

# $\beta$ -Cell Calcium-Independent Group VIA Phospholipase $A_2$ (iPLA $_2\beta$ )

## Tracking iPLA $_2\beta$ Movements in Response to Stimulation With Insulin Secretagogues in INS-1 Cells

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Evidence that group VIA cytosolic calcium-independent phospholipase  $A_2$  (iPLA $_2\beta$ ) participates in  $\beta$ -cell signal transduction includes the observations that inhibition of iPLA $_2\beta$  with the bromoenol lactone suicide substrate suppresses glucose-stimulated insulin secretion and that overexpression of iPLA $_2\beta$  amplifies insulin secretory responses in INS-1 insulinoma cells. Immunofluorescence analyses also reveal that iPLA $_2\beta$  accumulates in the perinuclear region of INS-1 cells stimulated with glucose and forskolin. To characterize this phenomenon further, iPLA $_2\beta$  was expressed as a fusion protein with enhanced green fluorescent protein (EGFP) in INS-1 cells so that movements of iPLA $_2\beta$  are reflected by changes in the subcellular distribution of green fluorescence. Stimulation of INS-1 cells overexpressing iPLA $_2\beta$ -EGFP induced greater insulin secretion and punctate accumulation of iPLA $_2\beta$ -EGFP fluorescence in the perinuclear region. To determine the identity of organelles with which iPLA $_2\beta$  might associate, colocalization of green fluorescence with fluorophores associated with specific trackers targeted to different subcellular organelles was examined. Such analyses reveal association of iPLA $_2\beta$ -EGFP fluorescence with the ER and Golgi compartments. Arachidonate-containing plasmenylethanolamine phospholipid species are abundant in  $\beta$ -cell endoplasmic reticulum (ER) and are excellent substrates for iPLA $_2\beta$ . Arachidonic acid produced by iPLA $_2\beta$ -catalyzed hydrolysis of their substrates induces release of  $Ca^{2+}$  from ER stores—an event thought to participate in glucose-stimulated insulin secretion. *Diabetes* 53 (Suppl. 1):S186–S189, 2004

**P**hospholipase  $A_2$  (PLA $_2$ ) is a diverse group of enzymes that catalyze hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (1,2). Among the PLA $_2$ s is an 84-kDa cytosolic PLA $_2$  that does not require  $Ca^{2+}$  for catalysis and is designated group VIA cytosolic calcium-independent phospholipase  $A_2$  (iPLA $_2\beta$ ) (3–5).

A role for iPLA $_2\beta$  in signal transduction in insulin-secreting  $\beta$ -cells is suggested by the observations that inhibition of iPLA $_2\beta$  activity with the bromoenol lactone (BEL) suicide substrate of iPLA $_2\beta$  suppresses insulin secretion, and overexpression of iPLA $_2\beta$  amplifies glucose- and forskolin-stimulated insulin secretion from pancreatic islets and INS-1 insulinoma cells (6,7). Stimulation of iPLA $_2\beta$ -overexpressing INS-1 cells with cAMP-elevating agents also induces translocation of iPLA $_2\beta$  to the perinuclear region (7). This is of interest because glucose promotes both  $\beta$ -cell insulin secretion and proliferation, and glucose-induced INS-1 cell mitogenesis is cAMP dependent (8). To characterize iPLA $_2\beta$  subcellular movements further, we developed INS-1 cell lines that overexpress iPLA $_2\beta$  as a fusion protein with enhanced green fluorescent protein (EGFP) so that green fluorescence associated with EGFP reflects the location of iPLA $_2\beta$ . Simultaneous monitoring of fluorescence associated with various tracking molecules allowed us to identify specific subcellular organelles with which iPLA $_2\beta$  associates when cells are stimulated.

### RESEARCH DESIGN AND METHODS

**Preparation of a construct for expressing iPLA $_2\beta$  as a fusion protein with EGFP and selection of stably transfected clones.** Full-length iPLA $_2\beta$  cDNA was amplified by PCR using the following primer set: sense, 5' AGCTTCGAATTCATGCAGTTCCTTGGACGC-3, and antisense, 5'-TTC GATATCGGGAGATAGCAGCAGCTGG-3'. The amplified full-length iPLA $_2\beta$  from the pMSCV-neo-iPLA $_2\beta$  constructs were then subcloned into the pEGFP-N2 (Clontech, Palo Alto, CA) after the immediate early promoter of cytomegalovirus major and before EGFP coding sequences in the same code-reading frame with EGFP. The EGFP-N2 control vector and the construct encoding iPLA $_2\beta$ -EGFP (FPN2) fusion protein were transfected into INS-1 cells with a Gene PORTER transfection system according to the manufacturer's instructions (Gene Therapy Systems, San Diego, CA). Stably transfected clones were selected using G418 (0.4 mg/ml). Stably transfected cells obtained with EGFP-N2 control vector and FPN2 construct are designed as N2 cells and FPN2 cells, respectively.

