 min of incubation with its acetoxymethyl ester (1 μM) in a medium containing 3 mM glucose, 1.3 mM Ca^{2+}, and 1 μM thapsigargin. The *trace* shows $[Ca^{2+}]_i$ derived from a recording of the 340/380 nm fluorescence excitation ratio in an individual cell. Removal of medium Ca^{2+} and addition of 2 mM EGTA are indicated by the *bar* and stimulation with 100 μM carbachol by the *arrow*. Data are representative of 57 cells. *B*, similar $[Ca^{2+}]_i$ recordings from two individual INS-1 cells not pre-exposed to thapsigargin. Responses are representative for 64 and 16 of 80 cells. *C*, $[Ca^{2+}]_i$ recording from an INS-1 cell during depolarization with 90 mM KCl in a medium containing 3 mM glucose and 1.3 mM Ca^{2+}. *D*, evanescent wave fluorescence recordings from a $PH_{PLC\delta1}$-GFP-expressing INS-1 cell during depolarization with 90 mM KCl in the presence of 1.3 mM extracellular Ca^{2+}. *E*, similar evanescent wave recording during depolarization in a Ca^{2+}-free medium containing 2 mM EGTA. Data are representative of 51 and 17 cells from 12 and 4 independent experiments in *D* and *E*, respectively.

$PH_{PLC\delta1}$-GFP demonstrated that the fluorescence was distributed predominantly in the plasma membrane under resting conditions. Weaker fluorescence was observed in the cytoplasm, but the construct was excluded from the nucleus (Fig. 1, *left panels*). Activation of PLC by stimulation with the muscarinic receptor agonist carbachol at 100 μM (Fig. 1*A, right*) or the purinergic agonist ATP (Fig. 1*B, right*) was associated with a pronounced translocation of $PH_{PLC\delta1}$-GFP from the plasma membrane to the cytoplasm.

We next investigated whether the $PH_{PLC\delta1}$-GFP translocation could be analyzed using evanescent wave microscopy. Fig. 2*A* shows cells expressing $PH_{PLC\delta1}$-GFP excited with an evanescent wave. A homogenous fluorescence was observed in the plasma membrane adhering to the coverslip. Dissociation of the $PH_{PLC\delta1}$-GFP construct from the plasma membrane upon stimulation with 100 μM carbachol was observed as a decrease in fluorescence, peaking at 27 ± 2% (n = 55) below initial intensity within 30 s (Fig. 2, *A* and *B*). In the continued presence of the agonist there was a partial recovery of the fluorescence to a level of 14 ± 1% (n = 55) below initial fluorescence within 2 min and a complete recovery upon removal of the stimulus (Fig. 2*B*). The drop in plasma membrane fluorescence was dose-dependent with half-maximal effect observed at about 10 μM carbachol (Fig. 2*C*). To verify that the changes in fluorescence reflected activation of PLC and not alterations in cell adhesion or morphology, we imaged cells expressing GFP targeted to the plasma membrane by a palmitoylation and myristoylation motif (PM-GFP (20)). Carbachol at a concentration of 100 μM was without effect on the evanescent wave-excited PM-GFP fluorescence (data not shown).

Depolarization and Elevation of $[Ca^{2+}]_i$ Activate PLC in Insulin-secreting Cells—Insulin-secreting INS-1 and pancreatic β-cells exhibit voltage-dependent Ca^{2+} influx upon stimulation with glucose. In INS-1 cells, $[Ca^{2+}]_i$ oscillations of varying amplitudes and duration are often observed in the 3–11 mM glucose range (Fig. 3, *A* and *B*). These oscillations immediately disappear after removal of extracellular Ca^{2+} but persist after depletion of ER Ca^{2+} stores with thapsigargin (Fig. 3, *A* and *B*; see also Refs. 23 and 24). Store depletion after thapsigargin pretreatment was verified by the lack of Ca^{2+} mobilization in response to 100 μM carbachol.

