Stimulation of insulin secretion and associated nuclear accumulation of iPLA$_2$$\beta$ in INS-1 insulinoma cells

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Ma, Zhongmin, Sheng Zhang, John Turk, and Sasanka Ramanadham. Stimulation of insulin secretion and associated nuclear accumulation of iPLA$_2$$\beta$ in INS-1 insulinoma cells. Am J Physiol Endocrinol Metab 282: E820–E833, 2002. First published November 27, 2001; 10.1152/ajpendo.00165.2001.—Accumulating evidence suggests that the cytosolic calcium-independent phospholipase A$_2$ (iPLA$_2$$\beta$) manifests a signaling role in insulin-secreting (INS-1) $\beta$-cells. Earlier, we reported that insulin-secretory responses to cAMP-elevating agents are amplified in iPLA$_2$$\beta$-overexpressing INS-1 cells (Ma Z, Ramanadham S, Bohrer A, Wohltmann M, Zhang S, and Turk J. J Biol Chem 276: 13198–13208, 2001). Here, immunofluorescence, immunoaffinity, and enzymatic activity analyses are used to examine distribution of iPLA$_2$$\beta$ in stimulated INS-1 cells in greater detail. Overexpression of iPLA$_2$$\beta$ in INS-1 cells leads to increased accumulation of iPLA$_2$$\beta$ in the nuclear fraction. Increasing glucose concentrations alone results in modest increases in insulin secretion, relative to parental cells, and in nuclear accumulation of the iPLA$_2$$\beta$ protein. In contrast, cAMP-elevating agents induce robust increases in insulin secretion and in time-dependent nuclear accumulation of iPLA$_2$$\beta$ fluorescence, which is reflected by increases in nuclear iPLA$_2$$\beta$ protein content and specific enzymatic activity. The stimulated effects are significantly attenuated in the presence of cell-permeable inhibitors of protein phosphorylation and glycosylation. These findings suggest that conditions that amplify insulin secretion promote translocation of $\beta$-cell iPLA$_2$$\beta$ to the nuclei, where it may serve a crucial signaling role.

immunofluorescence; immunoaffinity; enzymatic activity; insulin secretion; nuclear localization

PHOSPHOLIPASES A$_2$ (PLA$_2$) are a diverse group of enzymes that catalyze the hydrolysis of the sn-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (9, 12). Among the PLA$_2$s is an 85-kDa cytosolic PLA$_2$ that does not require Ca$^{2+}$ for catalysis. This PLA$_2$ has now been cloned from several sources (3, 23, 44), including rat and human pancreatic islet $\beta$-cells (23, 25), and has been proposed to be designated as the $\beta$-isoform (26, 43) of Group VIA calcium-independent PLA$_2$, or iPLA$_2$$\beta$ (22, 26). Several potential functions for iPLA$_2$$\beta$ have been proposed, including a housekeeping role (4) in phospholipid remodeling and a signaling role in secretion (22, 23, 29).

Because glucose-stimulated insulin secretion is associated with increased hydrolysis of the sn-2 substituent arachidonate from $\beta$-cell membrane phospholipids and its accumulation within the $\beta$-cells (45, 48), we initially investigated the possible participation of iPLA$_2$$\beta$ in the insulin-secretory pathway. Those studies (34, 37), performed in insulinoma cells and native pancreatic islets, revealed that inhibition of iPLA$_2$$\beta$ by the bromoenol lactone (BEL) suicide inhibitor of iPLA$_2$$\beta$ suppresses both glucose-stimulated arachidonate stimulation and insulin secretion. These findings raise the possibility that iPLA$_2$$\beta$ serves a signaling role in $\beta$-cells.

Subsequent findings in studies with murine P388D1 macrophage-like cells have led to the proposal that the iPLA$_2$$\beta$ enzyme serves a housekeeping role in phospholipid remodeling that involves generation of lysophospholipid acceptors for incorporation of arachidonic acid into phospholipids (4). However, detailed examination of this process by our group (35) indicated that inhibition of the iPLA$_2$$\beta$ enzyme does not influence incorporation of arachidonate or phospholipid remodeling in pancreatic islets or insulinoma cells but does inhibit secretagogue-stimulated insulin secretion. The potential signaling role of iPLA$_2$$\beta$ was further addressed in a recently reported study (22), in which the consequence of overexpressing iPLA$_2$$\beta$ on insulin secretion from INS-1 insulinoma cells and of phospholipid remodeling in these cells was examined. INS-1 cells after prolonged passaging become less responsive to glucose-stimulated insulin secretion (36). Late passage cells were transfected with iPLA$_2$$\beta$ cDNA, and the iPLA$_2$$\beta$-overexpressing INS-1 cells were subsequently incubated with glucose, either alone or in combination with cAMP-elevating agents. In contrast to the parental INS-1 cells, which were unresponsive to glucose and only mildly responsive to cAMP-elevating agents, a mild improvement in glucose responsiveness but robust responses to IBMX and forskolin were evident in...
the iPLA₂β-overexpressing INS-1 cells (22). Improvements in the insulin-secretory responses, however, were not associated with alterations in arachidonic acid incorporation into phospholipids of the transfected cells. Collectively, these findings reaffirm a signaling role for iPLA₂β in insulin-secreting β-cells.