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Received for publication 14 March 2003 and accepted in revised form 7 May 2003.

This article is based on a presentation at a symposium. The symposium and the publication of this article were made possible by an unrestricted educational grant from Les Laboratoires Servier.

BEL, bromoenol lactone; EGFP, enhanced green fluorescent protein; FITC, fluorescein isothiocyanate; iPLA $_2\beta$ , group VIA cytosolic calcium-independent phospholipase  $A_2$ ; PLA $_2$ , phospholipase  $A_2$ .

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**Preparation of INS-1 cell subcellular fractions and iPLA<sub>2</sub>β enzymatic activity assay.** INS-1 cells were washed with PBS and then detached by trituration. Subcellular fractions were prepared, and iPLA<sub>2</sub>β activity in aliquots of the fractions (25 μg protein) was assayed and quantitated, as previously described (6).

**Immunoblotting analyses of iPLA<sub>2</sub>β protein.** Western blot analyses were performed with INS-1 subcellular fractions, as previously described (6). The fusion protein was visualized by enhanced chemiluminescence after incubation with primary antibody, anti-green fluorescent protein (GFP) (IgG2α, 0.0002 μg/μl), or anti-iPLA<sub>2</sub>β (0.0015 μg/μl) and then the appropriate secondary antibody.

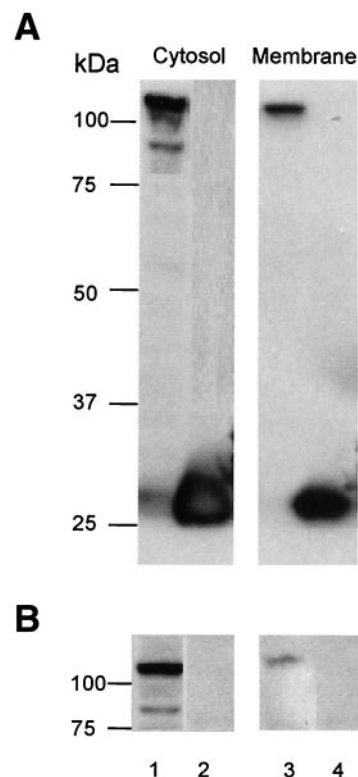
**Insulin secretion.** Cells were seeded in 24-well plates and allowed to proliferate to confluency. Insulin secretion in response to glucose (0–10 mmol/l) without or with forskolin (2.5 μmol/l) was determined, as described previously (9).

**Subcellular organelle tracking.** To identify subcellular organelles with which iPLA<sub>2</sub>β protein might be associated, EGFP fluorescence (fluorescein isothiocyanate [FITC], green) and fluorescence associated with various organelle markers were monitored in the FPN2-overexpressing cells, as described below. Golgi Tracker (BODIPY TR ceramide) was prepared according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Cells were first incubated (4°C) with 5 μmol/l ceramide-BSA for 30 min and then washed and incubated (37°C, 1 h) in culture medium containing no glucose and 0.1% BSA. The cells were then incubated (37°C) in medium supplemented with 2 mmol/l glucose and 2.5 μmol/l forskolin for up to 1 h. After various intervals, fluorescent signal from EGFP (FITC) and Golgi Tracker (rhodamine) were monitored.

For tracking of ER, mitochondria, or plasma membrane, cells were first washed and then incubated (37°C, 1 h) in medium containing no glucose and 0.1% BSA. That medium was then replaced with medium containing 2 mmol/l glucose and 2.5 μmol/l forskolin for up to 1 h. The ER tracker (Blue-White DPX, 600 nmol/l) or mitochondrial tracker (Deep Red 633, 100 nmol/l) was added during the final 30 min of incubation. The plasma membrane tracker (DiI D-282, 0.50 μmol/l) was added during the final 20 min of incubation. After various intervals, signals from these fluorophores were monitored.

