Involvement of Protein Kinase C-ε in Inositol Hexakisphosphate-induced Exocytosis in Mouse Pancreatic β-Cells*

Marianne Hoy†, Per-Olof Berggren‡, and Jesper Gromada¶

From the Laboratory of Islet Cell Physiology, Novo Nordisk A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark and The Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institutet, S-171 76 Stockholm, Sweden

Inositolhexakisphosphate (InsP₆) plays a pivotal role in the pancreatic β-cell stimulus-secretion coupling. We have used capacitance measurements to study the effects of InsP₆ on Ca²⁺-dependent exocytosis in single mouse pancreatic β-cells. In the presence of inhibitors of the protein phosphatase calcineurin to block endocytosis, intracellular application of InsP₆ produced a dose-dependent stimulation of exocytosis, and half-maximal effect was observed at 22 μM. The stimulatory effect of InsP₆ was dependent on protein kinase C (PKC) activity. Oligonucleotides directed against specific PKC isoforms (α, βII, δ, ε, ζ) revealed an involvement of PKC-ε in InsP₆-evoked exocytosis. Furthermore, expression of dominant negative PKC-ε abolished InsP₆-evoked exocytosis, whereas expression of wild-type PKC-ε led to a significant stimulation of InsP₆-induced exocytosis. These data demonstrate that PKC-ε is involved in InsP₆-induced exocytosis in pancreatic β-cells.

Inositol hexakisphosphate (InsP₆) levels transiently increase in pancreatic β-cells following an elevation of the ambient glucose concentration (1). This elevation in InsP₆ levels plays an important role in controlling exocytosis of the insulin-containing granules and subsequent retrieval of membrane by endocytosis (2, 3). These processes of exocytosis and endocytosis are dependent on protein kinase C (PKC) activity (2, 3). Several PKC isoforms are expressed in pancreatic β-cells and include PKC-α, -β, -δ, -ε, and -ζ (4, 5). However, the importance of a particular PKC isoform in controlling InsP₆-evoked exocytosis is unknown. The present study was designed to examine which PKC isoform mediates the stimulatory action of InsP₆ on Ca²⁺-induced exocytosis. Using whole-cell patch clamp techniques and capacitance measurements of exocytosis, we demonstrate that PKC-ε regulates InsP₆-evoked exocytosis in single mouse pancreatic β-cells.

**Experimental Procedures**

Preparation of Islet Cells—Pancreatic islets were isolated from fed female NMRI mice (18–23 g) by collagenase digestion as described previously (3). The local ethical committee in Copenhagen approved the methods of euthanasia. The islets were dispersed into single cells by shaking in a Ca²⁺-free solution, and the resulting cell suspension was plated on Nunc Petri dishes and maintained for up to 3 days in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 international units/ml penicillin, and 100 μg/ml streptomycin.

Electrophysiology—Exocytosis was measured as increases in cell membrane capacitance using an EPC-9 patch clamp amplifier and the Pulse software (v. 8.31; HEKA Elektronik, Lamprecht/Pfalz, Germany) as described previously (3). The interval between two successive points was 0.2 s, and the measurements of cell capacitance were initiated −5 s following establishment of the standard whole-cell configuration. The volume of the recording chamber was 0.4 ml, and the solution entering the bath (1.5 ml/min) was maintained at 33 °C. Exocytosis was elicited by infusion of an electrode solution consisting of 125 mM potassium glutamate, 10 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 3 mM Mg-ATP, 10 mM EGTA, and 0.01, 5, and 10 mM CaCl₂ (pH 7.15 with KOH). The free Ca²⁺ concentrations ([Ca²⁺]₀) of the resulting buffers were 0.03, 0.22, 0.87, and 2.0 μM, using the binding constants described in Ref. 6. The extracellular solution was composed of 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 5 mM HEPES (pH 7.4 with NaOH), and 5 mM D-glucose. Ins,1,4,5,6-P₄ was purchased from Alesis (San Diego, CA). All other chemicals were obtained from Sigma.