An intriguing finding in the study just described (22) was that stimulation of iPLA₂β-overexpressing INS-1 cells with cAMP-elevating agents was associated with increased accumulation of iPLA₂β in the nuclear region (22). In the present study, this phenomenon is further examined by use of immunocytofluorescence, immunoaffinity, and enzymatic activity analyses, and contributions of possible posttranslational modifications of the enzyme are addressed.

**METHODS**

*Materials.* INS-1 cells were generously provided by Dr. C. Newgard (University of Texas Southwestern Center for Diabetes Research, Dallas, TX). Other materials were obtained from the following sources: [γ-32P]ATP, (16:0/[14C]18:2)-glycerophosphocholine [1-palmitoyl-2-[14C]linoleoyl-3-2-phosphocholine (PLPC), 55 μCi/μmol] rainbow molecular mass standards, and enhanced chemiluminescence (ECL) reagent from Amersham (Arlington Heights, IL); H-89 and genistein, globulin-free bovine albumin, IBMX, NP-40, protein kinase A inhibitor (PKAI) from UBI (Lake Placid, NY); and Oct-1 (C-21) antibody from Zymed Laboratories (San Francisco, CA).

iPLA₂β immunoaffinity analyses of INS-1 cell iPLA₂β protein. iPLA₂β-overexpressing INS-1 cells, grown to confluence in T75 flasks, were washed with phosphate-buffered saline (PBS) and detached by incubation (3 min, 37°C) with 0.05% trypsin-0.02% EDTA. The cells were transferred to Tris-buffered saline (TBS) and pelleted by centrifugation (1,500 g for 5 min). Nuclear extracts were prepared, as described (42), by suspending the cells in 400 μl of cold buffer A (10 mM HEPE, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 10 μl PIC/10⁶ cells) by gentle trituration. The cells were allowed to swell on ice for 15 min, and then 10 μl of freshly prepared 1% NP-40 (final concentration 0.025%) were added. After vigorous vortexing for 10 s, the cell homogenate (CH) was centrifuged (16,000 g, 30 min, 4°C). The supernatant, containing cytoplasm and RNA, was saved and designated nonnuclear (NN) material. The nuclear pellet was resuspended in 50 μl of ice-cold buffer B (20 mM HEPE, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and PIC) and rocked vigorously (15 min, 4°C). The nuclear homogenate (NH) was centrifuged (16,000 g, 5 min, 4°C), the nuclear extract (NE) supernatant was saved, and the particulate (P) material was resuspended in buffer B (25 μl). Protein content in each fraction was determined by Bio-Rad assay against the BSA standard.

*Preparation of INS-1 cell nuclear fraction.* iPLA₂β-overexpressing INS-1 cells, grown to confluence in T75 flasks, were washed with phosphate-buffered saline (PBS) and detached by incubation (3 min, 37°C) with 0.05% trypsin-0.02% EDTA. The cells were transferred to Tris-buffered saline (TBS) and pelleted by centrifugation (1,500 g for 5 min). Nuclear extracts were prepared, as described (42), by suspending the cells in 400 μl of cold buffer A (10 mM HEPE, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 10 μl PIC/10⁶ cells) by gentle trituration. The cells were allowed to swell on ice for 15 min, and then 10 μl of freshly prepared 1% NP-40 (final concentration 0.025%) were added. After vigorous vortexing for 10 s, the cell homogenate (CH) was centrifuged (16,000 g, 30 min, 4°C). The supernatant, containing cytoplasm and RNA, was saved and designated nonnuclear (NN) material. The nuclear pellet was resuspended in 50 μl of ice-cold buffer B (20 mM HEPE, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and PIC) and rocked vigorously (15 min, 4°C). The nuclear homogenate (NH) was centrifuged (16,000 g, 5 min, 4°C), the nuclear extract (NE) supernatant was saved, and the particulate (P) material was resuspended in buffer B (25 μl). Protein content in each fraction was determined by Bio-Rad assay against the BSA standard.

*Verification of purity of nuclear preparation.* To ensure that the nuclear fraction did not contain mitochondria or plasma membrane fractions that might contribute to measured iPLA₂β activity, cytochrome c oxidase (mitochondrial marker enzyme) and 5’-nucleotidase (plasma membrane marker enzyme) activity assays were performed, as described (33, 42). In addition, aliquots of the cellular fractions were processed for immunoaffinity analyses, as we will describe, for the nuclear marker Oct-1. Incubations with primary (1:1,000) and secondary (1:40,000) antibodies were followed by visualization of immunoreactive bands by ECL.

iPLA₂β enzymatic activity assay. Enzymatic Ca²⁺-independent iPLA₂β activity in aliquots of cellular fractions (30 μg of protein) were assayed by ethanolic injection (5 μl) of the substrate PLPC (5 μCi in assay buffer (40 mM Tris, pH 7.5, 1 mM EGTA, total volume 200 μl). Assay mixtures were incubated (5 min, 37°C, with shaking), and the assay reaction was terminated with butanol (100 μl) addition and vigorous vortexing. The reaction mixture was centrifuged (2,000 g, 5 min), and products in the upper butanol layer were analyzed by silica gel G thin-layer chromatography (TLC) in petroleum ether-ethyl ether-acetic acid (80/20/1). The TLC plate region containing free fatty acid was identified with iodine vapor and scraped, and the released ¹⁴C fatty acid was quantitated by liquid scintillation spectrometry. Specific iPLA₂β activity was calculated from the dpm of released fatty acid and protein content as described (14). To verify that the measured activity reflected that of the iPLA₂β (35), the abilities of ATP (10 mM) to stimulate and of BEL (10 μM), a suicide inhibitor of iPLA₂β, to inhibit activity were examined. To test the effects of BEL on activity, the sample protein was preincubated with BEL (2 min at room temperature (RT)) before addition of substrate.