To test whether depolarization and elevation of $[Ca^{2+}]_i$ was sufficient to activate PLC in insulin-secreting cells, we challenged INS-1 cells with a high concentration of KCl. Isosmolarity was maintained by equimolar reduction of NaCl. Depolarization with 90 mM KCl resulted in a robust increase of $[Ca^{2+}]_i$ (Fig. 3*C*). In $PH_{PLC\delta1}$-GFP-expressing cells, the same stimulation resulted in a pronounced drop of evanescent wave-excited fluorescence (18 ± 1%; n = 51; Fig. 3*D*). Also in a Ca^{2+}-deficient medium containing 2 mM EGTA, KCl depolarization slightly reduced the $PH_{PLC\delta1}$-GFP fluorescence (8 ± 1%; n = 17; Fig 3*E*). Reintroduction of Ca^{2+} to the depolarized cells resulted in a further rapid drop in fluorescence. In contrast, strong depolarization had no effect on PM-GFP fluorescence (data not shown). 90 mM KCl depolarizes the membrane to the levels reached only during short periods of time under physiological conditions. We therefore tested the effect of a lower KCl concentration. At 30 mM KCl, activation of PLC was still detectable in 6 of 15 cells in a Ca^{2+}-deficient medium (3.5 ± 0.7% drop in $PH_{PLC\delta1}$-GFP fluorescence). These data indicate that elevation of $[Ca^{2+}]_i$ in a depolarized cell is a strong stimulus for PLC activation and that plasma membrane depolarization *per se* is sufficient for partial activation of the enzyme.

Oscillatory Activation of PLC by Periodic Depolarization and Ca^{2+} Influx—To investigate whether the influx-dependent $[Ca^{2+}]_i$ oscillations may generate oscillatory PLC activation, we simultaneously measured evanescent wave-excited fluorescence from the $[Ca^{2+}]_i$ indicator fura red and the $PH_{PLC\delta1}$-GFP probe. Fig. 4*A* shows such experiments demonstrating that $[Ca^{2+}]_i$ oscillations imposed by 3-min pulses of high $[K^+]$ repeated with 3-min intervals were paralleled by cyclic activation of PLC (14 ± 1% loss of $PH_{PLC\delta1}$-GFP fluorescence; n = 30 cells). In 11 of 19 cells exhibiting naturally occurring $[Ca^{2+}]_i$ oscillations in the presence of 3 or 11 mM glucose, PLC was activated in a periodic manner (6.3 ± 0.8% loss of $PH_{PLC\delta1}$-GFP fluorescence; Fig 4*B*). Increases of $[Ca^{2+}]_i$ coincided with drops in $PH_{PLC\delta1}$-GFP fluorescence. It was ascertained that cross-talk between the channels did not significantly influence the