## RESULTS

**Immunoblotting analyses of expression of the EGFP fusion protein in INS-1 cells.** To demonstrate that transfection of INS-1 cells with the vector containing the iPLA<sub>2</sub>β-EGFP construct causes overexpression of the fusion protein, cytosolic and membrane fractions were prepared from transfected INS-1 cells. Proteins in these fractions were analyzed by SDS-PAGE and transferred onto polyvinylidene difluoride membranes that were then used in immunoblotting analyses with antibodies directed against EGFP (Fig. 1A) or iPLA<sub>2</sub>β (Fig. 1B). Subcellular fractions prepared from cells transfected with the EGFP vector (N2) alone express only the EGFP-immunoreactive bands of 26 kDa (Fig. 1A, lanes 2 and 4). Transfection with the iPLA<sub>2</sub>β-EGFP fusion protein vector (FPN2) causes expression in both the cytosol and membrane fractions of an EGFP-immunoreactive protein with a molecular weight of 110–115 kDa (Fig. 1A, lanes 1 and 3) that represents full-length iPLA<sub>2</sub>β as a fusion protein. A second EGFP-immunoreactive band with a molecular weight of 90–95 kDa is also observed in cytosol of FPN2 transfected cells (Fig. 1A, lane 1). This molecular weight corresponds to an iPLA<sub>2</sub>β product of ~65–70 kDa plus the 26-kDa EGFP. Immunoblotting analyses with iPLA<sub>2</sub>β antibody (Fig. 1B) yield results similar to those observed with the GFP antibody. An iPLA<sub>2</sub>β-immunoreactive protein corresponding to an iPLA<sub>2</sub>β-EGFP fusion protein is not observed in cells transfected with vector containing only the N2 construct (Fig. 1B, lanes 2 and 4), but such a 110- to 115-kDa protein is observed in cells transfected with the vector containing the iPLA<sub>2</sub>β-EGFP construct (Fig. 1B, lanes 1 and 3).