*Immunocytochemistry analyses of INS-1 cell iPLA₂β protein.* INS-1 cellular fractions were diluted in an equal volume of sample buffer (1 M Tris-HCl, pH 6.8, 69 mM SDS, 10% glycerol, 0.01% bromphenol blue, and 1% BME) and boiled. Aliquots containing 25–50 μg of protein were analyzed by SDS-PAGE (7.5%) and transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membrane. The electroblot was sequentially blocked (3 h at RT) with TBS-Tween 20 (20 mM Tris-HCl, 137 mM NaCl, pH 7.6, and 0.05% Tween-20) containing 5% milk protein, washed (TBS-T, 5 × 5 min),
and then incubated (1 h, RT) with purified polyclonal (p) antibodies (0.0015 μg/μl in TBS-T containing 3% BSA) to iPLA2α generated by multiple antigen core technology (Research Genetics, Huntsville, AL) against peptides in the iPLA2α-deduced amino acid sequences. The iPLA2α peptides coupled to this core for immunizing rabbits were 35-KEVSLADYASSER41 and 489RMKDEVFRGSYP502. The membrane was again washed in TBS-T (5 × 5 min) and incubated (1 h, RT) with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:40,000 in TBS-T containing 3% BSA). The immunoreactive protein bands were visualized by ECL.

Visualization of iPLA2α protein by immunofluorescence staining and confocal microscopy. After removal of media, the attached cells were sequentially washed with PBS (2 × 5 min), fixed in 4% paraformaldehyde (10 min), washed (3 × 5 min), permeabilized in cold methanol (20 min, −20°C), washed (3 × 5 min), and blocked (in PBS containing 1% globulin-free BSA, 0.30% Triton X-100, and 3% normal goat serum). Next, p iPLA2α primary antibodies (0.003 μg/μl) were added, and the cells were incubated in a humidified chamber (O/N, 4°C). The cells were washed free of the primary antibodies (3 × 5 min) and incubated with fluorescent Cy3-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (1:400, 1 h). The cells were then washed (PBS, 3 × 5 min) and covered with a drop of antifade solution, and the slides were mounted with coverslips. Cell iPLA2α immunofluorescence was visualized by confocal microscopy at an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

Visualization of nuclear localization of iPLA2α by dual-labeling fluorescence. To examine whether the iPLA2α protein was accumulating in the nuclear region, after incubation with Cy3, the cells were washed (PBS, 2 × 5 min) and further incubated (12 min, RT) with bisbenzimide (1:20,000), a fluorescent DNA stain that intercalates in A-T regions of DNA. The cells were washed (PBS, 3 × 5 min) and covered with a drop of antifade solution, and the slides were mounted with coverslips. Rhodamine and DAPI filters attached to a Nikon Eclipse TE 300 Inverted Scope were used to visualize Cy3 (iPLA2α, red) and bisbenzimide (nuclei, blue) fluorescences, respectively, separately and in combination.

Effects of glucose, IBMX, and FSK stimulation on insulin secretion and iPLA2α protein localization. At confluence, iPLA2α-overexpressing cells were detached from T75 flasks, and aliquots of cells (2 × 106) were seeded in multichambered glass slides and allowed to attach overnight. The culture medium was then removed, and the cells were washed twice in Krebs-Ringer buffer (KRB), pH 7.3, containing (in mM): 115 NaCl, 24 NaHCO3, 5 KCl, 1 MgCl2, 25 HEPES, 1 glucose, and 0.1% BSA and were incubated in the same medium (1 h, 37°C, under an atmosphere of 95% air-5% CO2). The medium was then removed and replaced with KRB medium containing glucose (0–20 mM) without or with IBMX (100 μM) or FSK (2.5 μM). The cells were further incubated (0–60 min, 37°C, under an atmosphere of 95% air-5% CO2). At the end of the incubation period, the medium was removed for measurement of insulin by radiimmunoassay (65), and the cells were harvested for iPLA2α enzymatic activity assays, as we have described.

In vitro phosphorylation of iPLA2α. Islet iPLA2α was overexpressed in and purified from Sf9 insect cells by use of sequential (DEAE anion-exchange, ATP-affinity, and calmodulin-affinity) column chromatography, as previously described (49). Aliquots of the purified protein were incubated (30 min, 37°C) with buffer (10 mM Tris, 10 mM MgAc, 4 mM EGTA, pH 7.20), cPKA (10 U), and 10 μM [γ32P]ATP (total volume 110 μl), without and with either PKAI (25 μM), the tyrosine phosphorylation inhibitor genistein (100 μM), or staurosporine (100 nM), a nonspecific inhibitor of phosphorylation. After the assay period, the samples were immediately diluted in sample buffer and boiled. Aliquots of the samples were then analyzed by SDS-PAGE (7.5%), and the protein from gels was transferred onto Immobilon-P PVDF membrane. The transferred 32P-labeled proteins were visualized by autoradiography. Subsequently, the same blot was processed for iPLA2α immunofluorimnfinity analyses, as we have described.