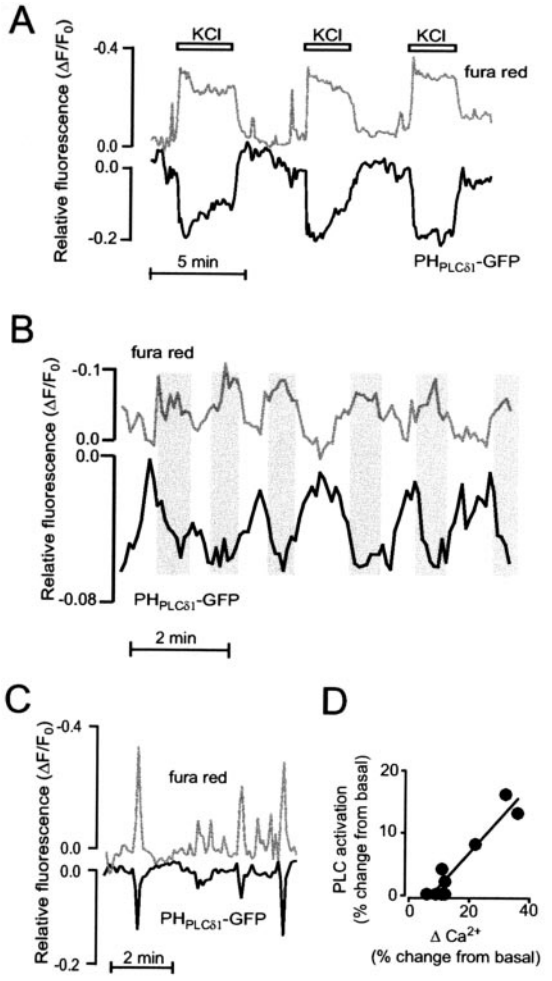

FIG. 4. **Oscillations of PLC activity triggered by periodic depolarization and Ca^{2+} influx in individual insulin-secreting cells.** Parallel evanescent wave microscopy recordings of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura red (*gray trace*, inverted scale) and PLC activity with $PH_{PLC\delta1}$-GFP (*black trace*). *A*, periodic depolarizations were imposed by raising extracellular [KCl] to 90 mM. Data are representative of 30 cells from eight independent experiments. *B*, correlation between spontaneous $[Ca^{2+}]_i$ oscillations and PLC activation observed during superfusion with medium containing 11 mM glucose. Rises of $[Ca^{2+}]_i$ occurred in synchrony with drops in $PH_{PLC\delta1}$-GFP fluorescence (*shadowed rectangles*). Data are representative for 11 of 19 cells in 10 independent experiments. *C*, correlation between the amplitude of $[Ca^{2+}]_i$ oscillations and activation of PLC. The relative change of $PH_{PLC\delta1}$-GFP fluorescence was plotted against the relative change of fura red fluorescence of each Ca^{2+} spike in the experiment shown on the *left*. The *line* is a fit of the data points to a linear equation. Data are representative of 11 cells in 10 independent experiments.

results. Accordingly, spontaneous oscillations in $PH_{PLC\delta1}$-GFP fluorescence were observed in the absence of fura red loading, and intensity oscillations of the Ca^{2+} indicator were observed also in non-transfected cells. The spontaneously occurring $[Ca^{2+}]_i$ oscillations exhibited varying amplitudes (on average 19 ± 2% change in fura red fluorescence, $n = 48$) and durations (30 ± 4 s width at half-maximum, $n = 48$). Fig. 4*C* shows an example of a cell with rapid $[Ca^{2+}]_i$ transients of varying amplitude, where only some of the transients resulted in detectable activation of PLC. When the amplitude of each $[Ca^{2+}]_i$ oscillation (relative change of fura red fluorescence) was plotted against the degree of PLC activation in the same cell (relative change of $PH_{PLC\delta1}$-GFP fluorescence), a strong correlation was found ($r = 0.95$; Fig 4*D*). This finding demonstrates that high amplitude $[Ca^{2+}]_i$ transients are the most efficient in activating PLC.

DISCUSSION

In the present study we have introduced a new approach for real-time monitoring of PLC activation dynamics in individual cells. Using evanescent wave microscopy, we have analyzed the membrane association of the PIP_2/IP_3-binding PH domain from $PLC\delta_1$ fused to GFP in parallel with measurements of $[Ca^{2+}]_i$ with the fluorescent indicator fura red in insulin-secreting INS-1 cells. The selective excitation of plasma membrane fluorescence in evanescent wave microscopy provides lower background, better signal-to-noise ratio, and minimal photobleaching and phototoxicity compared with other fluorescence microscopy approaches (18, 19). Moreover, the image analysis is greatly simplified, because plasma membrane association and dissociation of the fluorescent protein can be detected simply as an increase or decrease of overall fluorescence.

The translocation kinetics observed in response to the muscarinic M_3 receptor agonist carbachol resembled that observed using other microscopy techniques, different cell types, and various G protein-coupled receptor agonists (12, 13, 16, 17, 25). It is in debate as to whether $PH_{PLC\delta1}$-GFP translocation in response to PLC activation reflects the drop of membrane PIP_2 concentration or whether it is due to binding to the concomitantly formed IP_3 (15, 17). Some clarification was recently provided by a combined experimental and modeling study showing that both processes contribute to the dynamics of probe translocation in neuroblastoma cells (25). However, a definite distinction between PIP_2 and IP_3 is not required in order to reach the conclusions drawn about PLC in the present study.

INS-1 cells express at least five different isoforms of PLC ($PLC\beta_1$, $-\beta_2$, $-\beta_3$, $-\gamma_1$, and $-\delta_2$ (26)). Members of the $PLC\beta$ family are activated by heterotrimeric G proteins in response to activation of G protein-coupled receptors, and the $PLC\gamma$ isoforms are typically activated by receptor tyrosine kinases (8). Although all isoforms require Ca^{2+} for activity, the $PLC\delta$ family members are the most sensitive (8), suggesting that they may be regulated directly by $[Ca^{2+}]_i$ (27). Our finding that depolarization-induced $[Ca^{2+}]_i$ elevation triggers PLC activation in INS-1 cells is the first real-time demonstration of this mechanism in individual cells and is consistent with previous studies on islets and clonal insulin-secreting cells using radiotracer techniques (9–11). The observation that depolarization *per se* activates PLC is also in agreement with previous observations of stimulated IP_3 production in β-cells from *ob/ob* mice (6) and clonal βTC3 cells (28) under conditions in which Ca^{2+} influx was prevented. In contrast, depolarization was reported to inhibit PLC-mediated hydrolysis of phosphatidylinositol without effect on PIP_2 in rat islets (29). The different regulation of phosphatidylinositol and PIP_2 hydrolysis may reflect the involvement of different PLC isoforms with distinct substrate specificities (30). It is not known how PLC activity is modulated by membrane potential. Further experiments are required to specify the PLC isozyme(s) responsible for depolarization and Ca^{2+}-induced PIP_2 hydrolysis in insulin-secreting cells.

