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# IDENTIFICATION OF PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE IN PANCREATIC ISLETS AND INSULIN-SECRETING β-CELLS

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**Summary**: The signal transduction mechanisms involved in insulin secretion by the ß-cell are poorly understood. Glucose, the main physiological secretagogue, needs to be metabolized, but the identity of the intracellular messengers which couple glucose metabolism and insulin exocytosis is controversial. We now report the identification of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)$P_3$), the end-product of phosphatidylinositol 3-kinase phosphorylation of polyphosphoinositides, in islets and in an insulin-secreting clonal ß-cell line, RINm5F, using a combination of thin layer chromatography and high performance liquid chromatography analyses and by demonstrating that sequential deacylation and deglyceration of PtdIns(3,4,5)$P_3$ yields inositol 1,3,4,5-tetrakisphosphate. Unlike other cell types, significant levels of PtdIns(3,4,5)$P_3$ were detected in ß-cells under non-stimulatory conditions. Insulin secretagogues (28 mM glucose + 0.5 mM carbachol) caused a rapid and transient increase in PtdIns(3,4,5)$P_3$ levels which peaked at 2-5 min, corresponding to peak early phase insulin release. 

There are two major classes of secretagogues capable of stimulating insulin secretion from pancreatic islets: nutrient secretagogues and receptor-mediated agonists (1,2). The muscarinic agonist carbachol binds to a membrane receptor coupled to phospholipase C through a G-protein (3). In contrast, glucose, the major physiological secretagogue, must be metabolized by islets in order to induce insulin secretion (4,5). Phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$) generates the second messengers *myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$) and 1,2-diacyl-*sn*-glycerol (DAG), which have both

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The abbreviations used are: PtdIns(4,5)$P_2$, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)$P_3$, *myo*-inositol 1,4,5-trisphosphate; DAG, 1,2-diacyl-*sn*-glycerol; PtdIns(3,4,5)$P_3$, phosphatidylinositol 3,4,5-trisphosphate; Ins(1,3,4,5)$P_4$, *myo*-inositol 1,3,4,5-tetrakisphosphate; LysoPtdIns, lysophosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; SAX-HPLC, strong-anion exchange-HPLC; Gro-, glycero-; PI 3-kinase, phosphatidylinositol 3-kinase.

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been implicated in insulin secretion (6). Until recently, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)$P_3$) was believed not to exist in mammalian cells (7). Several groups have now demonstrated the presence of PtdIns(3,4,5)$P_3$, the product of a phosphatidylinositol 3-kinase (PI 3-kinase), in a number of cell lines including platelets, neutrophils, rat brain, T-cells, smooth muscle cells, Swiss 3T3 cells, and a rat pheochromocytoma cell line (8). Agonists (platelet-derived growth factor, f-Met-Leu-Phe (FMLP)), which stimulate neutrophils or platelets activate this pathway and cause a dramatic and rapid increase in PtdIns(3,4,5)$P_3$ (9,10). The function of PtdIns(3,4,5)$P_3$ is not well understood (8). Recently, however, PtdIns(3,4,5)$P_3$ was shown to activate an atypical isozyme ($\zeta$) of protein kinase C (11). In view of the controversy regarding the molecular mechanisms of insulin secretion, we examined whether this novel pathway may have a role in insulin secretion.

## MATERIALS AND METHODS

**Isolation of islets and insulin-secreting RINm5F cell line culture** - In a typical experiment, islets were isolated aseptically from 6-8 male Sprague-Dawley rats as previously described (12,13). Insulin-secreting RINm5F cells (passages 20-40) were cultured in RPMI 1640 (11 mM glucose) (14).

**Labeling of islets and RINm5F cells** - Islets (approx. 3,000) were labeled 24 hours with 20 μCi/ml of [$^3$H]inositol in CMRL-1066 (15). Following labeling, islets were washed five times in Krebs'-Hepes buffer supplemented with 3 mM D-glucose. RINm5F cells were labeled with 3.3 mCi/ml [$^3$H]inositol in RPMI-1640. In some experiments, islets were incubated with 1 mCi $^{32}$P for 90 min.

**Extraction of phospholipids** - After incubation, the reaction was stopped with 2 ml ice-cold chloroform-methanol (2:1,v/v) supplemented with butylated hydroxytoluene (0.25 mg/ml), vortexed for 1 min, and placed in a dry-ice/ethanol bath for 15 minutes. Carrier amounts (5 mg) of cold phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns(4)P), PtdIns(4,5)$P_2$, and lysophosphatidylinositol (LysoPtdIns) were added. The samples were sonicated, and chloroform (1.4 ml) and 2.4 N HCl (1.4 ml) were added. The organic phase was washed 6 times methanol/1N HCl (1:1 v/v).