In vitro glycosylation of purified iPLA2α. To detect the presence of O-GlcNac modification of the iPLA2α protein transfer of radiolabeled galactose onto the iPLA2α protein in the presence of β-d-galactosyltransferase was examined using reagents supplied in the GalNac kit. Aliquots of purified iPLA2α or standard hen egg albumin were incubated (4°C, 3 h, total volume of 50 μl) with reaction buffer B, consisting of 5 mM MnCl2, UDP [3H]galactose (sp act 5–20 μCi/μmol), and β-d-galactosyltransferase (0.025 U/ml). The reaction was terminated by the addition of sample buffer. Aliquots of the reaction mixtures were analyzed by SDS-PAGE, and the gel was fixed (30% MeOH/7% HAc, 15 min), soaked in Enhancer solution (30 min) and then in 10% glycerol (30 min), dried (80°C, 1 h), and cooled (10 min). Then followed visualization of labeled protein by autoradiography.

Effects of inhibition of PKA and O-glycosylation on insulin secretion, iPLA2α protein localization, and enzymatic activity. To examine whether phosphorylation or glycosylation events contribute to iPLA2α protein translocation in the cell, cellular permeable inhibitors of PKA (H-89) and of O-glycosylation (BG) were utilized. INS-1 cells overexpressing iPLA2α were seeded in 24-well plates (for insulin secretion assays), skls (for enzymatic activity assays), or glass slides (for immunofluorescence assays), and were pretreated with either H-89 (10–20 μM, 60 min) or BG (2–4 mM, 24 h), prepared in DMSO. The inhibitors were also included during stimulation of the cells with glucose (2 mM) in the absence and presence of FSK (2.5 μM). Control cells were incubated with the vehicle DMSO alone. After the incubation period, the medium was collected for measurement of insulin content, and the cells were processed either for enzymatic activity assays or immunofluorescence analyses.

Statistical analyses. The data were converted to mean ± SE values where appropriate, and the Student's t-test was applied to determine significant differences (at P < 0.05) between two groups.

RESULTS

Enzymatic activity and protein expression of iPLA2α in transfected INS-1 cells. To verify expression of active iPLA2α protein in INS-1 cells transfected with iPLA2α cDNA, homogenates were prepared from INS-1 cells transfected with either an empty retroviral (V) construct or with an iPLA2α cDNA (I)-containing construct. Aliquots of protein from these fractions were used to determine iPLA2α activity, by radiocchemical enzymatic assay, and iPLA2α protein expression, by immunoaffinity analyses. As expected, homogenates prepared from INS-1 cells transfected with iPLA2α cDNA expressed higher activity (nearly 10-fold) than cells transfected with the V construct (Fig. 1A). The increased expression of activity was reflected by the higher expression of iPLA2α protein in the INS-1 cells transfected with iPLA2α cDNA relative to V cells (Fig.
These results confirm that transfection of INS-1 cells with iPLA2β cDNA results in higher expression of catalytically active iPLA2β.

**Distribution of iPLA2β in overexpressing INS-1 cells.** Immunoaffinity, activity, and immunofluorescence analyses were next used to examine in greater detail the distribution of iPLA2β within the INS-1 cells (Fig. 2). Cellular fractions prepared from iPLA2β-overexpressing INS-1 cells, as described in Methods, were used in these analyses. As illustrated in Fig. 2A, left, abundant immunoreactive iPLA2β protein was evident in the CH, NN (containing cytosol), and NE fractions, with low iPLA2β protein content evident in the P fraction. The specificity of the antibody affinity for iPLA2β was then examined after incubations with secondary antibody alone (Fig. 2A, lane 1), preimmune IgG (lane 2), or piPLA2β antibodies plus antigenic peptide sequences of iPLA2β against which the antibodies were generated (lane 3). Under all three conditions, no signal for the iPLA2β protein was detected. These findings confirm that the piPLA2β antibodies generated by Research Genetics strategy are specific for the iPLA2β protein. Figure 2A, right, reflects iPLA2β enzymatic activity in the corresponding INS-1 cell fractions and illustrates an abundance of iPLA2β catalytic activity in the nuclear fraction.

The piPLA2β antibodies were next used to examine localization of the iPLA2β protein within the INS-1 cells by immunofluorescence analyses. As illustrated in Fig. 2B, in the presence of piPLA2β antibodies (a) a diffuse iPLA2β fluorescence signal is detected in the cytosol of cells along with a ring of fluorescence in the nuclear region. No fluorescence signal, however, is evident when preimmune IgG is used as the primary probe (b). The iPLA2β protein fluorescence is also completely neutralized when the cells are treated with piPLA2β antibodies in the presence of the peptide antigens (c). These findings confirm that the piPLA2β antibodies have specific affinity for the iPLA2β protein in both isolated cellular fractions and in intact cells.

