Feedback activation of phospholipase C via intracellular mobilization and store-operated influx of Ca^{2+} in insulin-secreting β-cells

Sophia Thore[1], Oleg Dyachok[1,2], Erik Gylfe[1] and Anders Tengholm[1,*]

[1]Department of Medical Cell Biology, Uppsala University, Biomedical Centre, Box 571, SE-75123 Uppsala, Sweden
[2]Department of Biophysics, National T. Shevchenko University of Kiev, Kiev, Ukraine
*Author for correspondence (e-mail: anders.tengholm@medcellbiol.uu.se)

Accepted 7 July 2005
Journal of Cell Science 118, 4463-4471 Published by The Company of Biologists 2005
doi:10.1242/jcs.02577

Summary

Phospholipase C (PLC) regulates various cellular processes by catalyzing the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol-4,5-bisphosphate (PIP_2). Here, we have investigated the influence of Ca^{2+} on receptor-triggered PLC activity in individual insulin-secreting β-cells. Evanescent wave microscopy was used to record PLC activity using green fluorescent protein (GFP)-tagged PIP_2/IP_3-binding pleckstrin homology domain from PLCδ1, and the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was simultaneously measured using the indicator Fura Red. Stimulation of MIN6 β-cells with the muscarinic-receptor agonist carbachol induced rapid and sustained PLC activation. By contrast, only transient activation was observed after stimulation in the absence of extracellular Ca^{2+} or in the presence of the non-selective Ca^{2+} channel inhibitor La^{3+}. The Ca^{2+}-dependent sustained phase of PLC activity did not require voltage-gated Ca^{2+} influx, as hyperpolarization with diazoxide or direct Ca^{2+} channel blockade with nifedipine had no effect. Instead, the sustained PLC activity was markedly suppressed by the store-operated channel inhibitors 2-APB and SKF96365. Depletion of intracellular Ca^{2+} stores with the sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitors thapsigargin or cyclopiazonic acid abolished Ca^{2+} mobilization in response to carbachol, and strongly suppressed the PLC activation in Ca^{2+}-deficient medium. Analogous suppressions were observed after loading cells with the Ca^{2+} chelator BAPTA. Stimulation of primary mouse pancreatic β-cells with glucagon elicited pronounced $[Ca^{2+}]_i$ spikes, reflecting protein kinase A-mediated activation of Ca^{2+}-induced Ca^{2+} release via IP_3 receptors. These $[Ca^{2+}]_i$ spikes were found to evoke rapid and transient activation of PLC. Our data indicate that receptor-triggered PLC activity is enhanced by positive feedback from Ca^{2+} entering the cytoplasm from intracellular stores and via store-operated channels in the plasma membrane. Such amplification of receptor signalling should be important in the regulation of insulin secretion by hormones and neurotransmitters.

Key words: Phospholipase C, Ca^{2+}, Pancreatic β-cell, Evanescent wave microscopy, Green fluorescent protein, Store-operated Ca^{2+} entry

Introduction

The ubiquitous enzyme phosphoinositide-specific phospholipase C (PLC) plays a key role in signal transduction by catalyzing the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol in response to various receptor stimuli. IP_3 mediates rapid mobilization of Ca^{2+} from the endoplasmic reticulum (ER), whereas diacylglycerol stimulates protein kinase C (Berridge et al., 2003). There are four families of PLC (PLC-β, -γ, -δ and -ϵ) with 11 different isoforms (Rhee, 2001). PLC-β is mainly activated by heterotrimeric G-proteins, PLC-γ by tyrosine kinases and PLC-ϵ by the small GTPase Ras. The activation mechanism for the δ isoforms is less clear, but it may be the elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) alone, as this isoform is particularly sensitive to Ca^{2+} (Rhee, 2001).

In pancreatic β-cells, PLC mediates the potentiating action on insulin secretion of many hormones and neurotransmitters. For example, it is well established that cholinergic stimulation of insulin secretion is associated with accumulation of IP_3 and diacylglycerol (Gilon and Henquin, 2001). This effect is due to activation of muscarinic M3 receptors, which, as in other tissues, are believed to stimulate PLC-β via the Gq family of heterotrimeric G proteins. It was recognized early on that phospholipid hydrolysis and inositol phosphate production after cholinergic stimulation was larger in β-cells maintained in Ca^{2+}-containing medium than in Ca^{2+}-deficient medium (Best, 1986; Biden et al., 1987; Garcia et al., 1988). This phenomenon is poorly understood but may be explained by Ca^{2+}-mediated activation of PLC (Biden et al., 1987). Analyses of $[Ca^{2+}]_i$ responses in β-cells have demonstrated that stimulation with the muscarinic-receptor agonist carbachol is associated with a biphasic increase of $[Ca^{2+}]_i$ with a rapid peak followed by a sustained plateau, sometimes with superimposed oscillations (Gylfe, 1991; Liu and Gylfe, 1997). Whereas the first phase reflects

rapid IP_3-mediated mobilization of intracellular Ca^{2+}, the second phase depends on Ca^{2+} influx through store-operated channels in the plasma membrane (Liu and Gylfe, 1997). As all PLC isoforms require Ca^{2+}, it is possible that such receptor-induced Ca^{2+} signals result in feedback activation of the enzyme.

Owing to difficulties in measuring PLC activity in individual cells, little is known about how physiological changes of $[Ca^{2+}]_i$ influence the activity of the lipase. Most studies of PLC have employed radiotracer techniques in populations of cells, but with the advent of phosphoinositide-specific fluorescent biosensors, it has become possible to measure the enzyme activity in individual living cells (Stauffer et al., 1998; Varnai and Balla, 1998). The most commonly used single-cell biosensor for PLC activity is the pleckstrin homology (PH) domain from PLCδ1 fused to the green fluorescent protein ($PH_{PLC\delta}$-GFP), which binds PIP_2 and IP_3 with high affinity and specificity (Stauffer et al., 1998; Varnai and Balla, 1998). In unstimulated cells, the construct is therefore located mainly to the plasma membrane. Upon PIP_2 hydrolysis and formation of IP_3, $PH_{PLC\delta}$-GFP dissociates from the membrane and binds to IP_3 in the cytoplasm. This $PH_{PLC\delta}$-GFP translocation can be used as an indicator of PLC activity. Using an evanescent wave microscopy approach for simultaneous measurements of PLC activity and $[Ca^{2+}]_i$, we recently demonstrated that PLC activity in the electrically excitable insulin-secreting β-cell is tightly controlled by $[Ca^{2+}]_i$ elevations that result from voltage-dependent Ca^{2+} entry (Thore et al., 2004). In the present paper, we test the hypothesis that elevations of $[Ca^{2+}]_i$ following receptor stimulation result in positive feedback activation of PLC. After stimulation of endogenous muscarinic receptors in insulin-secreting cells, two distinct phases of PLC activation were resolved: an initial transient phase that is amplified by mobilization of intracellular Ca^{2+}; and a second sustained phase, which is dependent on Ca^{2+} entry through store-operated channels in the plasma membrane. Moreover, activation of PLC by Ca^{2+} mobilized from intracellular stores was found to occur in primary mouse pancreatic β-cells after stimulation with the insulinotropic hormone glucagon.