Oligonucleotides—To investigate the role of PKC isoforms in InsP₆-evoked exocytosis, the following antisense and sense oligonucleotides were used: PKC-α antisense, 5′-CAGCCATGTTCCCCCAAC-3′ (7); PKC-β antisense, 5′-GTGGAGGTGTCCTCT-3′ (8); PKC-δ antisense, 5′-GCCAGCTACCGGCATTCT-3′ (7); PKC-ε antisense, 5′-GTCGACTGGACCTGGAGTCCTCT-3′ (7); PKC-ζ antisense, 5′-GCCCGTCCCGATCCTGGCCCTC-3′ (7); PKC-ε scrambled, 5′-GCCCGTCCCGATCCTGGCCCTC-3′ (7); PKC-ζ antisense, 5′-GCCCGTCCCGATCCTGGCCCTC-3′ (7); PKC-ε antisense, 5′-GCCCGTCCCGATCCTGGCCCTC-3′ (7). Cultures of β-cells were co-transfected with green fluorescent protein (1 μg/ml) and 5 μM PKC oligonucleotides using Oligofectamine (Invitrogen) and incubated in RPMI 1640 medium supplemented as described above for 48 h before use. The antisense and sense oligonucleotides were synthesized at TAG Copenhagen (Copenhagen, Denmark). The oligonucleotides were phosphothiolated at the underlined positions.

Plasmid Construction—The cDNAs for PKC-ε and PKC-β were kindly provided by Y. Nishizuka (Kobe University, Kobe, Japan). A dominant negative mutant of PKC-ε was obtained from K. Ridge (Northwestern University, Chicago, IL). Transfection of mouse β-cells was performed using 3 μg of PKC cDNA and 1 μg of green fluorescent protein using LipofectAMINE according to the manufacturer's instructions. Following transfection, cells were cultured for 48 h in RPMI 1640 supplemented as described above.

Data Analysis—Results are presented as mean values ± S.E. for the indicated number of experiments. The exocytotic rate (ΔC/Δt) is presented as the increase in cell capacitance measured 30–90 s following establishment of the whole-cell configuration. Statistical significance

Received for publication, April 15, 2003, and in revised form, June 26, 2003

Published, JBC Papers in Press, July 1, 2003, DOI 10.1074/jbc.M303927200

Cited in the JBC Papers in Press from May 2003

This paper is available online at http://www.jbc.org

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when measured after 30 s. The data are mean values ± S.E. of 5–10 different cells. *, p < 0.05; **, p < 0.01. Ins(1,3,4,6)P₄, inositol 1,3,4,6-tetrakisphosphate; Ins(3,4,5,6)P₄, inositol 3,4,5,6-tetrakisphosphate.

The graph shows increases in cell capacitance in the absence (−) and presence (+) of 100 μM InsP₆ in non-treated cells and following pretreatment with the calcineurin inhibitors cyclosporin A (1.5 μM for >15 min) and calphea C (1.5 μM for >15 min) or following inhibition of protein kinase A with (-)-cAMP (100 μM for >30 min). The rate of capacitance increase (ΔCm/Δt) was measured from 30 to 90 s after establishment of the whole-cell configuration. The line is the best fit of the average data to the Hill equation.

Fig. 2. InsP₆-induced exocytosis is dependent on PKC-activity.

The graph shows increases in cell capacitance in the absence (−) and presence (+) of 100 μM InsP₆ in cells treated with the calcineurin inhibitor cyclosporin A (1.5 μM for >15 min) and the PKC inhibitors calphostin C (1.5 μM for >15 min), bisindolylmaleimide (4 μM for >20 min), and staurosporine (100 nM for >10 min) or following inhibition of protein kinase A with (-)-cAMP (100 μM for >30 min). The rate of capacitance increase (ΔCm/Δt) was measured from 30 to 90 s. All inhibitors were included in the pipette-filling solution at the concentrations indicated above. The data are mean values ± S.E. for 5–8 different experiments. *, p < 0.05. /fF, femtofarads.
expressing wild-type PKC-β at 15 min. No significant stimulation of exocytosis was observed with cyclosporin A (1.5 μM). In all experiments, cells were treated infected with pcDNA alone (PKC-β II, -cells). As control, cells were transfected with pcDNA alone (mock). In all experiments, cells were treated with cyclosporin A (1.5 μM for >15 min). The data are mean values ± S.E. for 6–9 different experiments. *, p < 0.05.