**Immunofluorescence analyses of iPLA2β distribution in INS-1 cells after stimulation with glucose and IBMX.** To examine whether the iPLA2β protein undergoes translocation under conditions that promote insulin secretion, parental INS-1 cells and INS-1 cells transfected with iPLA2β cDNA were stimulated with glucose and IBMX, either alone or in combination. The cells were then sequentially fixed, permeabilized, and incubated with piPLA2β antibodies followed by Cy3 fluorescent secondary antibody. Localization of iPLA2β fluorescence was then visualized by confocal microscopy. Fluorescence recordings for iPLA2β in parental cells after a 60-min stimulation period with glucose in the absence and presence of IBMX are presented in Fig. 3. In the presence of no added glucose or IBMX, iPLA2β fluorescence is diffuse (A). At 2 mM glucose, the iPLA2β fluorescence appears as a ring (B) in the nuclear region. Higher glucose concentrations result in more prominent fluorescence, which is distributed throughout the cytoplasm and nuclear regions (C and D). Addition of IBMX by itself results in distinct iPLA2β fluorescent ring formation (E), which becomes more diffuse with increasing concentrations of glucose (F-H).

In contrast to parental cells, under basal conditions, a diffuse iPLA2β fluorescence distribution in the cytosol is accompanied by distinct rings of iPLA2β fluorescence around the nuclear region of INS-1 cells transfected with iPLA2β cDNA (Fig. 4A). Increases in the glucose concentration to 2 mM result in more...
distinct ring formation (B), reflecting increased accumulation of iPLA₂β in the nuclear region. Higher glucose concentrations, however, do not further promote increases in nuclear fluorescence of iPLA₂β (C and D). Addition of IBMX alone (E) results in robust increases in iPLA₂β fluorescence around the nuclear region. The intense iPLA₂β fluorescence with IBMX is modestly increased in the presence of glucose (F-H).

To monitor temporal changes in the distribution of the iPLA₂β protein during stimulation, INS-1 cells transfected with iPLA₂β cDNA were examined at various times during the 60-min exposures to either glucose or IBMX. Glucose alone (data not shown) at all concentrations promotes nuclear accumulation of iPLA₂β after 30 min of stimulation, which is reflected by the appearance of fluorescent rings in the nuclear region. However, by 60 min, iPLA₂β fluorescence at the nuclear perimeter disperses, and the signal becomes diffuse within the nuclei and in the cytosol. The relatively little effect of increasing concentrations of glucose on iPLA₂β distribution is associated with a diminished capacity to secrete insulin, relative to iPLA₂β-overexpressing cells, over the same range of glucose concentrations. This is illustrated in Fig. 4 (O data points in L), where secretion after a 60-min incubation period with 2 mM glucose in the absence and presence of IBMX is presented.

Stimulation of the cells with IBMX, in contrast, promotes increases in time-dependent accumulation of iPLA₂β fluorescence in the nuclear region (Fig. 4, I-K), which is reflected by the appearance of a diffused pattern by 15 min (J), relative to distribution at 0 min (A). After a 30-min period of stimulation with IBMX, reformation of distinct iPLA₂β fluorescent rings around the nuclear region is evident (J), and subsequently becomes more intense by 60 min (K). The IBMX-induced distribution of iPLA₂β is found to be similar in the absence and presence of glucose (2 mM). The time-dependent IBMX-induced accumulation of iPLA₂β in the nuclear region of iPLA₂β overexpressing INS-1 cells correlates well with the amplified temporal increase in IBMX-induced insulin secretion (■ data points in L) relative to parental responses (○, L).

Visualization of nuclear accumulation of iPLA₂β by dual-signal immunofluorescence. To verify that the stimulated iPLA₂β signal was indeed accumulating in the nuclear region, a dual-labeling fluorescence protocol was used. INS-1 cells overexpressing iPLA₂β were
stimulated with either glucose (2 mM) or IBMX (100 μM) alone and subsequently processed for dual-fluorescence analyses. The iPLA2 protein is visualized with Cy3 red fluorescence and the nuclear region with bisbenzimide blue fluorescence. An overlap of the two fluorescences would yield a pinkish hue and reflect the appearance of the iPLA2 protein in the nuclear region. This is illustrated in Fig. 5. In A, only the iPLA2 fluorescence with 2 mM glucose is visualized; in B, only the nuclear region in the corresponding cells is visualized. When both fluorescences are simultaneously monitored (C), a clear separation of the red iPLA2 from the blue nuclear fluorescence is evident. Figure 5D depicts the higher-intensity iPLA2 fluorescence in the presence of IBMX alone, and Fig. 5E reveals the nuclear region in the corresponding cells. When both fluorescences are simultaneously monitored in the IBMX-stimulated cells (F), the nuclear blue region is replaced by a pinkish coloring from the overlap of iPLA2 and the nuclear region, suggesting that, in the presence of IBMX, the iPLA2 protein accumulates in the nuclear region.