Materials and Methods

Materials

Materials of analytical grade and deionized water were used. Thapsigargin and the acetoxymethyl esters of the Ca^{2+} indicator Fura Red and the Ca^{2+} chelator BAPTA were from Molecular Probes (Eugene, OR). HEPES was obtained from Roche Diagnostics (Bromma, Sweden), and Invitrogen (Carlsbad, CA) provided foetal calf serum, DMEM and RPMI 1640 culture media. Cyclopiazonic acid (CPA) and SKF96365 were from Calbiochem (San Diego, CA); 2-aminoethyl diphenylborate (2-APB) was from Aldrich (Gillingham, UK). Diazoxide was a kind gift from Schering-Plough (Kenilworth, NJ). All other chemicals were from Sigma (St Louis, MO). Plasmids encoding the fusion construct between the PH domain of PLCδ1 and GFP (Stauffer et al., 1998), and GFP targeted to the plasma membrane via covalent lipid modification (GFP-CAAX) were kindly provided by Professor Tobias Meyer, Stanford University.

Cell culture and transfection

Insulin-secreting MIN-6 β-cells (passage 16-30) (Miyazaki et al., 1990) were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM containing 25 mM glucose and supplemented with 15% foetal calf serum, 2 mM glutamine, 70 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. After 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 with Lipofectamine 2000 (Invitrogen) in a 1:2.5 DNA:lipid ratio (according to the manufacturer's protocol) and further cultured for 24-48 hours.

Mouse pancreatic β-cells were obtained from collagenase-isolated islets of Langerhans from *ob*/*ob* mice. Free cells were prepared by shaking the islets in a Ca^{2+}-deficient medium (Lernmark, 1974). The cells were then suspended in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 30 μg/ml gentamycin, and allowed to attach to 25 mm coverslips for 1-3 days in culture at 37°C in a humidified atmosphere of 5% CO_2. The *ob*/*ob* mouse islets contain more than 90% β-cells (Hellman, 1965), which respond normally to glucose and to other regulators of insulin release (Hahn et al., 1974). Transfection of the primary mouse β-cells was performed by electroporation of in vitro transcribed mRNA, as outlined below.

In vitro transcription and processing of mRNA

As an alternative to conventional plasmid and viral methods commonly applied to transfect pancreatic β-cells, we used an mRNA transfection technique (Yokoe and Meyer, 1996) to express $PH_{PLC\delta}$ fused to yellow fluorescent protein (YFP) in the *ob*/*ob* mouse β-cells. First, we generated the vector pCS2-$PH_{PLC\delta}$-YFP. The PH domain from $PH_{PLC\delta}$-GFP was PCR amplified using primers 5′-TTTTGGATCCACCATGGGCCTACAGGATGATGAGGA-3′ (forward) and 5′-TTTTTCTAGAGCCTGGATGTTGAGCTCCTTCAG-3′ (reverse) containing *Bam*HI and *Xba*I restriction sites, respectively (underlined). The product was subsequently ligated into the corresponding sites of the transcription vector pCS2-YFP (Tengholm and Meyer, 2002). The plasmid was linearized with *Not*I and subsequent in vitro transcription with SP6 RNA polymerase and poly-A tail addition were performed according to the manufacturers' protocol using commercial kits [mMESSAGE mMACHINE, and Poly(A) Tailing kit, respectively, Ambion Europe]. After purification of the mRNA by column chromatography (RNeasy, Qiagen), the eluent was dried and the mRNA dissolved at 2 μg/μl in phosphate-buffered saline (PBS; pH 7.00).

Electroporation

Electroporation of the adherent mouse pancreatic β-cells was performed using a custom-built small-volume electroporator (Teruel and Meyer, 1997). After replacement of the medium with electroporation buffer (PBS supplemented with 20 mM glucose at pH 7.00), 1-2 μl of the 2 μg/μl mRNA sample was applied to the ~15 μl electroporation chamber. Electroporation was performed at 220 V/cm, using three voltage pulses, each 30 mseconds and 40 seconds apart. After transfection, the electroporation buffer was replaced with RPMI 1640 medium and the cells were kept in culture for 10-20 hours to allow for expression of $PH_{PLC\delta}$-YFP.

Fluorescence microscopy

Before experiments, the cells were transferred to a buffer containing 125 mM NaCl, 4.8 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 3 mM glucose and 25 mM HEPES with pH adjusted to 7.40 with NaOH. Where indicated, transfected cells were loaded with the Ca^{2+} indicator Fura Red by a 40 minute incubation at 37°C with 10 μM of its acethoxymethyl ester. The coverslips were used as

exchangeable bottoms of a 50 μl open chamber and superfused with buffer at a rate of 0.3 ml/minute. All experiments were performed at 37°C.

GFP, YFP and Fura Red fluorescence was measured using an evanescent wave microscopy setup built around an Eclipse TE2000 microscope (Nikon) as previously described (Thore et al., 2004). The 488 nm beam of an argon ion laser (Creative Laser Production, Munich, Germany) was homogenized, expanded and refocused onto the periphery of the back focal plane of a 60× 1.45-NA objective (Nikon) to achieve total internal reflection at the interface between the coverslip and the adherent cells. The fluorescence excited by the evanescent field was detected using an IEEE1394 Orca-ER camera (Hamamatsu) controlled by MetaMorph or MetaFluor software (Universal Imaging). Selection of emission wavelength was made with interference (525/25 nm for GFP; 550/30 for YFP) and long-pass (>630 nm for Fura Red) filters (Chroma Technology) mounted in a Lambda 10-2 filter wheel (Sutter Instruments) capable of changing positions within 60 mseconds. Images (or image pairs) were acquired every 5 seconds, except for in the experiments in Fig. 4, where image pairs were acquired at ~1.5 Hz in the data-streaming mode of MetaFluor. 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) softwares. GFP, YFP and Fura Red fluorescence intensities are expressed as changes relative to initial fluorescence ($\Delta F/F_o$) after subtraction of background. All data are presented as mean values±s.e.m. Statistical significances were evaluated using Student's *t*-test.

Results

Ca^{2+}-dependent, biphasic PLC activation in insulin-secreting cells

When insulin-secreting MIN6 β-cells were stimulated with 100 μM of the muscarinic-receptor agonist carbachol, there was a rapid and pronounced activation of PLC, detected as a decrease in evanescent wave excited $PH_{PLC\delta}$-GFP fluorescence (Fig. 1A). This activation consisted of two phases: an initial rapid peak (16±1% loss of fluorescence reached at 31±2 seconds; *n*=33), followed within 3 minutes by a sustained plateau (6.4±0.8% below baseline, *n*=33) that lasted throughout the stimulation period (more than 10 minutes; Fig. 1B,F). After washout of carbachol, the fluorescence returned to initial intensity, sometimes after temporarily overshooting the baseline. When the cells were stimulated in Ca^{2+}-deficient medium containing 2 mM EGTA, the initial peak response to carbachol was indistinguishable from that in control cells (15.5±1.0% decrease, *n*=10), but there was no sustained plateau and fluorescence gradually returned to basal levels (Fig. 1C,F). Likewise, when extracellular Ca^{2+} was removed during the plateau phase of carbachol stimulation, fluorescence rapidly returned towards baseline. This effect was readily reversible with restoration of the plateau upon re-addition of Ca^{2+} (Fig. 1D). Irrespective of whether Ca^{2+} was present, carbachol produced no effect in control experiments with cells expressing GFP alone in the cytoplasm or targeted to the plasma membrane (data not shown). To verify that the Ca^{2+} requirement for sustained receptor-triggered PLC activity involved influx of the ion, we applied the non-selective Ca^{2+} channel inhibitor La^{3+}. At 0.5 mM, this ion strongly enhanced evanescent wave excited $PH_{PLC\delta}$-GFP fluorescence (45±4% increase of fluorescence, *n*=18; Fig. 1E, inset). A similar effect was observed with GFP when targeted to the plasma membrane (data not shown), indicating that La^{3+} might affect membrane properties and/or cell adhesion. Nevertheless, carbachol still triggered translocation. Although the initial peak translocation was unaffected by La^{3+}, there was no sustained plateau (Fig. 1E,F). Thus, receptor-triggered PLC activity is enhanced by Ca^{2+} influx through the plasma membrane.

