Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4435–4439, April 1997
Cell Biology

Inositol hexakisphosphate stimulates non-Ca^{2+}-mediated and primes Ca^{2+}-mediated exocytosis of insulin by activation of protein kinase C

(insulin exocytosis/HIT T15 cells)

ALEXANDRE M. EFANOV*†‡, SERGEI V. ZAITSEV*†‡, AND PER-OLOF BERGGREN*

*The Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institute, Karolinska Hospital, S-17176 Stockholm, Sweden; and †Belozersky Institute of Physico-Chemical Biology, 119889 Moscow State University, Moscow, Russia

Communicated by Viktor Mutt, Karolinska Institutet, Stockholm, Sweden, March 3, 1997 (received for review November 18, 1996)

ABSTRACT **D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$), formed via complex pathways of inositol phosphate metabolism, composes the main bulk of inositol polyphosphates in the cell. Relatively little is known regarding possible biological functions for $InsP_6$. We now show that $InsP_6$ can modulate insulin exocytosis in permeabilized insulin-secreting cells. Concentrations of $InsP_6$ above 20 μM stimulated insulin secretion at basal Ca^{2+}-concentration (30 nM) and primed Ca^{2+}-induced exocytosis (10 μM), both effects being due to activation of protein kinase C. Our results suggest that $InsP_6$ can play an important modulatory role in the regulation of processes such as exocytosis in insulin-secreting cells. The specific role for $InsP_6$ can then be to recruit secretory granules to the site of exocytosis.**

Highly phosphorylated inositol phosphates are the major inositol phosphates present in the cell (1). Specifically, the intracellular concentration of D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$) reaches levels corresponding to 40–60 μM in several cell types, including insulin-secreting cells (2–4). In some cell types the concentration of these inositol polyphosphates can be rapidly altered upon cell stimulation (5, 6).

Inositol polyphosphates may be involved in the control of several cellular functions (1), one of which is regulation of vesicle trafficking, processes of endocytosis and vesicle recycling. D-*myo*-inositol 1,3,4,5-tetrakisphosphate, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) and $InsP_6$ bind to the C2B domain of synaptotagmin (7), a protein believed to be a main Ca^{2+}-sensor in regulated exocytosis in neurons. The injection of inositol polyphosphates into squid giant synapse preterminal caused blocking of synaptic transmission, due to binding of injected compounds to the C2B domain of synaptotagmin (8). Inositol polyphosphates binding to clathrin assembly proteins, adaptor proteins AP-2 (9), and AP-3 (10) inhibit clathrin assembly, suggesting the influence of the compounds upon vesicle trafficking (10).

The aim of the present study was to evaluate a possible role for $InsP_6$ in the regulation of insulin exocytosis.

MATERIALS AND METHODS

Materials. $InsP_5$ and $InsP_6$ were from Calbiochem–Novabiochem. Streptolysin O was obtained from Difco. All other reagents were from Sigma.

Cell Culture. Hamster insulinoma tumor HIT T15 cells were cultured at 37°C in RPMI medium 1640 containing 11 mM glucose and supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 10^{-7} M selenous acid, and 10 μg/ml glutathione, as described (11). All experiments were carried out in HIT T15 cells of passage numbers between 73–80.

Cell Permeabilization and Measurements of Insulin Secretion. Two different procedures were used to permeabilize cells, namely permeabilization by electric field and streptolysin O-induced permeabilization. The permeabilization buffer consisted of 140 mM potassium glutamate, 5 mM NaCl, 1.2 mM $MgCl_2$, 10 mM EGTA, 25 mM Hepes, 0.025% albumin (pH 7.0). For streptolysin O-induced permeabilization, 1 mM dithiothreitol was added to the buffer. The electropermeabilization and permeabilization with streptolysin O were performed as described (12, 13).

Both types of permeabilization have their own benefits and drawbacks. Electropermeabilization creates only small holes in the plasma membrane, that prevents leakage of molecules >5 kDa from the cell. These holes reseal with time (14). By contrast, cells permeabilized with streptolysin O are leaking not only small molecules but also large proteins. Cells permeabilized by this procedure do not reseal (15). The use of one or the other of the procedures was dependent on the aim of each particular experiment.