Verification of purity of nuclear preparation. To determine whether the accumulation of iPLA2 protein in the nuclear region was accompanied by increases in nuclear iPLA2 activity, nuclear fractions were prepared from iPLA2-overexpressing INS-1 cells according to the procedure of Schreiber et al. (41). Because we have previously observed the presence of iPLA2 activity in mitochondrial and plasma membrane compartments of β-cells (14), the possibility that the nuclear preparation is contaminated with these cellular components was first assessed. As illustrated in Fig. 6, mitochondria-associated cytochrome c oxidase (A) and plasma membrane-associated 5’-nucleotidase (B) activities are enriched in the NN fraction relative to the nuclear fraction. Furthermore, immunoreactive nuclear transcription factor Oct-1 is evident in the nuclear fraction (C) but not in the NN fraction. These findings verify that the NE fraction is not contaminated with mitochondria or plasma membrane and contains nuclei. To verify that the Ca2+-independent PLA2 activity expressed in NN and NE fractions is manifested by the iPLA2 protein, the ability of ATP to stimulate and of BEL, a suicide inhibitor of iPLA2 (35), to inhibit activity was determined. The β-cell iPLA2 enzymatic activity has previously been shown to be uniquely sensitive to ATP and BEL (35–37). As demonstrated in Fig. 6D, the enzymatic activity expressed in both fractions is found to be stimulated by ATP and completely inhibited by BEL; arrows indicate absence of measurable activity. These findings therefore confirm the presence of iPLA2 protein in the INS-1 cell fractions of interest.
Stimulated accumulation of nuclear iPLA₂ enzymatic activity. To examine more directly the influence of cAMP-elevating agents on the distribution of iPLA₂ in overexpressing cells, the adenylate cyclase stimulator FSK was utilized in subsequent studies. Hence, iPLA₂ activity was determined in the NN and NE fractions after treatment of iPLA₂-overexpressing INS-1 cells with glucose (2 mM) in the absence and presence of FSK. As illustrated in Fig. 7A, NN-associated specific iPLA₂ activity is relatively unchanged under 2 G ± FSK conditions. This is confirmed in immunoaffinity analyses, which reveals no significant change in the iPLA₂ content in this fraction (data not presented). In contrast, nuclear-associated iPLA₂ activity increases only modestly (Fig. 7B) with 2 G, but significantly (nearly 2-fold) with the addition of FSK. Such an increase in activity is reflected by increased accumulation of the iPLA₂ protein (Fig. 7C) in the NE fraction after stimulation with FSK. Potential phosphorylation and glycosylation of iPLA₂. Previous reports suggest that phosphorylation stimulates nuclear association of catalytic (c) PLA₂ (13, 40), and that cytosolic and nuclear proteins undergo stimulated glycosylation (7, 15). In view of this, it was of interest to examine the potential involvement of these two processes in nuclear accumulation of iPLA₂ in INS-1 cells. Elevations in cAMP, as with FSK, induce cAMP-dependent PKA-induced phosphorylation of serine and threonine residues of proteins (8). As such, the ability of cPKA to phosphorylate iPLA₂ was
Fig. 5. Visualization of nuclear accumulation of iPLA2β by dual-signal immunofluorescence. INS-1 cells overexpressing iPLA2β were incubated with either glucose (G, 2 mM) or IBMX (100 µM) for 1 h, as described in Fig. 4, and processed for immunofluorescence analyses, as described in Fig. 3. After incubation with the Cy3 secondary antibody, cells were incubated with bisbenzimide (12 min at room temperature [RT]). iPLA2β fluorescence (red) was visualized with a rhodamine filter, and the nuclear fluorescence (blue) was visualized with a DAPI filter with a Nikon Inverted Scope. A–C, 2 G; D–F, 100 µM IBMX. A and D: Cy3 (iPLA2β) fluorescence; B and E: bisbenzimide (nuclear region) fluorescence; C and F: dual Cy3 and bisbenzimide fluoroscences.

Fig. 6. Verification of purity of nuclear preparation and presence of of iPLA2β activity in the nuclear fraction. Cellular fractions from the iPLA2β-overexpressing INS-1 cells were prepared, as described in METHODS, and were assayed for mitochondria-associated cytochrome c oxidase (A) and plasma membrane-associated 5′-nucleotidase (B) activities and nuclear Oct-1 immunoaffinity (C). D: iPLA2β enzymatic activity in the absence (Con) and presence of either ATP (10 mM) or BEL (10 µM) in the non-nuclear and nuclear fractions, respectively. Data in A, B, and D represent means ± SE of specific enzymatic activities of cytochrome c oxidase (mU/mg protein), 5′-nucleotidase (pmol·mg protein⁻¹·min⁻¹), and iPLA2β (pmol·mg protein⁻¹·min⁻¹), respectively (n = 4). *P < 0.05, ATP-treated groups significantly different from control groups. Arrows, complete inhibition of iPLA2β enzymatic activity after BEL treatment. CH, cellular homogenate; NN, non-nuclear homogenate; NH, nuclear homogenate; NE, nuclear extract; P, particulate fraction.
examined. Treatment of purified iPLA2β with cPKA promotes incorporation of $^{32}$P into the protein (Fig. 8A, top, lane 1). Such PKA-induced phosphorylation of iPLA2β is completely blocked by a specific peptide inhibitor of PKA (PKAI, lane 2) and by the nonspecific inhibitor of phosphorylation, staurosporine (lane 5) but not by genistein (lane 3), which inhibits tyrosine phosphorylation. As shown (bottom), immunoaffinity analysis of the same blot verifies that all lanes are loaded with similar iPLA2β protein amounts. As illustrated in Fig. 8B, incubation of purified iPLA2β with galactosyltransferase results in glycosylation of the protein. These findings raise the possibility that β-cell iPLA2β is a candidate for phosphorylation and glycosylation.