PLC activity is maintained by Ca^{2+} influx through store-operated channels

We investigated the nature of the Ca^{2+} influx pathway for sustained PLC activation in response to muscarinic-receptor stimulation. As carbachol-mediated stimulation of β-cells is associated with slight depolarization, which may activate voltage-dependent influx of Ca^{2+} through the plasma membrane (Gilon and Henquin, 2001), we tested the possible involvement of such a mechanism for the sustained PLC activation. The K_{ATP} channel opener diazoxide, which hyperpolarizes the β-cells, did not affect the plateau $PH_{PLC\delta}$-GFP fluorescence (Fig. 2A,E). Likewise, 10 μM of the L-type Ca^{2+} channel inhibitor nifedipine was without effect on $PH_{PLC\delta}$-GFP fluorescence (Fig. 2B,E), although the same concentration suppressed the elevation of $[Ca^{2+}]_i$ induced by depolarization with 90 mM KCl (data not shown), indicating that voltage-gated Ca^{2+} influx is not required for the sustained carbachol-induced activation of PLC.

Receptor-induced mobilization of intracellular Ca^{2+} in β-cells, as in most other cells, is associated with activation of Ca^{2+} entry through a store-operated pathway (Liu and Gylfe, 1997; Dyachok and Gylfe, 2001). To test the involvement of such a mechanism, we applied the relatively specific store-operated Ca^{2+} channel inhibitors 2-APB and SKF96365 at 100 and 50 μM, respectively. Both agents markedly reduced the sustained $PH_{PLC\delta}$-GFP translocation in response to 100 μM carbachol (Fig. 2B,C,E,F), but they had no effect on the fluorescence of GFP in the cytoplasm (data not shown) or targeted to the plasma membrane (a representative experiment with 2-APB is shown in Fig. 2D). When carbachol was applied after 2-APB, there was transient initial $PH_{PLC\delta}$-GFP translocation of the same magnitude as in control cells (18±4% fluorescence change, *n*=7; compare with 20±5% in control, *n*=7), but no sustained plateau (Fig. 2F).

To verify the role of store-operated Ca^{2+} entry for activation of PLC, we compared the influence of extracellular Ca^{2+} on membrane $PH_{PLC\delta}$-GFP fluorescence under control conditions and after activating this Ca^{2+} influx pathway by depletion of the intracellular Ca^{2+} stores. Under control conditions, Ca^{2+} removal with addition of 2 mM EGTA and the following reintroduction of 1.28 and 2.56 mM of the ion resulted in less than 2% changes in $PH_{PLC\delta}$-GFP fluorescence (*n*=9; Fig. 2G). Subsequent activation of the store-operated pathway by addition of 100 μM of the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor CPA was without significant effect on $PH_{PLC\delta1}$-GFP fluorescence in Ca^{2+}-deficient medium. By contrast, there was an immediate translocation of $PH_{PLC\delta}$-GFP upon reintroduction of 1.28 mM Ca^{2+} to the medium (5.4±0.7% loss of fluorescence, *n*=9, *P*<0.01 for difference

