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

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\textsuperscript{2+} is well recognized, but it is not known how PLC activity is affected by physiological variations of the cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i). Here, we applied evanescent wave microscopy to monitor PLC activity in parallel with [Ca\textsuperscript{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 PLC\textsubscript{δ} fused to green fluorescent protein (PH\textsubscript{PLC\textsubscript{δ}}-GFP) and the Ca\textsuperscript{2+} indicator fura red. In resting cells, PH\textsubscript{PLC\textsubscript{δ}}-GFP was located predominantly at the plasma membrane. Activation of PLC by muscarinic or purinergic receptor stimulation resulted in PH\textsubscript{PLC\textsubscript{δ}}-GFP translocation from the plasma membrane to the cytoplasm, detected as a decrease in evanescent wave-excited PH\textsubscript{PLC\textsubscript{δ}}-GFP fluorescence. Using this translocation as a measure of PLC activity, we found that depolarization by raising extracellular [K\textsuperscript{+}] triggered activation of the enzyme. This effect could be attributed both to a rise of [Ca\textsuperscript{2+}]i and to depolarization per se, because some translocation persisted during depolarization in a Ca\textsuperscript{2+}-deficient medium containing the Ca\textsuperscript{2+} chelator EGTA. Moreover, oscillations of [Ca\textsuperscript{2+}]i, resulting from depolarization with Ca\textsuperscript{2+} influx evoked concentration-dependent periodic activation of PLC. We conclude that PLC activity is under tight dynamic control of [Ca\textsuperscript{2+}]i.

Processes, including the secretion of insulin from pancreatic \(\beta\)-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 KATP channels with membrane depolarization, and Ca\textsuperscript{2+} influx through voltage-dependent channels (4). The resulting rise in cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) triggers exocytosis of the insulin-containing granules. The elevation of [Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+}]i are associated with periodic activation of PLC with resulting generation of IP\textsubscript{3} and release of Ca\textsuperscript{2+} from internal stores.

One of the most widely used methods for measuring PLC activity is labeling cells with myo-[\textsuperscript{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 PLC\textsubscript{δ} fused to green fluorescent protein (PH\textsubscript{PLC\textsubscript{δ}}-GFP) (12, 13). This PH domain binds specifically to 4,5-phosphorylated PI\textsubscript{3}-P and PI\textsubscript{3}-P, but only weakly to other phospholipids or inositol polyphosphates (14). PH\textsubscript{PLC\textsubscript{δ}}-GFP was introduced to monitor plasma membrane PI\textsubscript{3}-P concentration during PLC activation with receptor agonists or Ca\textsuperscript{2+} ionophores (12, 13), but was later employed it as a probe for the cytoplasmic PI\textsubscript{3}-P concentration (15, 16). Upon activation of PLC, PH\textsubscript{PLC\textsubscript{δ}}-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\textsubscript{PLC\textsubscript{δ}}-GFP by applying evanescent wave microscopy, also known as total internal reflection fluorescence...
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 activity in individual insulin-secreting INS-1 cells. Simultaneous measurements of PLC activation and [Ca^{2+}]_{cytosol} resulted from perivascular 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 PLCδ1 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^{3}/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+}]_{cytosol} imaging of fura-2 loaded cells was performed using an epifluorescence imaging system as described previously (22).

The subcellular distribution of the PHPLCδ1-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 lines (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) 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.

RESULTS

Confocal and Evanescent Wave Microscopy Imaging of PLC Activity—Confocal microscopy of INS-1 cells expressing
PH\textsubscript{PLC\textsubscript{CA1}}-GFP demonstrated that the fluorescence was distributed predominately 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 \mu M (Fig. 1A, right) or the purinergic agonist ATP (Fig. 1B, right) was associated with a pronounced translocation of PH\textsubscript{PLC\textsubscript{CA1}}-GFP from the plasma membrane to the cytoplasm.

We next investigated whether the PH\textsubscript{PLC\textsubscript{CA1}}-GFP translocation could be analyzed using evanescent wave microscopy. Fig. 2A shows cells expressing PH\textsubscript{PLC\textsubscript{CA1}}-GFP excited with an evanescent wave. A homogenous fluorescence was observed in the plasma membrane adhering to the coverslip. Dissociation of the PH\textsubscript{PLC\textsubscript{CA1}}-GFP construct from the plasma membrane upon stimulation with 100 \mu M carbachol was observed as a decrease in fluorescence, peaking at 27 \pm 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 \pm 1% (n = 55) below initial fluorescence within 2 min and a complete recovery upon removal of the stimulus (Fig. 2B). The drop in plasma membrane fluorescence was dose-dependent with half-maximal effect observed at about 10 \mu M carbachol (Fig. 2C). 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 \mu M was without effect on the evanescent wave-excited PM-GFP fluorescence (data not shown).