mimicked by permethrin, an inactive analogue of deltamethrin (Fig. 1B). In the presence of permethrin, InsP6-induced exocytosis was inhibited by 65% (p < 0.05; n = 5), which is not different from that observed under control conditions (Fig. 1B). Finally, okadaic acid, an inhibitor of protein phosphatases 1, 2A, and 3, did not affect the InsP6-induced reduction of exocytosis (Fig. 1B). These data suggest that InsP6 stimulates both exocytosis and endocytosis in pancreatic β-cells and that the endocytic process dominates under the actual experimental conditions as suggested by the overall decrease in the rate of capacitance increase. In this respect, it is important to emphasize that capacitance measurements reflect net changes in plasma membrane area resulting from the summed activity of all endocytic and exocytotic processes. The stimulatory action of InsP6 on exocytosis was revealed following inhibition of endocytosis.

The stimulatory action of InsP6 on exocytosis was dependent on dose. No significant stimulation of exocytosis was observed at <10 μM InsP6, but higher concentrations elicited a dose-dependent acceleration of secretion (Fig. 1C). Approximating the average data points to the Hill equation yielded a half-maximal stimulatory effect at 22 μM and a cooperativity factor of 1.4. Maximal stimulation of exocytosis was seen at concentrations of InsP6 ≥100 μM (Fig. 1C), at which exocytosis was nearly doubled over the control rate of capacitance increase.

The ability of InsP6 to stimulate exocytosis depends on the [Ca2+]i in the pipette-filling solution (Fig. 1D). No stimulation was observed at 30 nM [Ca2+]i, but increasing concentrations of Ca2+ elicited a progressive increase in the rate of exocytosis, which was further accelerated in the presence of InsP6. The lack of effect of InsP6 at low [Ca2+]i contrasts previous findings in HIT insulinoma cells (2). This difference in Ca2+ sensitivity of the stimulatory action of InsP6 on exocytosis most likely reflects the use of a clonal cell line versus primary β-cells but could also result from different experimental approaches (permeabilized cells and single cell capacitance measurements).

Fig. 1E shows that the stimulatory action of InsP6 on exocytosis was also evoked, although to a lesser extent, by inositol tetakisphosphate compounds and the inositol pentakisphosphate, Ins(1,3,4,5,6)P5. These inositol polyphosphates stimulated exocytosis by only 48–66%, as compared with 86% increase in the presence of InsP6 (Fig. 1D). It is noteworthy that the required stimulatory concentrations of InsP6, but not InsP5, are 50–100 times higher than those measured in insulin-secreting cells (11). No stimulation of exocytosis was observed in the presence of 100 μM Ins(1,3,4)P3 and Ins(1,4,5)P3 (Fig. 1D). These data suggest that a minimum of four phosphate groups are required for stimulation of exocytosis and that these phosphate groups can be randomly placed on the inositol ring. Furthermore, the data show that all six phosphate groups are required for maximal stimulation of exocytosis.

InsP6 stimulates exocytosis by activation of PKC in permeabilized insulinoma cells (2). Here we extend this observation to mouse β-cells since the stimulatory action of InsP6 on exocytosis was abolished by the PKC inhibitors calphostin C (1.5 μM for >15 min), bisindolylmaleimide (4 μM for 20 min), and staurosporine (100 nM for >10 min) (Fig. 2). On the contrary, no effect was observed on the stimulatory action of InsP6 in the presence of the protein kinase A inhibitor (R)-cAMP (100 μM for >30 min). Under these conditions, InsP6 stimulated exocytosis by 90% (p < 0.05; n = 5; Fig. 2).

To investigate which isofrom of PKC mediates the stimulatory action of InsP6 on exocytosis, cells were treated for 48 h with antisense oligonucleotides against PKC-α, -βII, -δ, -ε, and -ζ. Fig. 3A shows that InsP6 failed to increase exocytosis in cells exposed to antisense oligonucleotides against PKC-ε. However, InsP6 retained its stimulatory action when cells were treated with wild-type PKC-β.