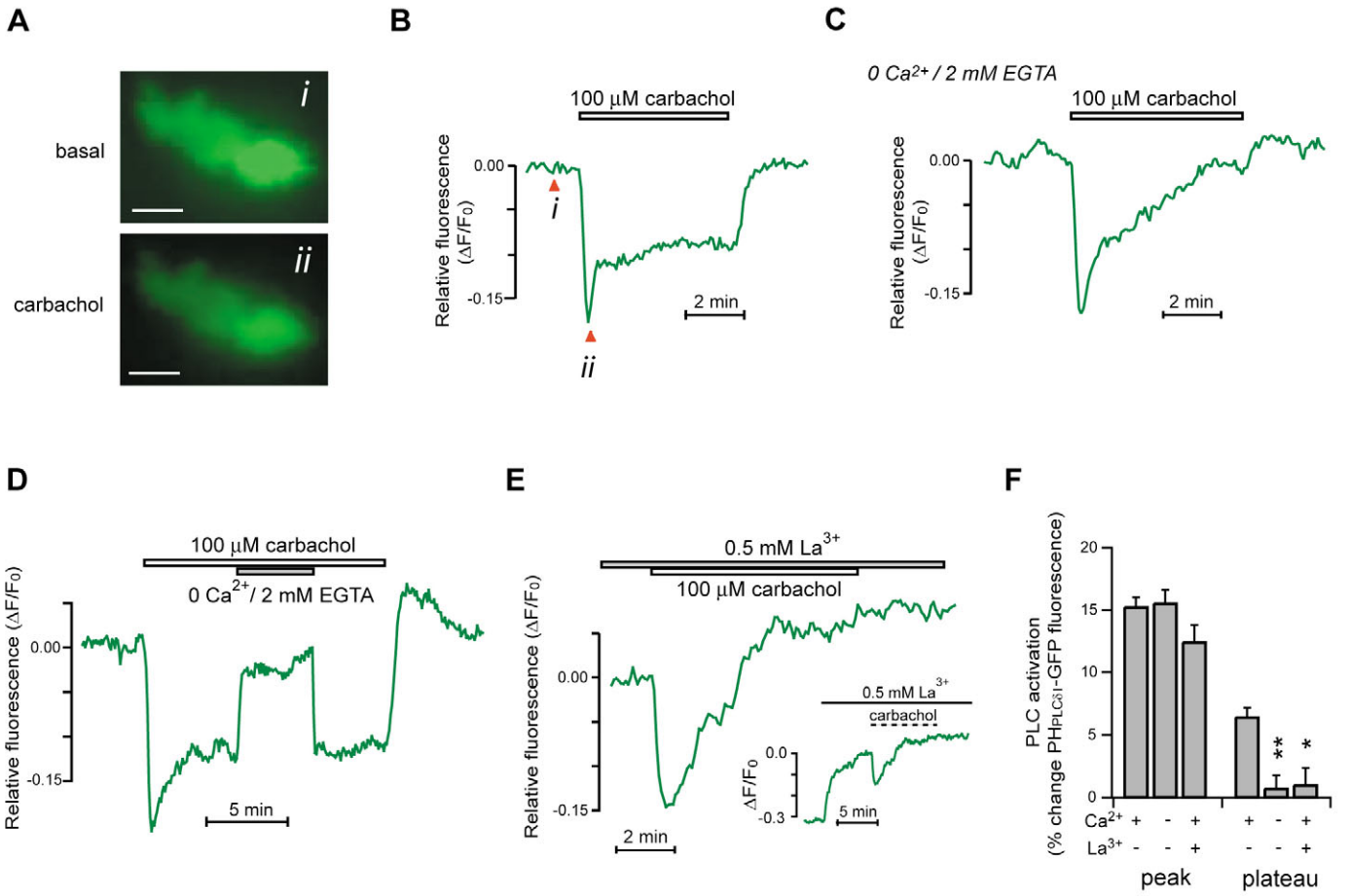

Fig. 1. Ca^{2+}-dependent, biphasic activation of PLC in insulin-secreting cells. (A) MIN6 cells were transiently transfected with $PH_{PLC\delta}$-GFP and visualized 24 hours later with evanescent wave microscopy under basal conditions and after stimulation with 100 μM carbachol at the time points *i* and *ii* indicated in B. Bars 5 μm. (B) Time-course of $PH_{PLC\delta}$-GFP translocation in the cell shown in A after stimulation with 100 μM carbachol. (C) Time-course of $PH_{PLC\delta}$-GFP translocation in response to 100 μM carbachol in Ca^{2+}-deficient medium containing 2 mM EGTA. (D) Effect of Ca^{2+} removal with addition of 2 mM EGTA on the steady-state $PH_{PLC\delta}$-GFP fluorescence during stimulation with 100 μM carbachol. (E) Time-course of $PH_{PLC\delta}$-GFP translocation in response to 100 μM carbachol in the presence of 0.5 mM La^{3+}. The inset shows the entire experiment, including the rise of $PH_{PLC\delta}$-GFP fluorescence that occurs upon addition of La^{3+}. The fluorescence was normalized against the level prior to carbachol stimulation. (F) Mean±s.e.m. for the effects of Ca^{2+} removal and La^{3+} addition on $PH_{PLC\delta}$-GFP fluorescence. The peak amplitude was defined as the maximal change from initial fluorescence and the plateau amplitude was calculated from the off-response upon washout of carbachol. $^{**}P<0.001$; $^{*}P<0.01$.

from Ca^{2+} addition before CPA; Fig. 2G). No further change of fluorescence was observed when raising Ca^{2+} to 2.56 mM. The Ca^{2+} effect was specific, as there was no change in the fluorescence in control cells expressing cytoplasmic or membrane-targeted GFP alone (data not shown). Taken together, the results indicate that sustained PLC activation depends on Ca^{2+} influx through store-operated channels.

PLC activation involves positive feedback from intracellular Ca^{2+} mobilization

In view of the potent effect of Ca^{2+} influx on PLC activity, we investigated the influence of Ca^{2+} mobilized from intracellular stores on receptor-triggered PLC activity. Simultaneous recording of PLC activity with $PH_{PLC\delta}$-GFP and $[Ca^{2+}]_i$ with Fura Red demonstrated that the transient PLC activation induced by carbachol in Ca^{2+}-deficient medium containing EGTA was associated with a rapid and pronounced spike of $[Ca^{2+}]_i$ (Fig. 3A). Increasing the Ca^{2+} buffering capacity of the cytoplasm by loading the cells with 1 mM of the acetoxymethyl ester of the Ca^{2+} chelator BAPTA resulted in altered response patterns for both $[Ca^{2+}]_i$ and $PH_{PLC\delta}$-GFP translocation (Fig. 3B). The $[Ca^{2+}]_i$ response had a lower amplitude and longer duration in the BAPTA-loaded cells (Fig. 3B,E), and similar differences were observed for $PH_{PLC\delta}$-GFP translocation (Fig. 3B,D), indicating that the $[Ca^{2+}]_i$ response is important for PLC activation kinetics. Further support for this idea was obtained from experiments in which intracellular Ca^{2+} stores had been depleted by SERCA inhibition with 100 μM CPA (Fig. 3C-E) or 1 μM thapsigargin (Fig. 3D,E). Accordingly, both agents not only abolished the $[Ca^{2+}]_i$ elevation induced by carbachol, but also strongly suppressed the $PH_{PLC\delta}$-GFP translocation (Fig. 3C-E). These findings indicate that PLC activation in response to muscarinic-receptor stimulation is enhanced by positive feedback from Ca^{2+} that is mobilized from intracellular stores.

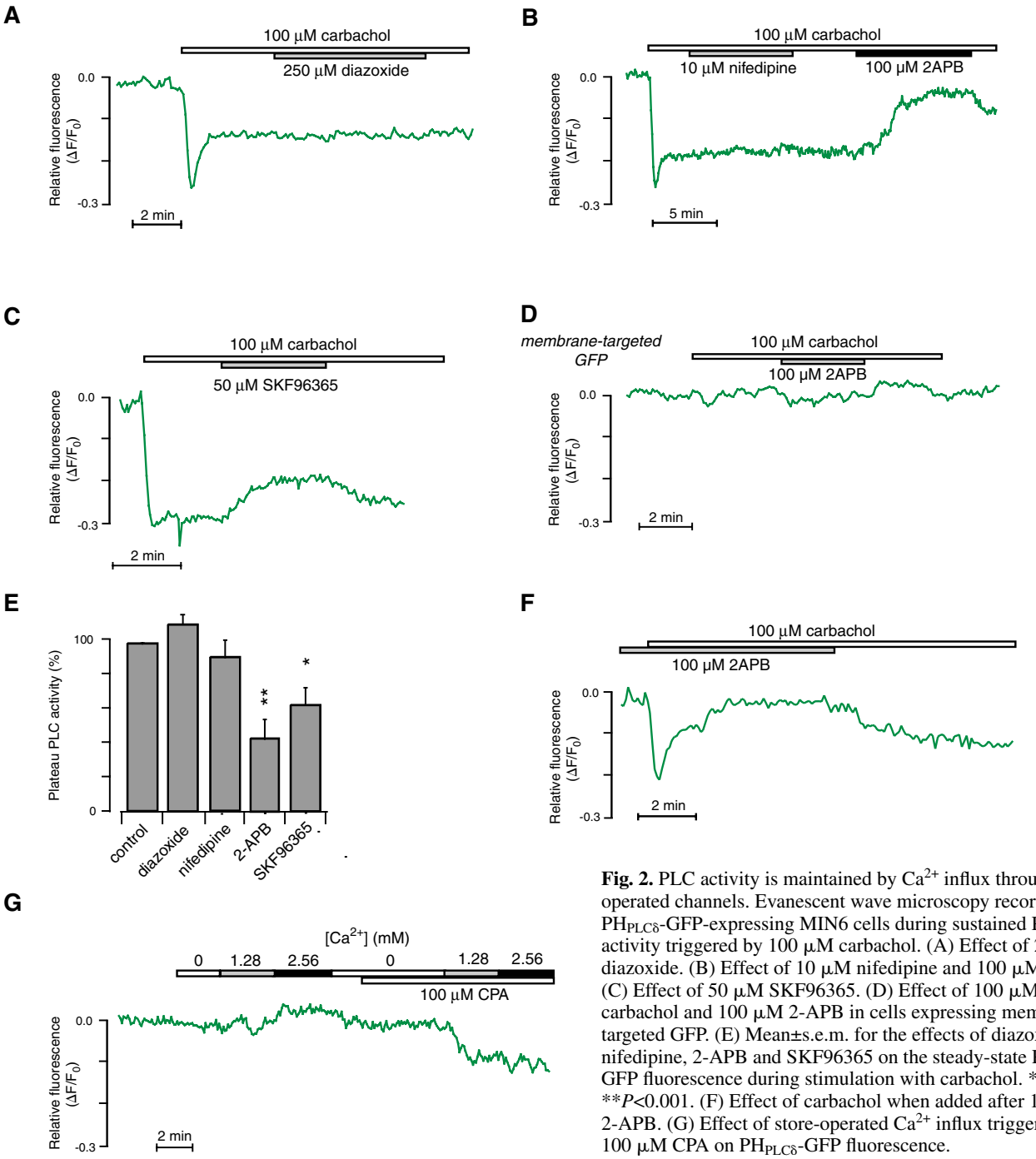

Fig. 2. PLC activity is maintained by Ca^{2+} influx through store-operated channels. Evanescent wave microscopy recording of $PH_{PLC\delta}$-GFP-expressing MIN6 cells during sustained PLC activity triggered by 100 μM carbachol. (A) Effect of 250 μM diazoxide. (B) Effect of 10 μM nifedipine and 100 μM 2-APB. (C) Effect of 50 μM SKF96365. (D) Effect of 100 μM carbachol and 100 μM 2-APB in cells expressing membrane-targeted GFP. (E) Mean±s.e.m. for the effects of diazoxide, nifedipine, 2-APB and SKF96365 on the steady-state $PH_{PLC\delta}$-GFP fluorescence during stimulation with carbachol. *P<0.01; **P<0.001. (F) Effect of carbachol when added after 100 μM 2-APB. (G) Effect of store-operated Ca^{2+} influx triggered by 100 μM CPA on $PH_{PLC\delta}$-GFP fluorescence.