**Methylamine deacylation of polyphosphoinositides** - Extracted phospholipids were resuspended in 3 ml of methylamine reagent (40% aqueous methylamine/methanol/water/n-butanol (67:114:40:29, v/v/v/v)) and vortexed 1 min (16). Samples were incubated 45 min at 53°C, evaporated, and washed with water. Glycerophospholipids were extracted with 2 ml of n-butanol/petroleum ether (bp 40-60 °C)/ethyl formate (20:4:1 v/v/v), the top aqueous layer was collected, evaporated and either reconstituted in 0.35 ml of 1 mM EDTA (pH 7.0) which contained 6 μmol each of AMP, ADP, and ATP for HPLC, or subjected to deglyceration. Controls consisting of [$^3$H]inositol-labeled PtdIns, PtdIns(4)P, PtdIns(4,5)$P_2$ were deacylated in parallel (to yield the corresponding glycero-phosphoinositides) and analyzed by HPLC.

**Deglyceration of glycero-phosphoinositides** - Glycerophospholipids were resuspended in 1 ml of 1 mM $NaIO_4$, vortexed, and incubated at room temperature in the dark for 75 min (17,18). Ethylene glycol (0.1% in water) was added (30 μl) for 10 min followed by 1,1-

dimethylhydrazine (1% in water, pH 4.0 (formic acid), 400 µl), vortexed, incubated at room temperature for 1 h, and cleaned with a 1 ml slurry of Dowex-50 Cation exchange resin prior to SAX-HPLC as described below. Controls consisting of [$^3$H]inositol labeled PtdIns, PtdIns(4)P, PtdIns(4,5)$P_2$ were deacylated and deglycerated in parallel to yield the corresponding inositol phosphates (recovery of 58% after deacylation and deglyceration, n=4).

**Thin layer chromatography (TLC) analysis of intact phospholipids** - Extracted phospholipids were analyzed on potassium oxalate (1%) coated Silica gel H plates (20 x 20 cm, 250 mm thick, Analtech, Inc, Newark, DE.). Plates were pre-activated for 45 minutes at 110°C and developed in methanol/chloroform/water/14.8N ammonium hydroxide (100/70/25/15, v/v/v/v). The radioactivity of the chromatogram was quantitated with a Berthold Linear Analyzer 284 (Nashua, NH) as described (19-21).

**SAX-HPLC of glycero-phosphoinositides and inositol phosphates** - HPLC analysis was performed using a strong-anion-exchange column (SAX) (Whatman Partisil 10 SAX, 0.46 x 25 cm) on a Varian system: Variable Wavelength UV-VIS Detector (9050), Solvent Delivery System (9010), and Autosampler (9095) (300 µl per injection). A pre-column packed with Whatman pellicular media and a SAX guard cartridge (Whatman) were also installed. The gradient of solvent A (2.5 M $NaH_2PO_4$, pH 3.8 with 10N NaOH) over 100 minutes was as follows: 0 min 100% water, 30 min 5% solvent A, 32 min 12% solvent A, 59 min 17% solvent A, 61 min 22% solvent A, 81 min 35% solvent A, 83 min 100% solvent A maintained until 100 min, followed by at least 40 min of water prior to the next injection. The flow rate was 1 ml/min and fractions were collected every min. For each HPLC run, the retention times of unlabeled AMP, ADP, and ATP were monitored (15, 42, 66 min, respectively) by UV spectrophotometry (254 nm) to control for day to day variation. Four ml of Inflow BD scintillation cocktail was added to each sample, and radioactivity was measured by liquid scintillation spectrometry.

## RESULTS

In the first approach, islets were labeled with [$^3$H]inositol and separated by TLC (Figure 1). The relative mobilities of polyphosphoinositides were 0.79 (PtdIns), 0.60 (PtdIns(4)P), and 0.49 (PtdIns(4,5)$P_2$). A slow-moving [$^3$H]inositol labeled peak with a $R_f$ of 0.13 was systematically observed (n=12) which was presumed to be PtdIns(3,4,5)$P_3$ based on its known mobility in this TLC system (22). A smaller peak with $R_f$ of 0.25 was also seen which may correspond to the 3-phosphorylated isomer of PtdIns(4,5)$P_2$, PtdIns(3,4)$P_2$. Since islets are composed of a heterogenous population of α, β, and δ cells, an insulin-secreting clonal ß-cell line, RINm5F, was also examined. Studies with RINm5F cells showed similar peaks with $R_f$'s of 0.13 and 0.20 (n=5) consistent with PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ (data not shown). These data strongly suggest the presence of a highly polar inositol-containing phospholipid in insulin secreting ß-cells. Further identification was performed by HPLC analysis of the products formed after deacylation and deglyceration.