The most important finding in our study is that periodic depolarization and Ca^{2+} influx result in the oscillatory activation of PLC. Via generation of IP_3, such oscillations will likely give rise to periodic release of Ca^{2+} from the endoplasmic reticulum and thus provide a link between Ca^{2+} entry and intracellular release. The resulting enhancement of the initial $[Ca^{2+}]_i$ elevation by mobilization of the ion from the stores is often referred to as Ca^{2+}-induced Ca^{2+} release. This term is most commonly used to denote the process by which Ca^{2+} promotes its own release by a direct interaction with the intracellular Ca^{2+} release channels (31). However, it is possible that Ca^{2+}-induced Ca^{2+} release mediated by IP_3 receptors depends,

at least in part, on Ca^{2+}-stimulated generation of IP_3. Although there is evidence that $[Ca^{2+}]_i$ oscillations in β-cells (32, 33) and INS-1 cells (present data and Refs. 23 and 24) may occur without involvement of intracellular Ca^{2+} stores, various observations indicate that release of Ca^{2+} from the ER influences the dynamics and shaping of the $[Ca^{2+}]_i$ oscillations (33, 34). IP_3-mediated release of Ca^{2+} from the ER may affect membrane potential and thereby feedback on the voltage-dependent Ca^{2+} influx. In mouse β-cells, IP_3-induced Ca^{2+} release has been shown to activate a K^+ current that contributes to repolarization during action potential firing (5, 35). Ca^{2+} release from the ER also results in activation of a depolarizing store-operated current that may be important in the generation of $[Ca^{2+}]_i$ oscillations (36, 37). Oscillations of $[Ca^{2+}]_i$ are often observed in INS-1 cells at lower glucose concentrations than in primary β-cells. Despite the possible limitations associated with the use of the INS-1 cell line, it is tempting to believe that depolarization and Ca^{2+}-induced oscillations of PLC activity is not unique to INS-1 cells. Primary β-cells and INS-1 cells exhibit comparable patterns of periodic depolarization and influx of Ca^{2+}. Although the phenomenon still remains to be shown in primary β-cells, we have observed oscillations in PLC activity also in murine insulin-secreting MIN6 cells, which exhibit a glucose sensitivity similar to that of primary β-cells.[2]

In addition to contributing to the generation and shaping of Ca^{2+} signals, PLC activation can be anticipated to play a role in regulating the kinetics and magnitude of insulin secretion via generation of DAG and activation of PKC (3). PKCβII has been shown to translocate to the membrane in response to $[Ca^{2+}]_i$ oscillations in MIN6 cells (38). Generation of DAG could then activate PKC recruited to the membrane during each $[Ca^{2+}]_i$ oscillation. The activation of PLC by voltage-dependent Ca^{2+} influx may therefore result in a tight Ca^{2+}-controlled temporal regulation of PKC substrate phosphorylation important in the regulation of insulin secretion.

Acknowledgment—We thank Dr. Tobias Meyer for providing cDNA constructs.