Ca^{2+} mobilization activates PLC in primary mouse pancreatic β-cells

The requirement for intracellular Ca^{2+} mobilization for maximal PLC activation in response to carbachol raises the issue of whether intracellular Ca^{2+} release is sufficient for activation of PLC. This hypothesis was tested in primary mouse pancreatic β-cells, which show a Ca^{2+}-induced Ca^{2+} release mechanism mediated by protein kinase A-dependent sensitization of IP_3 receptors (Liu et al., 1996; Dyachok and Gylfe, 2004). Mouse β-cells were transfected with $PH_{PLC\delta}$-YFP and loaded with the Ca^{2+} indicator Fura Red. When the cells were exposed to medium containing 20 mM glucose and the voltage-gated Ca^{2+} influx was prevented by a combination of 250 μM diazoxide and 50 μM of the L-type Ca^{2+} channel blocker methoxyverapamil, $[Ca^{2+}]_i$ was low and stable, and evanescent wave-excited $PH_{PLC\delta}$-YFP exhibited steady fluorescence. As previously described (Liu et al., 1996; Dyachok and Gylfe, 2004), addition of glucagon resulted in the appearance of pronounced spikes of $[Ca^{2+}]_i$ occurring from the baseline. Simultaneous recording of PLC activity demonstrated that the high $[Ca^{2+}]_i$ spikes were paralleled by transient drops of $PH_{PLC\delta}$-YFP fluorescence (7.8±0.7 % loss of fluorescence, n=12; Fig. 4A). When these events were captured at a high time resolution (>1.5 Hz), it became evident

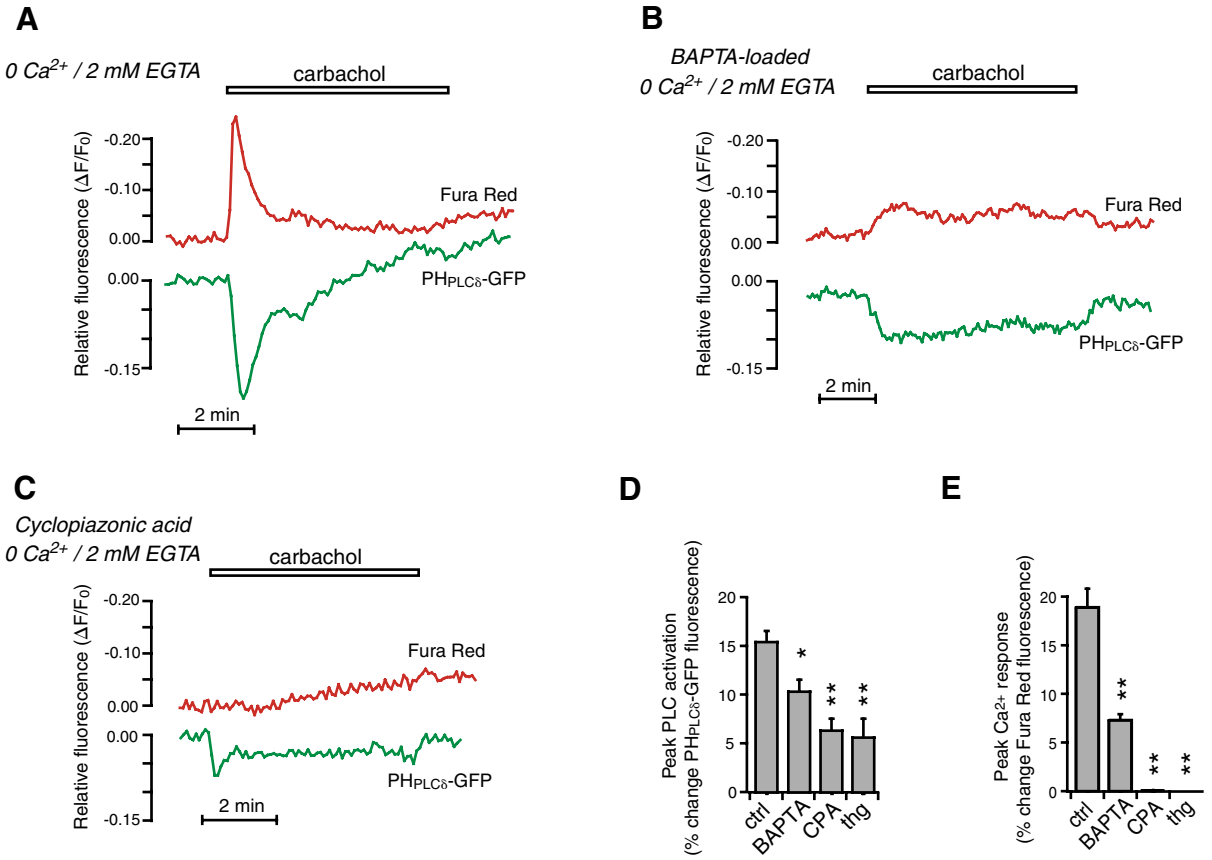

Fig. 3. PLC activation involves positive feedback from intracellular Ca^{2+} mobilization. (A) Simultaneous evanescent wave microscopy fluorescence recording of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) with Fura Red (red trace) and PLC activity with $PH_{PLC\delta}$-GFP (green trace) in a MIN6 cell stimulated with 100 μM carbachol in Ca^{2+}-deficient medium (0 Ca^{2+}/2 mM EGTA). The Fura Red trace has been inverted to show $[Ca^{2+}]_i$ increases as upward deflections. (B) Carbachol-induced $PH_{PLC\delta}$-GFP translocation and $[Ca^{2+}]_i$ response in a cell preincubated for 40 minutes with 1 mM BAPTA acetoxymethyl ester and maintained in Ca^{2+}-deficient medium from 5 minutes prior to stimulation. (C) $PH_{PLC\delta}$-GFP translocation and $[Ca^{2+}]_i$ response in a cell maintained in Ca^{2+}-deficient medium containing 100 μM cyclopiazonic acid. (D,E) Mean±s.e.m. for peak $PH_{PLC\delta}$-GFP translocation and $[Ca^{2+}]_i$ responses. *P<0.01; **P<0.001.

that the $[Ca^{2+}]_i$ elevations preceded each increase of PLC activity by 0.85±0.08 seconds (difference in time to half-maximal change of fluorescence; n=12; Fig. 4B). These results indicate that intracellular Ca^{2+} mobilization can trigger PLC activation and that this mechanism operates in primary pancreatic β-cells.