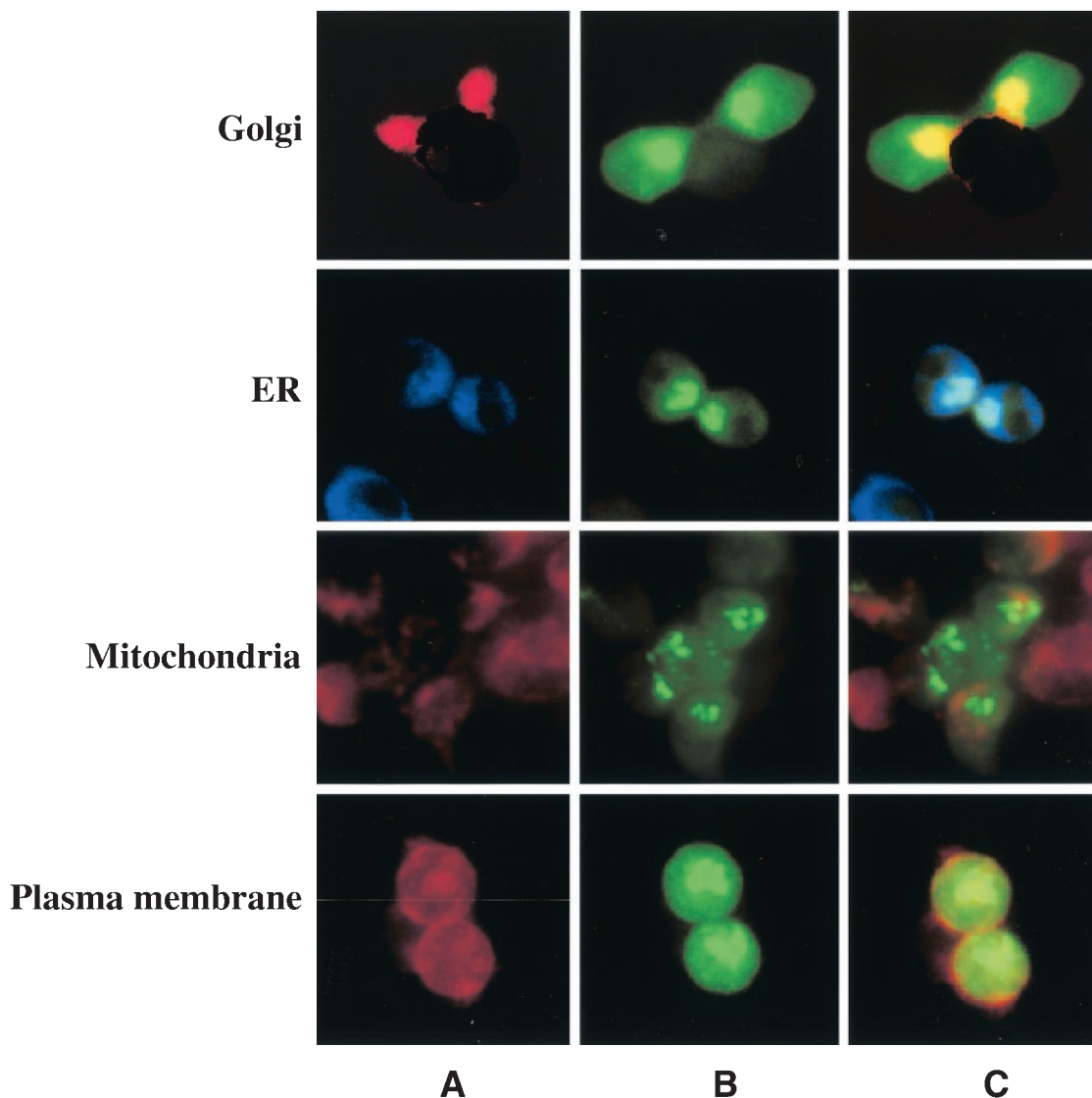


**FIG. 1.** Immunoblotting analyses of expression of the EGFP-iPLA<sub>2</sub>β fusion protein in INS-1 cells. Cytosol and membrane protein fractions were prepared, analyzed by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were probed with antibodies directed against GFP (A) or iPLA<sub>2</sub>β (B), and the immunoreactive protein bands were visualized by enhanced chemiluminescence. Lanes 1 and 3: FPN2 cells; lanes 2 and 4: N2 cells.

**iPLA<sub>2</sub>β enzymatic activity in INS-1 cells overexpressing iPLA<sub>2</sub>β as a fusion protein.** To demonstrate that the FPN2-transfected cells express iPLA<sub>2</sub>β enzymatic activity, cytosolic and membrane-associated iPLA<sub>2</sub>β activities were assayed in the absence and presence of ATP or BEL. FPN2-transfected cells were found to express calcium-independent PLA<sub>2</sub> activity in cytosol and membranes nearly four- to fivefold higher than that expressed in N2 cells (data not shown). The calcium-independent PLA<sub>2</sub> activity in FPN2 cells is stimulated by ATP and inhibited by the BEL suicide substrate, as is the case for native iPLA<sub>2</sub>β (3). These findings and those in Fig. 1 demonstrate that transfection of INS-1 cells with the iPLA<sub>2</sub>β-EGFP fusion protein vector causes overexpression of an enzymatically active iPLA<sub>2</sub>β protein.

**Amplification of glucose-induced insulin secretion by forskolin.** INS-1 cells stably overexpressing iPLA<sub>2</sub>β exhibit greater insulin secretory responses to cAMP-elevating agents in the presence of glucose (3,6). Similarly, when INS-1 cells that overexpress iPLA<sub>2</sub>β as a fusion protein with EGFP are stimulated with the adenylate cyclase activator forskolin, insulin secretory responses are about threefold higher than those of control cells (data not shown).

**Secretagogue-stimulated subcellular redistribution of iPLA<sub>2</sub>β.** Movements of the iPLA<sub>2</sub>β-EGFP were monitored in INS-1 cells transfected with FPN2 vectors after secretagogue stimulation for 0.5–2 h. Under basal conditions, the EGFP fluorescence is dispersed. After addition



**FIG. 2.** Secretagogue-stimulated subcellular redistribution of iPLA<sub>2</sub>β-EGFP (FPN2). The iPLA<sub>2</sub>β-EGFP overexpressing cells (FPN2) were incubated with glucose (2 mmol/l) plus forskolin (2.5 μmol/l) with organelle trackers for Golgi (5 μmol/l), ER (600 nmol/l), mitochondria (100 nmol/l), or plasma membrane (0.50 μmol/l). One hour after stimulation, the EGFP (FITC)-green fluorescent signal was monitored separately from rhodamine-red (Golgi and mitochondrial) and DAPI (4',6-diamidino-2-phenylindole dihydrochloride)-blue (ER) tracker fluorescent signals. *A*: Organelle tracker fluorescence alone. *B*: EGFP fluorescence alone. *C*: Overlay of EGFP and organelle marker fluorescent signals.

of glucose and forskolin, punctuate accumulations of green fluorescence appear as a halo around the perinuclear region in the FPN2 cells illustrated in the companion article (10). These findings are analogous to our previous observations where antibodies directed against iPLA<sub>2</sub>β were used to visualize localization of iPLA<sub>2</sub>β (7).