FIG. 3. PKC-ε controls InsP6-induced exocytosis in mouse β-cells. A, histogram depicting average rates of capacitance increase (ΔC/cm²) measured from 30 to 90 s after establishment of the whole-cell configuration in the absence (−) and presence (+) of 100 μM InsP6. The cells were treated for 48 h with 5 μM antisense oligonucleotides against PKC-α, -βII, -δ, -ε, and -ζ or sense oligonucleotide against PKC-β. B, same as in panel A, except that cells were transiently transfected with pcDNA alone (mock). In all experiments, cells were treated with cyclosporin A (1.5 μM for >15 min). The data are mean values ± S.E. for 6–9 different experiments. *, p < 0.05.

FIG. 4. Model for InsP6-regulated exocytosis and endocytosis in pancreatic β-cells. See “Results and Discussion” for details. KATP, ATP-sensitive K+ channel; Calci, calcineurin; PLC, phospholipase C; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate; PIP4, phosphatidylinositol tetrasphosphate; PIP5, phosphatidylinositol pentasphosphate; PIP6, phosphatidylinositol hexaphosphate.
with antisense oligonucleotides against PKC-α, -βII, -δ, -ε, and sense PKC-ε oligonucleotides. These data argue that InsP₆ stimulates exocytosis by a mechanism that involves activation of PKC-ε.

The involvement of PKC-ε in InsP₆-induced exocytosis was further supported by studies in mouse β-cells transiently transfected with either wild-type or a dominant negative mutant of PKC-ε. Fig. 3B shows that InsP₆ (100 μM) evoked a robust increase in cell capacitance in mock-transfected cells and in cells expressing wild-type PKC-ε. On the contrary, the dominant negative PKC-ε isoform abolished the ability of InsP₆ to stimulate exocytosis. Interestingly, the wild-type PKC-ε isoform significantly increased Ca²⁺-induced exocytosis, whereas the dominant negative isoform reduced the exocytotic response in the absence of InsP₆ by 50% (Fig. 3B). Transfection of the wild-type PKC-βI isoform did not influence Ca²⁺- and InsP₆-induced exocytosis (Fig. 3B).

The results from this study show for the first time that PKC-ε is involved in the stimulatory action of InsP₆ on Ca²⁺-evoked exocytosis. The molecular mechanism for the activation of PKC-ε by InsP₆ remains to be explored. However, it is pertinent to emphasize that an InsP₆-activated protein kinase has been identified in rat brain (12) and that InsP₆ enhances L-type Ca²⁺ channel activity in vascular smooth muscle cells by a PKC-dependent mechanism (13). The substrate(s) for PKC-ε are poorly understood, but recent data from our laboratories demonstrate that PKC-ε associates with insulin-containing granules in response to glucose and clinically used sulfonylureas.² These data suggest that PKC-ε represents an important component of the secretory network in pancreatic β-cells.

A model for the effects of glucose and InsP₆ on exocytosis and endocytosis is presented in Fig. 4. Glucose acts to stimulate ATP synthesis, leading to closure of ATP-sensitive K⁺ channels, cell depolarization, and Ca²⁺ influx. The resulting increase in cytoplasmic Ca²⁺ concentration stimulates exocytosis. Not only activation of the phospholipase C system but also glucose stimulation result in InsP₆ production, which leads to enhanced PKC-ε activity. PKC-ε potentiates Ca²⁺-induced exocytosis. An increase in cytoplasmic free Ca²⁺ concentration is also required for stimulation of endocytosis. This process involves activation of calcineurin and PKC-ε, most likely resulting in a sequential phosphorylation and dephosphorylation of proteins involved in endocytosis (for details, see Ref. 3). This suggests that InsP₆ has an important integral role in pancreatic β-cell membrane trafficking, being part of the molecular mechanisms linking exocytosis and endocytosis.

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J. Biol. Chem. 2003, 278:35168-35171.
doi: 10.1074/jbc.M303927200 originally published online July 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303927200

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