Discussion

In the present study, we used a novel evanescent wave microscopy approach to investigate the involvement of Ca^{2+} in regulating receptor-triggered PLC activation. Dissociation of $PH_{PLC\delta}$-GFP from the plasma membrane has become a well-established single-cell assay for PIP_2 hydrolysis and IP_3 formation (Stauffer et al., 1998; Varnai and Balla, 1998; Hirose et al., 1999), now used as a measure of PLC activity in insulin-secreting β-cells. Detection of plasma membrane-associated fluorescence with evanescent wave microscopy, rather than with confocal or conventional epifluorescence microscopy, has several advantages, including lower background and less photobleaching and phototoxicity (Steyer and Almers, 2001). Using cells loaded with a fluorescent Ca^{2+} indicator allows simultaneous measurements of $[Ca^{2+}]_i$ and PLC activation dynamics. Using this approach, we recently showed that both membrane depolarization and the concomitant voltage-dependent influx of Ca^{2+} are sufficient to trigger PLC activation in insulin-secreting cells (Thore et al., 2004).

We now extend these findings by showing that also PLC activity triggered by a muscarinic-receptor agonist is tightly regulated by Ca^{2+}. Our data indicate that Ca^{2+} exerts pronounced amplification of both the initial and sustained receptor-triggered PLC activity. Whereas the initial activation of the enzyme in response to carbachol was unaffected by omission of extracellular Ca^{2+}, it was markedly suppressed after depletion of Ca^{2+} from the ER. The latter effect is probably explained by the failure of carbachol to mobilize Ca^{2+} from intracellular stores. A similar effect was thus observed when the $[Ca^{2+}]_i$ elevation in response to carbachol was prevented by increasing the cytoplasmic Ca^{2+} buffering capacity with BAPTA. Depletion of ER Ca^{2+} has previously been found to suppress α1B-adrenoceptor-mediated oscillations of IP_3 in CHO cells, indicating a role for Ca^{2+} feedback on PLC for periodic generation of IP_3 (Young et al., 2003). Direct support for the idea that Ca^{2+} mobilization from

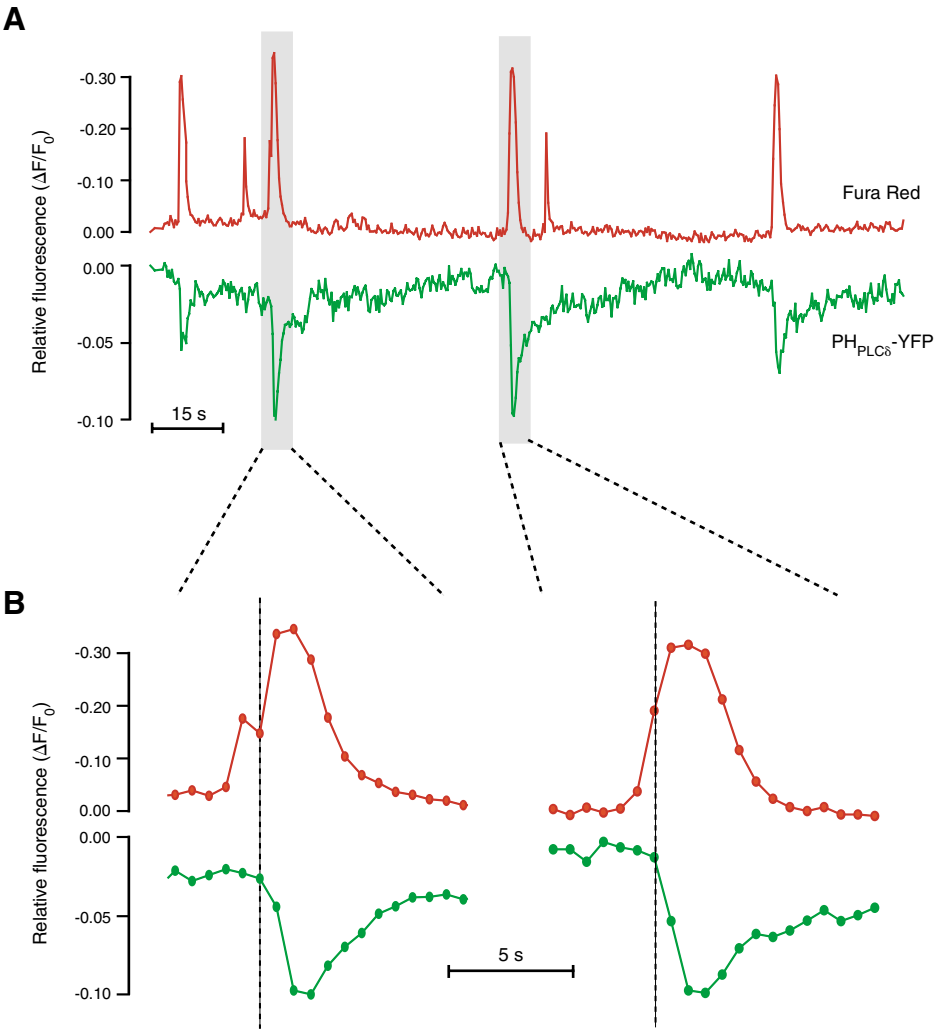

Fig. 4. Ca^{2+} mobilization activates PLC in primary mouse pancreatic β-cells. (A) Mouse pancreatic β-cells transiently transfected with $PH_{PLC\delta}$-YFP and loaded with Fura Red were transferred to medium containing 20 mM glucose, 250 μM diazoxide, 50 μM verapamil and 100 nM glucagon. PLC activity ($PH_{PLC\delta}$-YFP fluorescence, green trace) and $[Ca^{2+}]_i$ response (red trace) were recorded simultaneously with an image pair every 0.7 seconds. The Fura Red trace has been inverted to show $[Ca^{2+}]_i$ increases as upward deflections. (B) Time expansions of the shaded regions in A showing that the $[Ca^{2+}]_i$ responses precede PLC activation. The recording is representative for six cells from five independent experiments.

intracellular stores enhances PLC activity was now provided by the finding in primary mouse pancreatic β-cells that cAMP-sensitized intracellular Ca^{2+} mobilization is rapidly followed by activation of PLC. It is important to note that the glucagon receptor does not activate PLC in β-cells (S.T. and A.T., unpublished) and that the pronounced transients of $[Ca^{2+}]_i$ seen in this cell type after stimulation with cAMP-elevating agents is due to Ca^{2+}-induced Ca^{2+} release via PKA-mediated sensitization of IP_3 receptors (Liu et al., 1996; Dyachok and Gylfe, 2004).

Whereas the initial PLC activation was amplified by intracellular Ca^{2+} mobilization, the second sustained phase of PLC activity after receptor stimulation crucially depended on Ca^{2+} influx from the extracellular medium. Although carbachol under some conditions may depolarize the β-cell sufficiently to reach the activation threshold for voltage-dependent Ca^{2+} entry (Gilon and Henquin, 2001), the involvement of such a mechanism is not required, as neither hyperpolarization with diazoxide nor direct inhibition of the Ca^{2+} channels with nifedipine had any effect on the sustained PLC activity. Instead, the PLC activity was suppressed by La^{3+} and commonly used inhibitors of store-operated Ca^{2+} channels.

None of the currently available inhibitors of the store-operated Ca^{2+} influx pathway is entirely specific. Although 2-APB was originally described as a membrane-permeable IP_3-receptor inhibitor (Maruyama et al., 1997), later studies have shown that this effect is weak and that, instead, 2-APB is a reliable inhibitor of store-operated Ca^{2+} entry in various types of cells, including pancreatic β-cells (Bootman et al., 2002; Dyachok and Gylfe, 2001). If the currently observed effect of 2-APB on $PH_{PLC\delta}$-GFP fluorescence were due to inhibition of IP_3 receptors, the early and late phase of the carbachol response should have been affected equally. However, 2-APB only inhibited the late sustained PLC activation upon carbachol-mediated stimulation, consistent with a negligible effect on IP_3 receptors. The inhibition of PLC activity with unrelated blockers of store-operated channels and the stimulation obtained after activation of store-operated Ca^{2+} entry together support the conclusion that this pathway is involved in receptor-triggered PLC activation in insulin-secreting cells. In this context, it is worth noting that an inhibitory effect of 2-APB on acetylcholine-induced IP_3 production in pancreatic acinar cells was attributed to a novel unknown action of this drug (Wu et al., 2004). In view of the present data, this observation may represent suppression of IP_3 production after inhibition of store-operated Ca^{2+} entry.