Depolarization and Elevation of [Ca\textsuperscript{2+}].—To investigate whether the influx-dependent [Ca\textsuperscript{2+}], oscillations may generate oscillatory PLC activation, we simultaneously measured evanescent wave-excited fluorescence from the [Ca\textsuperscript{2+}] indicator fura red and the PH\textsubscript{PLC\textsubscript{CA1}}-GFP probe. Fig. 4A shows such experiments demonstrating that [Ca\textsuperscript{2+}], oscillations imposed by 3-min pulses of high [K\textsuperscript{+}] repeated with 3-min intervals were paralleled by cyclic activation of PLC (14 \pm 1% loss of PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence; n = 30 cells). In 11 of 19 cells exhibiting naturally occurring [Ca\textsuperscript{2+}], oscillations in the presence of 3 or 11 mM glucose, PLC was activated in a periodic manner (6.3 \pm 0.8% loss of PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence; Fig 4B). Increases of [Ca\textsuperscript{2+}], coincided with drops in PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence. It was ascertained that crosstalk between the channels did not significantly influence the

pretreatment was verified by the lack of Ca\textsuperscript{2+} mobilization in response to 100 \mu M carbachol.

To test whether depolarization and elevation of [Ca\textsuperscript{2+}], was sufficient to activate PLC in insulin-secreting cells, we challenged INS-1 cells with a high concentration of KCl. Isomolarity was maintained by equimolar reduction of NaCl. Depolarization with 90 mM KCl resulted in a robust increase of [Ca\textsuperscript{2+}], (Fig. 3C). In PH\textsubscript{PLC\textsubscript{CA1}}-GFP-expressing cells, the same stimulation resulted in a pronounced drop of evanescent wave-excited fluorescence (18 \pm 1%; n = 51; Fig. 3D). Also in a Ca\textsuperscript{2+}-deficient medium containing 2 mM EGTA, KCl depolarization slightly reduced the PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence (8 \pm 1%; n = 17; Fig. 3E). Reintroduction of Ca\textsuperscript{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\textsuperscript{2+}-deficient medium (3.5 \pm 0.7% drop in PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence). These data indicate that elevation of [Ca\textsuperscript{2+}], 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\textsuperscript{2+} Influx.—To investigate whether the influx-dependent [Ca\textsuperscript{2+}], oscillations may generate oscillatory PLC activation, we simultaneously measured evanescent wave-excited fluorescence from the [Ca\textsuperscript{2+}] indicator fura red and the PH\textsubscript{PLC\textsubscript{CA1}}-GFP probe. Fig. 4A shows such experiments demonstrating that [Ca\textsuperscript{2+}], oscillations imposed by 3-min pulses of high [K\textsuperscript{+}] repeated with 3-min intervals were paralleled by cyclic activation of PLC (14 \pm 1% loss of PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence; n = 30 cells). In 11 of 19 cells exhibiting naturally occurring [Ca\textsuperscript{2+}], oscillations in the presence of 3 or 11 mM glucose, PLC was activated in a periodic manner (6.3 \pm 0.8% loss of PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence; Fig 4B). Increases of [Ca\textsuperscript{2+}], coincided with drops in PH\textsubscript{PLC\textsubscript{CA1}}-GFP fluorescence. It was ascertained that crosstalk between the channels did not significantly influence the
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 PIP2/PIP3-binding PH domain from PLCδ1 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 PHPLC1-GFP translocation in response to PLC activation reflects the drop of membrane PIP2 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 PIP2 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\(\beta_1\), \(\beta_2\), \(\beta_4\), \(\gamma_1\), and \(\gamma_2\) (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 activation, the PLC\(\gamma\) 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 \(\beta\)-cells from \(ob\)ob mice (6) and clonal \(\beta\)TC3 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 IP\(_3\) 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,

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**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\(^{2+}\)]) with fura red (gray trace, inverted scale) and PLC activity with PHPLC1-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\(^{2+}\)] oscillations and PLC activation observed during superfusion with medium containing 11 mM glucose. Rises of [Ca\(^{2+}\)] occurred in synchrony with drops in PHPLC1-GFP fluorescence (shadowed rectangles). Data are representative for 11 of 19 cells in 10 independent experiments. C, correlation between the amplitude of [Ca\(^{2+}\)] oscillations and activation of PLC. The relative change of PHPLC1-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.

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results. Accordingly, spontaneous oscillations in PHPLC1-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\(^{2+}\)] 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. 4C shows an example of a cell with rapid [Ca\(^{2+}\)] transients of varying amplitude, where only some of the transients resulted in detectable activation of PLC. When the amplitude of each [Ca\(^{2+}\)] oscillation (relative change of fura red fluorescence) was plotted against the degree of PLC activation in the same cell (relative change of PHPLC1-GFP fluorescence), a strong correlation was found (\(r = 0.95\); Fig 4D). This finding demonstrates that high amplitude [Ca\(^{2+}\)] transients are the most efficient in activating PLC.
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). PKC\(\beta II\) 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.

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REFERENCES


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