Islets were labeled with 5 mCi of $^{32}$P to achieve high incorporation into phospholipids, which were then extracted, deacylated with methylamine, and subjected to SAX-HPLC. The results of a typical experiment from unstimulated islets are depicted in Figure 2. In this system, [$^3$H]glycero-

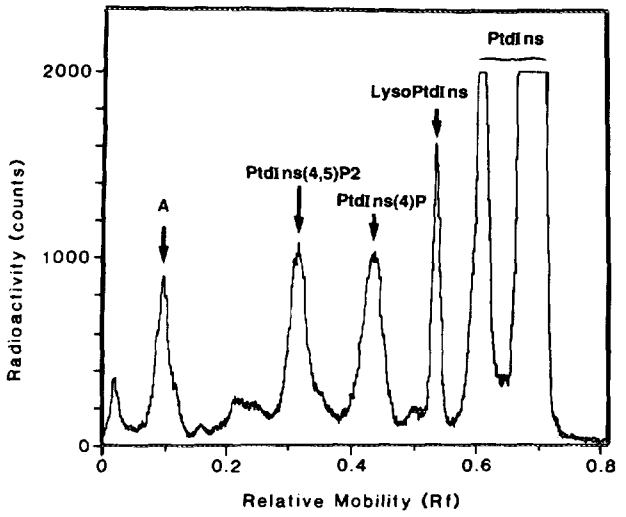


**Figure 1. TLC of polyphosphoinositides in islets labeled with [$^3$H]inositol.** Isolated pancreatic islets (400 per condition) were labeled with [$^3$H]inositol. After chloroform/methanol extraction, the organic phase was analyzed on a $K^+$-oxalate Silica Gel plate and developed in methanol/chloroform/water/ammonium hydroxide (14.8N) (100/70/25/15, v/v/v/v). Tritiated standards were run in parallel. The peak marked "A" was systematically found in both unstimulated islets (n=12) and in RINm5F cells (n=5) and has the expected mobility of PtdIns(3,4,5)$P_3$.

PtdIns, [$^3$H]glycero-PtdIns(4)P, and [$^3$H]glycero-PtdIns(4,5)$P_2$ were well resolved (retention times of 15, 32, and 59 min respectively). In material derived from $^{32}$P-labeled islets, in addition to the expected peaks of glycero-PtdIns, glycero-PtdIns(4)P, and glycero-PtdIns(4,5)$P_2$, two peaks were consistently found (n=14). Based on their mobility in this HPLC system, the first peak (48 min) is glycero-PtdIns(3,4)$P_2$, while the second peak (78 min) is glycero-PtdIns(3,4,5)$P_3$. In order to further demonstrate the existence of PtdIns(3,4,5)$P_3$ in islets, the glycero-phosphoinositides were further deglycerated to their corresponding inositol phosphates.

$^{32}$P-labeled phospholipids were deacylated, deglycerated, and analyzed by SAX-HPLC (Figure 3). The system was calibrated with [$^3$H]-labeled inositol phosphates: [$^3$H]Ins(1,4)$P_2$ (42 min), [$^3$H]Ins(1,4,5)$P_3$ (79 min), and [$^3$H]Ins(1,3,4,5)$P_4$ (92 min). The SAX-HPLC analysis of deacylated and deglycerated phospholipids derived from unstimulated islets (n=4) revealed a peak at 92 min, consistent with Ins(1,3,4,5)$P_4$ (Figure 3). Although Ins(1,3,4,5)$P_4$ is a known adduct of islet inositol phosphates, the peak seen at 92 min is unlikely to be an inositol tetrakisphosphate contaminant from the phospholipid extraction for two reasons. First, in our extraction procedure, the organic phase was washed six times with methanol/1 N HCl which removes more than 99% of the inositol phosphates (as assessed with [$^3$H]Ins(1,4,5)$P_3$ and