**Secretagogue-stimulated redistribution of iPLA<sub>2</sub>β to specific organelles.** To determine whether iPLA<sub>2</sub>β associates with specific organelles in the cell after stimulation, dual fluorescence analyses using organelle trackers were performed. The targeted organelles were the Golgi apparatus, ER, mitochondria, and plasma membrane. FPN2 cells were treated with glucose and forskolin and then loaded with various organelle-specific trackers. The EGFP (green) and organelle tracker fluorescence signals were then recorded in a field of cells. The individual tracker and EGFP fluorescence signals recorded in FPN2 cells are shown in Fig. 2*A* and *B*, respectively, and the overlay of the two recordings is shown in Fig. 2*C*. As illustrated, green

fluorescence associated with the iPLA<sub>2</sub>β-EGFP protein appears yellow after overlay with the Golgi-rhodamine fluorescence and as blue-white after overlay with the ER-DAPI (4',6-diamidino-2-phenylindole dihydrochloride) fluorescence. These color changes occur when the two fluorophores colocalize, but not otherwise. There is no apparent overlap of the iPLA<sub>2</sub>β-EGFP green fluorescence with the red fluorescence associated with either the mitochondrial or plasma membrane tracker, and the two fluorescent signals remain separate in the overlay image.

#### DISCUSSION

Many potential biological functions have been proposed for the cytosolic calcium-independent iPLA<sub>2</sub>β enzyme (3). Among the proposed functions are its participation in phospholipid remodeling in P388D1 macrophage-like cells (5,11), cell proliferation (12,13), apoptosis (3), and signal transduction in β-cells and in other cells (12).

In  $\beta$ -cells, inhibition of  $iPLA_2\beta$  suppresses glucose-induced insulin secretion, and overexpression of the enzyme enhances secretion, but neither inhibition nor overexpression of  $iPLA_2\beta$  affects incorporation of fatty acids into  $\beta$ -cell phospholipids (12). These findings indicate that  $iPLA_2\beta$  has a signaling role in insulin secretion but does not serve a housekeeping role in phospholipid remodeling in  $\beta$ -cells.

Immunofluorescence analyses using polyclonal antibodies directed against  $iPLA_2\beta$  also indicate that stimulation of  $\beta$ -cells with insulin secretagogues cause redistribution of  $iPLA_2\beta$  within the  $\beta$ -cell. There is accumulation of an  $iPLA_2\beta$  immunofluorescence signal in the perinuclear region of INS-1 insulinoma cells that are stimulated with glucose and the cAMP-elevating agent forskolin (7). To examine this phenomenon further by an approach not confounded by the potential cross-reactivity of  $iPLA_2\beta$  antibodies,  $iPLA_2\beta$  was overexpressed in INS-1 cells as a fusion protein with EGFP in the studies described here. The location of the fusion protein is reflected by green fluorescence associated with EGFP and facilitates tracking of the subcellular location of  $iPLA_2\beta$ .

In INS-1 cells that overexpress  $iPLA_2\beta$ -EGFP, stimulation with glucose alone or in combination with forskolin induces the appearance of punctate accumulations of green fluorescence as a halo around the perinuclear region. Studies to identify subcellular organelles with which  $iPLA_2\beta$  associates were performed with fluorescent trackers targeted to specific organelles, and these experiments reveal colocalization of  $iPLA_2\beta$ -EGFP-associated green fluorescence with ER and Golgi markers.

Because membranes of the nucleus and ER are contiguous (14,15), perinuclear accumulation of  $iPLA_2\beta$  is consistent with association with a subcellular compartment that is likely to include ER (15). Arachidonate-containing plasmenylethanolamine phospholipid molecular species are abundant in  $\beta$ -cell ER and are excellent substrates for  $iPLA_2\beta$  (16). Arachidonic acid and lysophospholipids produced by  $iPLA_2\beta$ -catalyzed hydrolysis of these substrates induce  $Ca^{2+}$  release from  $\beta$ -cell ER, which is thought to participate in induction of insulin secretion (16,17). Arachidonic acid hydrolyzed from plasmenylethanolamine molecular species (18) and/or arachidonate metabolites have been proposed to participate in the  $\beta$ -cell secretory process (17,18), and stimulated association of  $iPLA_2\beta$  with a perinuclear membrane would cause release of arachidonic acid in close proximity to enzymes that catalyze eicosanoid generation and are located in the nuclear envelope and ER (19,20).

#### ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (R37-DK34388, P41-RR00954, P01-HL57278, P60-DK20579, and P30-DK56341) and by an Award (to S.R.) from the American Diabetes Association.

The authors thank Alan Bohrer and Dr. Mary Wohltmann for expert technical assistance.

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