REFERENCES

1. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 517–529
2. Hellman, B., Gylfe, E., Grapengiesser, E., Lund, P.-E., and Marcström, A. (1992) in *Nutrient Regulation of Insulin Secretion* (Flatt, P. R., ed) pp. 213–246, Portland Press, London
3. Zawalich, W. S., and Zawalich, K. C. (1996) *Am. J. Physiol.* **271,** E409–E416
4. Henquin, J. C. (2000) *Diabetes* **49,** 1751–1760
5. Dryselius, S., Grapengiesser, E., Hellman, B., and Gylfe, E. (1999) *Am. J. Physiol.* **276,** E512–E518
6. Liu, Y. J., Grapengiesser, E., Gylfe, E., and Hellman, B. (1996) *Arch. Biochem. Biophys.* **334,** 295–302
7. Gilon, P., and Henquin, J. C. (2001) *Endocr. Rev.* **22,** 565–604
8. Rhee, S. G. (2001) *Annu. Rev. Biochem.* **70,** 281–312
9. Mathias, P. C., Best, L., and Malaisse, W. J. (1985) *Cell Biochem. Funct.* **3,** 173–177
10. Best, L., Tomlinson, S., Hawkins, P. T., and Downes, C. P. (1987) *Biochim. Biophys. Acta* **927,** 112–116
11. Biden, T. J., Peter-Riesch, B., Schlegel, W., and Wollheim, C. B. (1987) *J. Biol. Chem.* **262,** 3567–3571
12. Stauffer, T. P., Ahn, S., and Meyer, T. (1998) *Curr. Biol.* **8,** 343–346
13. Varnai, P., and Balla, T. (1998) *J. Cell Biol.* **143,** 501–510
14. Lemmon, M. A., Ferguson, K. M., O'Brien, R., Sigler, P. B., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10472–10476
15. Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H., and Iino, M. (1999) *Science* **284,** 1527–1530
16. Nash, M. S., Young, K. W., Willars, G. B., Challiss, R. A., and Nahorski, S. R. (2001) *Biochem. J.* **356,** 137–142
17. van der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. (2001) *J. Biol. Chem.* **276,** 15337–15344
18. Steyer, J. A., and Almers, W. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 268–275
19. Axelrod, D. (2001) *Traffic* **2,** 764–774
20. Teruel, M. N., Blanpied, T. A., Shen, K., Augustine, G. J., and Meyer, T. (1999) *J. Neurosci. Methods* **93,** 37–48
21. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A., and Wollheim, C. B. (1992) *Endocrinology* **130,** 167–178
22. Dyachok, O., Tufveson, G., and Gylfe, E. (2004) *Cell Calcium*, in press
23. Dorff, G., Grapengiesser, E., and Hellman, B. (2002) *Biochem. Biophys. Res. Commun.* **293,** 842–846
24. Herbst, M., Sasse, P., Greger, R., Yu, H., Hescheler, J., and Ullrich, S. (2002) *Cell Calcium* **31,** 115–126
25. Xu, C., Watras, J., and Loew, L. M. (2003) *J. Cell Biol.* **161,** 779–791
26. Gasa, R., Trinh, K. Y., Yu, K., Wilkie, T. M., and Newgard, C. B. (1999) *Diabetes* **48,** 1035–1044
27. Allen, V., Swigart, P., Cheung, R., Cockcroft, S., and Katan, M. (1997) *Biochem. J.* **327,** 545–552
28. Gromada, J., Frokjaer-Jensen, J., and Dissing, S. (1996) *Biochem. J.* **314,** 339–345
29. Biden, T. J., Davison, A. G., and Prugue, M. L. (1993) *J. Biol. Chem.* **268,** 11065–11072
30. Mitchell, C. J., Kelly, M. M., Blewitt, M., Wilson, J. R., and Biden, T. J. (2001) *J. Biol. Chem.* **276,** 19072–19077
31. Roderick, H. L., Berridge, M. J., and Bootman, M. D. (2003) *Curr. Biol.* **13,** R425
32. Liu, Y. J., Grapengiesser, E., Gylfe, E., and Hellman, B. (1995) *Cell Calcium* **18,** 165–173
33. Liu, Y. J., Tengholm, A., Grapengiesser, E., Hellman, B., and Gylfe, E. (1998) *J. Physiol.* **508,** 471–481
34. Fridlyand, L. E., Tamarina, N., and Philipson, L. H. (2003) *Am. J. Physiol.* **285,** E138–E154
35. Ämmälä, C., Larsson, O., Berggren, P. O., Bokvist, K., Juntti-Berggren, L., Kindmark, H., and Rorsman, P. (1991) *Nature* **353,** 849–852
36. Worley, J. F., III, McIntyre, M. S., Spencer, B., Mertz, R. J., Roe, M. W., and Dukes, I. D. (1994) *J. Biol. Chem.* **269,** 14359–14362
37. Gilon, P., Arredouani, A., Gailly, P., Gromada, J., and Henquin, J. C. (1999) *J. Biol. Chem.* **274,** 20197–20205
38. Pinton, P., Tsuboi, T., Ainscow, E. K., Pozzan, T., Rizzuto, R., and Rutter, G. A. (2002) *J. Biol. Chem.* **277,** 37702–37710

[2] S. Thore and A. Tengholm, unpublished observations.