Involvement of protein phosphorylation and glycosylation during insulin secretion. To examine whether protein phosphorylation or glycosylation has a role during stimulation of insulin secretion from iPLA2β-overexpressing INS-1 cells, the effects of H-89 and BG, cell-permeable specific inhibitors of PKA (50) and O-glycosylation (20), respectively, were examined. As shown in Fig. 9, insulin secretion from the overexpressing INS-1 cells is modestly elevated in the presence of 5 mM glucose alone, but robustly amplified with additions of cAMP-elevating agents IBMX and FSK. Both H-89 and BG significantly attenuate the IBMX- and FSK-induced responses, suggesting that phosphorylation and glycosylation events participate in insulin-
secretory responses amplified by cAMP-elevating agents.

Effects of inhibitors of phosphorylation and glycosylation on iPLA2 localization. Immunofluorescence analyses were performed next to examine whether inhibition of protein phosphorylation or glycosylation affects stimulated nuclear accumulation of iPLA2. As illustrated in Fig. 10, relative to the 0 G condition (A), presence of 2 G alone promotes appearance of a modest ring-like accumulation of iPLA2 in the nuclear region (B). Neither H-89 (C) nor BG (D) appears to affect such accumulation. FSK alone (E), similar to IBMX (Fig. 4), increases nuclear accumulation of the iPLA2 protein, and addition of 2 G further promotes this association into ring-like formation (F). After treatment with H-89 (G) or BG (H), FSK-amplified increases in nuclear immunofluorescence of iPLA2 are markedly reduced. When iPLA2 enzymatic activity was determined in the corresponding groups, FSK-stimulated increase in nuclear iPLA2 activity was found to return to basal levels (Fig. 11). Collectively, these findings suggest that phosphorylation and glycosylation events participate in stimulated nuclear accumulation of iPLA2 in INS-1 β-cells.

DISCUSSION

Potential functions proposed for the β isoform of calcium-independent Group VIA PLA2 (iPLA2) include generation of substrate for eicosanoid synthesis (28), membrane degradation during apoptosis (2), regulation of phosphatidylcholine composition and content (4, 5), and involvement in signaling events (19, 46). Studies in murine P388D1 macrophage-like cells have led to the suggestion that iPLA2 participates in phospholipid remodeling and therefore serves a housekeeping role (4). In that study, they observed that
inhibition of iPLA2 activity with either BEL or with antisense oligonucleotide treatments led to suppressed incorporation of arachidonic acid into the phosphatidylycholine pool and also reduced the generation of lysophosphatidylcholine (4). Subsequent studies in our laboratory, however, indicate that suppression of iPLA2 inhibits insulin secretion without affecting arachidonic acid incorporation into the β-cell phosphatidylycholine pool (35). Furthermore, when the proposed role of iPLA2 in phospholipid remodeling was examined in INS-1 insulinoma cells overexpressing iPLA2, the rate or extent of arachidonic acid incorporation into INS-1 cell phospholipids was not found to be altered, whereas the insulin-secretory response was markedly greater in the iPLA2-overexpressing INS-1 cells (22). These findings strongly suggest that the iPLA2 enzyme manifests a signaling, rather than a housekeeping, role in β-cells.

The iPLA2-overexpressing INS-1 cells studied in the present study were found to be more sensitive to cAMP-elevating agents than parental INS-1 cells. Increasing concentrations of glucose produced only modest increases in insulin secretion, but IBMX (a phosphodiesterase inhibitor) and FSK (an adenylyl cyclase activator) promoted robust increases in the insulin secretory response. Attenuated responses to glucose alone have previously been recognized in native β-cells that are stimulated after separation from the non-β-cell population of pancreatic islets (29). Glucose stimulation of isolated islet β-cells promotes only minor increases in insulin secretion, but costimulation with cAMP-elevating agents or reintroduction of non-β-cells restores the insulin-secretory capacity (31). The ability of cAMP-elevating agents to amplify glucose-stimulated insulin secretion has also been noted in insulinoma cell lines (32). These observations highlight the requirement for maintaining a critical level of cAMP for glucose-stimulated insulin secretion.

Overexpression of iPLA2 in INS-1 cells promotes accumulation of the enzyme in the nuclear region, even under basal conditions. Additions of increasing concentrations of glucose do not influence this accumulation significantly, but cAMP-elevating agents dramatically enhance accumulation of iPLA2 in the nuclear region, as reflected by immunofluorescence, immunoadfinity, and enzymatic activity analyses. This effect is time dependent and very closely correlates with the secretory response seen in the iPLA2-overexpressing cells. Such a stimulated translocation of iPLA2 provides further evidence to support its potential role in signaling during secretory events.