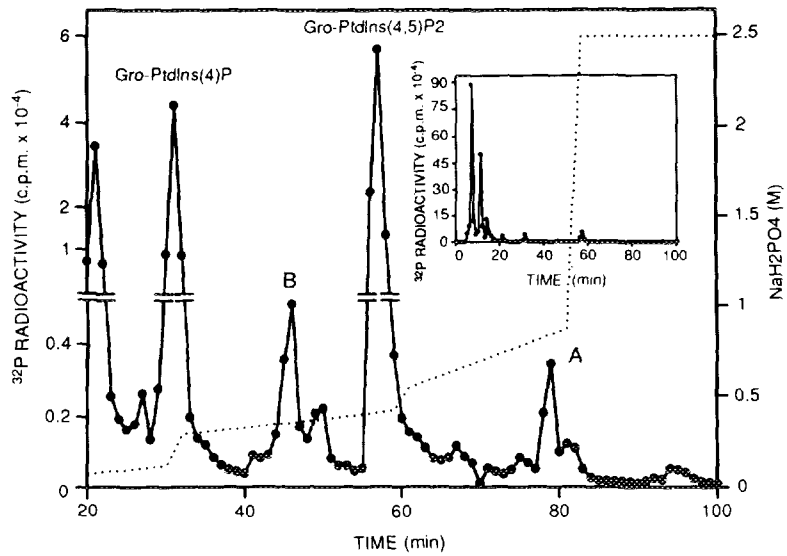


**Figure 2. SAX-HPLC analysis of glycero-phosphoinositides in islets labeled with $^{32}P$.** Isolated rat islets were labeled with 5 mCi $^{32}P$ for 90 minutes in Krebs'-Hepes buffer (3 mM glucose). After extraction of the phospholipids, lipids were deacylated with methylamine reagent to the resulting glycero-phosphoinositides. Samples were purified by SAX-HPLC with a gradient of 2.5 *M* $NaH_2PO_4$ *over 100 minutes (dotted line). One-ml fractions (flow rate 1 ml/min) were* collected and counted by liquid scintillation spectrometry (closed circles). The column was standardized with deacylated tritiated standards. The peaks marked "A" and "B" have the expected retention time of glycero-PtdIns(3,4,5)$P_3$ and glycero-PtdIns(3,4)$P_2$ (representative of 14 experiments). The inset shows the full-scale chromatogram.

[$^3$H]Ins(1,3,4,5)$P_4$). Second, islets (approx. 1,200) labeled with $^{32}P$ were deacylated, and a small fraction analyzed by SAX-HPLC: under these conditions, no significant peak was detected at 92 min (Figure 3A). The remaining fraction was then deglycerated. The resulting SAX-HPLC chromatogram showed a substantial peak at 92 min (Figure 3B), demonstrating that Ins(1,3,4,5)$P_4$ was not present until after deglyceration and that Ins(1,3,4,5)$P_4$ is a moiety of islet PtdIns(3,4,5)$P_3$. These studies were repeated using the ß-cell clonal line, RINm5F. Phospholipids from $^{32}P$-labeled RINm5F cells which were deacylated and deglycerated again demonstrated the presence of Ins(1,3,4,5)$P_4$ derived from PtdIns(3,4,5)$P_3$ (data not shown, n=4). These results localize PtdIns(3,4,5)$P_3$ to the insulin secreting ß-cell.

$^{32}P$-labeled islets were stimulated with 28 mM glucose and 0.5 mM carbachol for either 0, 2, 5, 15, or 30 min. Phospholipids were deacylated and analyzed by SAX-HPLC. There was a significant and transient secretagogue-induced rise in PtdIns(3,4,5)$P_3$ levels which peaked at the same time as the peak insulin secretion: 1.7 fold increase at 2 min, 10 fold increase at 5 min, and 4.1 fold increase at 15 min (n=3). In contrast PtdIns(4,5)$P_2$ levels did not increase, or in some cases, showed a decrease (data not shown). Insulin (0.3 µM, n=2) and insulin-like growth factor 1 (100 ng/ml) did not affect PtdIns(3,4,5)$P_3$ or PtdIns(4,5)$P_2$ levels (data not shown).