The maintenance of PLC activity by store-operated Ca^{2+} entry can explain early observations that carbachol-stimulated inositol phosphate production is larger in β-cells maintained in Ca^{2+}-containing than in Ca^{2+}-deficient medium (Best, 1986; Biden et al., 1987; Garcia et al., 1988). Regulation of receptor-triggered PLC activity by Ca^{2+} entry has been described also in other types of cells. Using CHO cells expressing heterologous G-protein-coupled receptors, Nash et al. (Nash et al., 2001; Nash et al., 2002) have reported that Ca^{2+} influx stimulates PLC activity triggered by muscarinic M3 receptors, α1B adrenoceptors and mGlu1a metabotropic glutamate receptors, but inhibits that triggered by mGlu5 receptors. Receptor-activated Ca^{2+} entry has also been reported to promote PLC activity in bradykinin-stimulated PC12 cells (Kim et al., 1999), in B-cell receptor-ligated DT40 B lymphocytes (Nishida et al., 2003) and in rabbit gastric smooth muscle cells stimulated with some $G_{i/o}$-coupled receptor agonists (Murthy et al., 2004). These findings suggest that store-operated Ca^{2+} entry in various types of cells, including insulin-secreting β-cells, serves to amplify Ca^{2+} signalling not only by elevating $[Ca^{2+}]_i$ and replenishing intracellular stores, but also by directly stimulating PLC.

Although store-operated Ca^{2+} entry in pancreatic β-cells causes a rather modest elevation of $[Ca^{2+}]_i$ (Liu and Gylfe, 1997; Dyachok and Gylfe, 2001), it was associated with pronounced activation of PLC. Such high Ca^{2+} sensitivity may be explained by increased susceptibility of PLC to regulation by Ca^{2+} after activation by G-proteins (Rana and Hokin, 1990). It is also possible that PLC colocalizes with the sites for Ca^{2+} entry and senses the high Ca^{2+} concentration close to the store-operated channels. Indeed, via a PDZ domain-containing scaffold protein, some PLC isoforms have been found to associate with the mTRP4 channel, which forms store-operated channels in several types of cells (Tang et al., 2000).

It still remains to be clarified which PLC isoforms account for the different phases of receptor activation. All PLC isoforms depend on Ca^{2+} for activity (Rhee, 2001) and PLCδ has been suggested to be most sensitive (Allen et al., 1997). The store-operated Ca^{2+} entry following bradykinin-induced PLCβ activation in PC12 cells (Kim et al., 1999) and that induced by $G_{i/o}$-coupled receptor agonists in smooth muscle cells (Murthy et al., 2004) were thus found to stimulate PLCδ1, whereas B-cell receptor signalling in DT40 lymphocytes is amplified by Ca^{2+}-mediated activation of PLCγ2 (Nishida et al., 2003). When overexpressed in MIN6 β-cells, the PLC-β1 and -δ1 isoforms were reported to be equally sensitive to elevation of $[Ca^{2+}]_i$ (Ishihara et al., 1999), while the overexpressed β1, but not the δ1, isoform was stimulated by $[Ca^{2+}]_i$ elevation in insulin-secreting RINm5F cells (Kelley et al., 2001).

In summary, we demonstrate that activation of PLC by endogenous muscarinic receptors in electrically excitable insulin-secreting β-cells is enhanced by positive feedback from Ca^{2+} entering the cytoplasm from intracellular Ca^{2+} stores and via store-operated channels in the plasma membrane (Fig. 5). Agonist binding to the G-protein-coupled receptor leads to partial activation of PLC and production of sufficient amounts of IP_3 to trigger Ca^{2+} release from the ER. The resulting elevation of $[Ca^{2+}]_i$ leads to marked amplification of PLC activity with further IP_3 production and elevation of $[Ca^{2+}]_i$. The reduction of Ca^{2+} in the ER leads to opening of store-operated channels in the plasma membrane with entry of Ca^{2+}, which also stimulates PLC and serves to maintain enzyme activity during sustained stimulation. Finally, we have shown that $[Ca^{2+}]_i$ spikes occurring as a result of PKA-mediated sensitization of IP_3 receptors, induce transient activation of PLC in primary mouse pancreatic β-cells. Thus, amplification of receptor signalling by feedback activation of PLC is involved in the physiological regulation of insulin secretion by hormones and neurotransmitters.

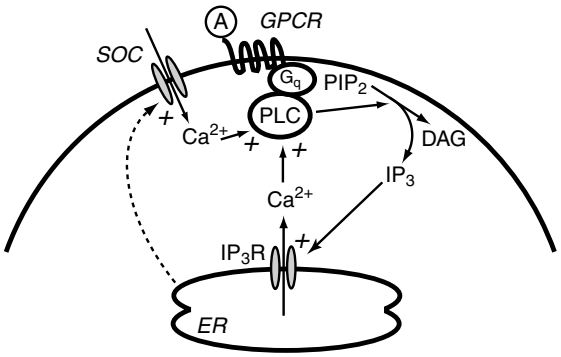

Fig. 5. Model for Ca^{2+} regulation of receptor-triggered PLC activity in insulin-secreting cells. Agonist (A) stimulation of G-protein-coupled receptors (GPCR) leads to partial activation of PLC via the Gq family of heterotrimeric G proteins. The resulting hydrolysis of membrane PIP_2 leads to formation of diacylglycerol (DAG) and sufficient amounts of IP_3 to mobilize Ca^{2+} via IP_3 receptors (IP_3R) in the endoplasmic reticulum (ER). The elevation of $[Ca^{2+}]_i$ further activates PLC to stimulate formation of more IP_3, which leads to further elevation of $[Ca^{2+}]_i$, etc. The drop of Ca^{2+} concentration inside the ER triggers activation of store-operated channels (SOC) in the plasma membrane. This store-operated Ca^{2+} entry acts to increase $[Ca^{2+}]_i$ and to stimulate PLC activity during prolonged receptor stimulation.

The authors are indebted to Professor Tobias Meyer (Stanford University) for generously making an electroporator available and for providing cDNA constructs. Professor Jun-Ichi Miyazaki (Osaka University) is acknowledged for providing MIN6 β-cells and Ms Elina Sandwall for skilful technical assistance. This work was supported by grants from Åke Wiberg's foundation, Carl Trygger's Foundation for Scientific Research, the Family Ernfors Fund, the European Foundation for the Study of Diabetes/Novo Nordisk, the Novo Nordisk Foundation, the Swedish Diabetes Association, the Swedish Research Council (32X-14643, 32BI-15333, 32P-15439 and 12X-6240) and the Wenner-Gren Foundations.

References

Allen, V., Swigart, P., Cheung, R., Cockcroft, S. and Katan, M. (1997). Regulation of inositol lipid-specific phospholipase Cδ by changes in Ca^{2+} ion concentrations. *Biochem. J.* **327**, 545-552.

Berridge, M. J., Bootman, M. D. and Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell. Biol.* **7**, 517-529.