Nuclear association of iPLA2 induced by cAMP-elevating agents in INS-1 cells is of interest, because glucose promotes β-cell insulin secretion and proliferation, and glucose-induced INS-1 cell mitogenesis is cAMP dependent (18). As membranes of the nucleus and endoplasmic reticulum (ER) are contiguous (17, 40), perinuclear accumulation of iPLA2 is consistent with association with a subcellular compartment that is likely to include ER (17). The β-cell ER is known to contain an abundance of arachidonate-containing plasmenelethanolamine molecular species (33), and products of PLA2 action induce Ca2+ release from β-cell ER (48), which is thought to participate in induction of insulin secretion (10). cAMP-mediated increases in cytosolic [Ca2+]i, via Ca2+ entry and mobilization of intracellular Ca2+ stores (11, 17, 51), and sensitization of the exocytotic apparatus to Ca2+ (1) are among the mechanisms by which cAMP augments the insulin-secretory responses. In view of earlier observations suggesting involvement of arachidonic acid, in particular of arachidonic acid hydrolyzed from plasmenelethanolamine molecular species (33, 34) and of its metabolites in the β-cell secretory process (45), the nuclear association of iPLA2 after stimulation with cAMP-elevating agents might be consistent with a process that results in the generation of arachidonic acid in close proximity to enzymes, which catalyze eicosanoid generation, localized in the nuclear envelope and ER (27, 39).

The nuclear association of cPLA2 after cellular stimulation with the calcium ionophore A-23187 has been reported to be associated with increased phosphorylation of the cPLA2 (13, 40). Although nuclear accumulation of iPLA2 has been reported during myocardial ischemia (47), it is not known whether nuclear accumulation is associated with phosphorylation of the iPLA2. Because stimulation with IBMX or FSK increases cAMP content in INS-1 cells (22), it might be speculated that, as a consequence, a cAMP-dependent PKA, which phosphorylates serine and threonine residues, is activated and that the iPLA2 serves as a candidate for PKA-catalyzed phosphorylation. The likelihood that the iPLA2 becomes associated with the nuclear region is enhanced by the recognition of a...
bipartite nuclear localization sequence (511)KREFGEHTKMDVKKPK(527) in the deduced amino acid sequence of iPLA$_2$B (24) that is similar to the sequence in other nuclear proteins (10, 38).

To examine the possibility that iPLA$_2$B can undergo PKA-stimulated phosphorylation, islet iPLA$_2$B overexpressed in s9f cells and purified was incubated with cPKA. Under the conditions studied, PKA promotes phosphorylation of iPLA$_2$B in a protein concentration-dependent manner (data not shown). Such phosphorylation is completely blocked by a specific peptide inhibitor of PKA and by the general inhibitor of phosphorylation staurosporine, but not by genistein, which inhibits tyrosine phosphorylation. These findings raise the possibility that iPLA$_2$B is a candidate for PKA-induced phosphorylation. In view of this, effects of inhibiting cellular PKA activity with H-89 (a cell-permeable inhibitor) on iPLA$_2$B distribution were examined. The data obtained in the presence of H-89 reveal that FSK-stimulated accumulation of iPLA$_2$B immunofluorescence in the nuclear region is dramatically attenuated and that this is accompanied by reductions in FSK-stimulated nuclear accumulation of iPLA$_2$B enzymatic activity and by inhibition of cAMP-elevating agent-stimulated insulin secretion.

Another potential modification of the iPLA$_2$B protein that might affect its localization is glycosylation, a modification that has been reported with several cytosolic and nuclear proteins (7, 15). Although it is not clear what effect glycosylation of the iPLA$_2$B protein might have on its function or localization, it might be noted that the iPLA$_2$B contains a consensus sequence site for O-GlcNacylation. Interestingly, iPLA$_2$B also contains ankyrin-like repeat sequences (25) that bind tubulin and integral membrane proteins (30), and O-GlcNacylation is involved in neurofilament assembly (16). A number of agents that interfere with microtubule assembly or disassembly inhibit insulin secretion (21). In view of these observations, it might be speculated that glycosylation of the iPLA$_2$B promotes its binding to the neurofilament processes through the ankyrin-like repeats and facilitates its trafficking into the nuclear region. Such a possibility was examined using purified iPLA$_2$B and intact cells. Incubation of purified iPLA$_2$B with galactosyltransferase resulted in an increase in the incorporation of labeled galactose, suggesting that the protein was a candidate for O-GlcNacylation. After treatment of iPLA$_2$B-overexpressing cells with BG, a cell-permeable competitive inhibitor of galactosaminyltransferase and O-glycosylation, decreases in FSK-stimulated nuclear accumulation of iPLA$_2$B fluorescence are observed. As with H-89, FSK-stimulated nuclear accumulation of iPLA$_2$B activity and insulin secretion are also significantly attenuated by BG. Collectively, the data obtained with H-89 and BG raise the possibility that phosphorylation and glycosylation of the iPLA$_2$B protein itself, or of associated proteins involved in the secretory pathway, might participate in promoting translocation of iPLA$_2$B to the nuclear region.

In summary, in the present study we report that the β-cell iPLA$_2$B becomes associated with the nuclear region under conditions that promote increases in insulin secretion. Posttranslational modifications such as phosphorylation and glycosylation may potentially participate in promoting its association with the nuclear region and facilitate its signaling role in β-cells. Detailed analyses are still required to identify the sites in the iPLA$_2$B protein that might be modified during stimulatory conditions, and such studies are currently underway.

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