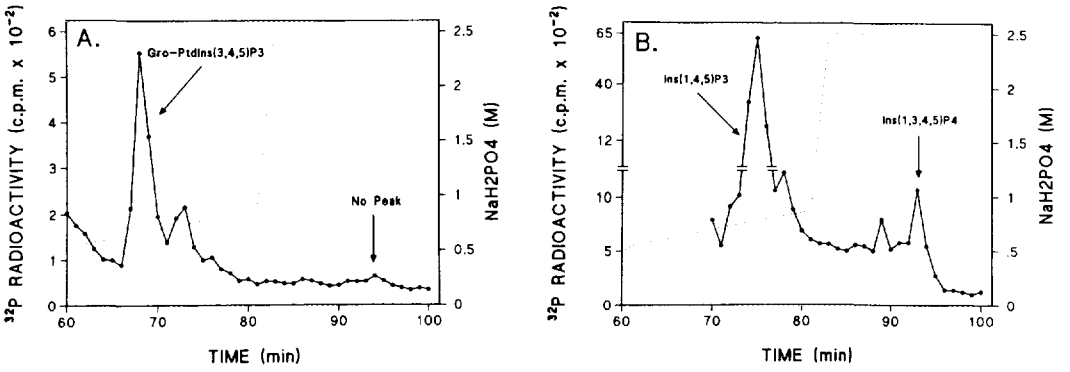


**Figure 3. SAX-HPLC analysis of inositol phosphates derived from polyphosphoinositides after deacylation and deglyceration in islets labeled with $^{32}P$.** Islets were labeled with $^{32}P$ as described in Figure 2. *Figure 3A:* Representative SAX-HPLC chromatogram of a fraction of sample (from approx. 1,200 islets) analyzed after methylamine deacylation (n=4). *Figure 3B:* SAX-HPLC chromatogram of the remaining fraction from Figure 3A which was further deglycerated. Peak identity was determined by analyzing [$^3$H]-inositol phosphates as well as [$^3$H]-phosphoinositides which were deacylated and deglycerated in parallel.

## DISCUSSION

We have identified the presence of the novel inositol phospholipid, PtdIns(3,4,5)$P_3$, a product of PI 3-kinase phosphorylation of PtdIns(4,5)$P_2$, in pancreatic islets and in the insulin-secreting clonal ß-cell line, RINm5F. Chromatographic identification was based on 1) the expected TLC mobility of a [$^3$H]inositol containing peak derived from islets labeled with [$^3$H]inositol, 2) the detection of a glycero-PtdIns(3,4,5)$P_3$ peak by SAX-HPLC of deacylated phospholipids labeled with $^{32}P$, and 3) the demonstration that the PtdIns(3,4,5)$P_3$ peak contains Ins(1,3,4,5)$P_4$. A similar chromatographic approach has been used to convincingly identify PtdIns(3,4,5)$P_3$ in neutrophils and platelets (9,10). Since pancreatic islets of Langerhans have a heterogeneous cellular composition (approximately 85% insulin-secreting ß-cells, remainder glucagon and somatostatin-secreting cells), it was important to identify PtdIns(3,4,5)$P_3$ in insulin-secreting ß-cells. RINm5F ß-cells are derived from a radiation-induced insulinoma in rats, and secrete insulin in response to the nutrient glyceraldehyde and to the muscarinic agonist carbachol (14). Using the same chromatographic approaches as for islets, we also identified PtdIns(3,4,5)$P_3$ in RINm5F ß-cells. Thus, these observations strongly suggest that PtdIns(3,4,5)$P_3$ is localized in insulin-secreting ß-cells.

PtdIns(3,4,5)$P_3$ has now been identified in a variety of cell lines which include FMLP and mastoparan-stimulated human neutrophils, thrombin-stimulated platelets, T-cells, growth factor-stimulated Swiss 3T3 fibroblasts, and growth factor-stimulated PC12 cell line (8). Our

study is the first demonstration of its presence in an endocrine cell. Furthermore, in most other cells, PtdIns(3,4,5)$P_3$ was only detected after agonist stimulation of the cell (9,10,23). In contrast, in the ß-cell, PtdIns(3,4,5)$P_3$ was clearly detected under non-stimulatory conditions, although it represents a minor fraction of the total phospholipid pool (<0.2%). Stimulation of the ß-cell with glucose and the muscarinic agonist carbachol caused a transient and dramatic increase in PtdIns(3,4,5)$P_3$ levels which correlated with the peak of insulin release. In most cells, agonist stimulation of PtdIns(3,4,5)$P_3$ accumulation is very transient with peak increases which vary from 30 sec for thrombin-stimulated platelets to 3 min for GM-CSF-challenged neutrophils and U937 cells (23,24).

There is a substantial body of indirect evidence (reviewed in (8)) suggesting that PtdIns(3,4,5)$P_3$ is a novel intracellular messenger, although its intracellular target is unknown. Recently, PtdIns(3,4,5)$P_3$ was shown to activate an atypical isoform (ζ) of protein kinase C, and it was suggested that this protein kinase C isoform may be the link between activation of PI 3-kinase and a biological response (11). In islets, there has been conflicting evidence as to the role of protein kinase C in insulin secretion. Several groups have failed to demonstrate glucose activation of total protein kinase C activity, while others have shown that glucose can stimulate the translocation of protein kinase Cα using immunological methods (25-27). Whether protein kinase Cζ is present in islets and activated by secretagogues via increases in PtdIns(3,4,5)$P_3$ remains to be determined.

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