Best, L. (1986). A role for calcium in the breakdown of inositol phospholipids in intact and digitonin-permeabilized pancreatic islets. *Biochem. J.* **238**, 773-779.

Biden, T. J., Peter-Riesch, B., Schlegel, W. and Wollheim, C. B. (1987). Ca^{2+}-mediated generation of inositol 1,4,5-triphosphate and inositol 1,3,4,5-tetrakisphosphate in pancreatic islets. Studies with K^+, glucose, and carbamylcholine. *J. Biol. Chem.* **262**, 3567-3571.

Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge,

M. J. and Peppiatt, C. M. (2002). 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. *FASEB J.* **16**, 1145-1150.

Dyachok, O. and Gylfe, E. (2001). Store-operated influx of Ca^{2+} in pancreatic beta-cells exhibits graded dependence on the filling of the endoplasmic reticulum. *J. Cell Sci.* **114**, 2179-2186.

Dyachok, O. and Gylfe, E. (2004). Ca^{2+}-induced Ca^{2+} release via inositol 1,4,5-trisphosphate receptors is amplified by protein kinase A and triggers exocytosis in pancreatic beta-cells. *J. Biol. Chem.* **279**, 45455-45461.

Garcia, M. C., Hermans, M. P. and Henquin, J. C. (1988). Glucose-, calcium- and concentration-dependence of acetylcholine stimulation of insulin release and ionic fluxes in mouse islets. *Biochem. J.* **254**, 211-218.

Gilon, P. and Henquin, J. C. (2001). Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. *Endocr. Rev.* **22**, 565-604.

Gylfe, E. (1991). Carbachol induces sustained glucose-dependent oscillations of cytoplasmic Ca^{2+} in hyperpolarized pancreatic beta cells. *Pflügers Arch.* **419**, 639-643.

Hahn, H. J., Hellman, B., Lernmark, Å., Sehlin, J. and Täljedal, I. B. (1974). The pancreatic beta-cell recognition of insulin secretogogues. Influence of neuraminidase treatment on the release of insulin and the islet content of insulin, sialic acid, and cyclic adenosine 3′:5′-monophosphate. *J. Biol. Chem.* **249**, 5275-5284.

Hellman, B. (1965). Studies in obese-hyperglycemic mice. *Ann. New York Acad. Sci.* **131**, 541-558.

Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H. and Iino, M. (1999). Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science* **284**, 1527-1530.

Ishihara, H., Wada, T., Kizuki, N., Asano, T., Yazaki, Y., Kikuchi, M. and Oka, Y. (1999). Enhanced phosphoinositide hydrolysis via overexpression of phospholipase C β1 or δ1 inhibits stimulus-induced insulin release in insulinoma MIN6 cells. *Biochem. Biophys. Res. Commun.* **254**, 77-82.

Kelley, G. G., Ondrako, J. M. and Reks, S. E. (2001). Fuel and hormone regulation of phospholipase C β1 and δ1 overexpressed in RINm5F pancreatic beta cells. *Mol. Cell. Endocrinol.* **177**, 107-115.

Kim, Y. H., Park, T. J., Lee, Y. H., Baek, K. J., Suh, P. G., Ryu, S. H. and Kim, K. T. (1999). Phospholipase C-δ1 is activated by capacitative calcium entry that follows phospholipase C-β activation upon bradykinin stimulation. *J. Biol. Chem.* **274**, 26127-26134.

Lernmark, Å. (1974). The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* **10**, 431-438.

Liu, Y. J. and Gylfe, E. (1997). Store-operated Ca^{2+} entry in insulin-releasing pancreatic beta-cells. *Cell Calcium* **22**, 277-286.

Liu, Y. J., Grapengiesser, E., Gylfe, E. and Hellman, B. (1996). Crosstalk between the cAMP and inositol trisphosphate-signalling pathways in pancreatic beta-cells. *Arch. Biochem. Biophys.* **334**, 295-302.

Maruyama, T., Kanaji, T., Nakade, S., Kanno, T. and Mikoshiba, K. (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca^{2+} release. *J. Biochem. (Tokyo)* **122**, 498-505.

Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y. and Yamamura, K. (1990). Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* **127**, 126-132.

Murthy, K. S., Zhou, H., Huang, J. and Pentyala, S. N. (2004). Activation of PLC-δ1 by $G_{i/o}$-coupled receptor agonists. *Am. J. Physiol. Cell Physiol.* **287**, C1679-C1687.

Nash, M. S., Saunders, R., Young, K. W., Challiss, R. A. and Nahorski, S. R. (2001). Reassessment of the Ca^{2+} sensing property of a type I metabotropic glutamate receptor by simultaneous measurement of inositol 1,4,5-trisphosphate and Ca^{2+} in single cells. *J. Biol. Chem.* **276**, 19286-19293.

Nash, M. S., Schell, M. J., Atkinson, P. J., Johnston, N. R., Nahorski, S. R. and Challiss, R. A. (2002). Determinants of metabotropic glutamate receptor-5-mediated Ca^{2+} and inositol 1,4,5-trisphosphate oscillation frequency. Receptor density versus agonist concentration. *J. Biol. Chem.* **277**, 35947-35960.

Nishida, M., Sugimoto, K., Hara, Y., Mori, E., Morii, T., Kurosaki, T. and Mori, Y. (2003). Amplification of receptor signalling by Ca^{2+} entry-mediated translocation and activation of PLCgamma2 in B lymphocytes. *EMBO J.* **22**, 4677-4688.

Rana, R. S. and Hokin, L. E. (1990). Role of phosphoinositides in transmembrane signaling. *Physiol. Rev.* **70**, 115-164.

Rhee, S. G. (2001). Regulation of phosphoinositide-specific phospholipase. *Annu. Rev. Biochem.* **70**, 281-312.

Stauffer, T. P., Ahn, S. and Meyer, T. (1998). Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* **8**, 343-346.

Steyer, J. A. and Almers, W. (2001). A real-time view of life within 100 nm of the plasma membrane. *Nat. Rev. Mol. Cell. Biol.* **2**, 268-275.

Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V. and Zhu, M. X. (2000). Association of mammalian trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. *J. Biol. Chem.* **275**, 37559-37563.

Tengholm, A. and Meyer, T. (2002). A PI3-kinase signaling code for insulin-triggered insertion of glucose transporters into the plasma membrane. *Curr. Biol.* **12**, 1871-1876.

Teruel, M. N. and Meyer, T. (1997). Electroporation-induced formation of individual calcium entry sites in the cell body and processes of adherent cells. *Biophys. J.* **73**, 1785-1796.

Thore, S., Dyachok, O. and Tengholm, A. (2004). Oscillations of phospholipase C activity triggered by depolarization and Ca^{2+} influx in insulin-secreting cells. *J. Biol. Chem.* **279**, 19396-19400.

Varnai, P. and Balla, T. (1998). Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* **143**, 501-510.

Wu, J., Takeo, T., Suga, S., Kanno, T., Osanai, T., Mikoshiba, K. and Wakui, M. (2004). 2-aminoethoxydiphenyl borate inhibits agonist-induced Ca^{2+} signals by blocking inositol trisphosphate formation in acutely dissociated mouse pancreatic acinar cells. *Pflügers Arch.* **448**, 592-595.

Yokoe, H. and Meyer, T. (1996). Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat. Biotechnol.* **14**, 1252-1256.

Young, K. W., Nash, M. S., Challiss, R. A. and Nahorski, S. R. (2003). Role of Ca^{2+} feedback on single cell inositol 1,4,5-trisphosphate oscillations mediated by G-protein-coupled receptors. *J. Biol. Chem.* **278**, 20753-20760.