Measurements of insulin secretion were performed in a permeabilization buffer containing additionally 2 mM MgATP and an ATP-regenerating system, 2 mM creatine phosphate, 10 unit/ml creatine phosphokinase, and Ca^{2+} concentrations in the range from 3×10^{-8} M to 1×10^{-5} M. Priming effects of $InsP_6$ on insulin secretion were measured as follows. Cells were treated with different concentrations of $InsP_6$ for 15 min in ATP-containing buffer and then insulin secretion was measured in ATP-free buffer without $InsP_6$ at 30 nM or at 10 μM Ca^{2+} during 15 min. Insulin content was determined by radioimmunoassay using rat insulin as a standard. The level of insulin secretion at 30 nM Ca^{2+}, ranging from 30–50 μunits/10^5 cells/15 min in different experiments, was taken as 100%.

Free Ca^{2+}-concentration in solutions was measured with a Ca^{2+}-selective electrode (model 93-20, Orion, Boston). As standard solutions, Ca^{2+}-buffers (World Precision Instruments, Sarasota, FL) with known Ca^{2+} concentrations were used.

Measurements of Protein Kinase C (PKC) Activity. PKC activity was assayed by measuring the transfer of ^{32}P to a specific enzyme substrate. PKC Assay system (Promega) provided high specificity of the assay by using biotinylated peptide substrate and streptavidin-coated filters. Briefly, cells were washed several times in PBS buffer, resuspended in extraction buffer (25 mM Tris·HCl, pH 7.4/0.5 mM EDTA/0.5 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/944435-5$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: $InsP_5$, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate; PKC, protein kinase C; PP-$InsP_5$ and $(PP)_2$-$InsP_4$, inositol polyphosphate pyrophosphates; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate.

‡A.M.E. and S.V.Z. contributed equally to this paper.

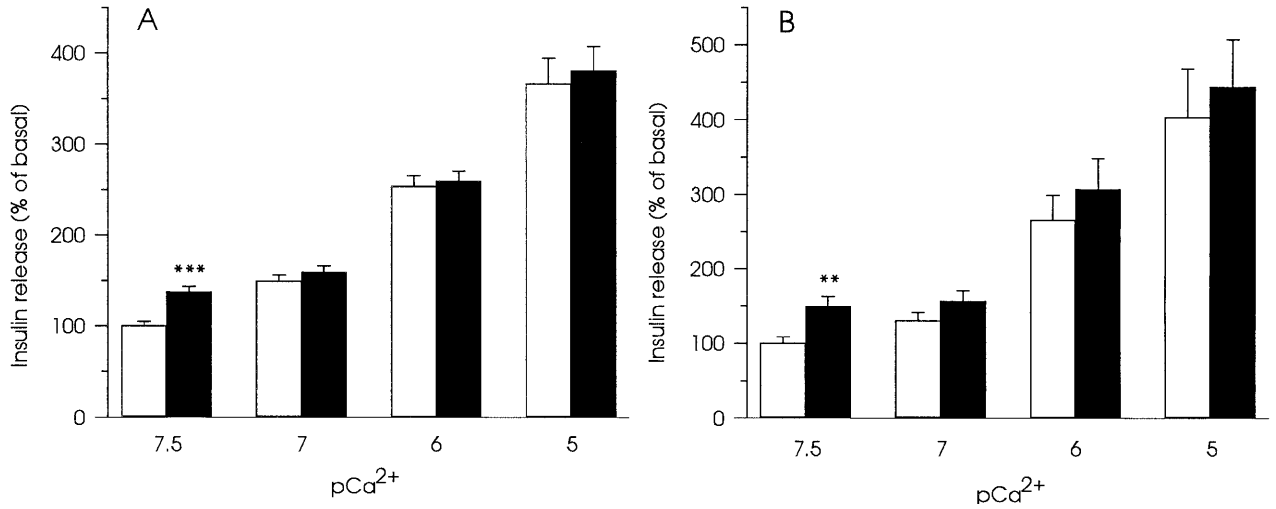

FIG. 1. Effects of 20 μM $InsP_5$ (*A*) and 20 μM $InsP_6$ (*B*) on insulin secretion in electropermeabilized HIT T15 cells at different free Ca^{2+}-concentrations. Secretion without inositol polyphosphates is denoted by □ and with inositol polyphosphates by ■. Data represent mean ± SEM for 16 observations from three separate experiments. **, $P < 0.01$ and ***, $P < 0.001$, relative to insulin secretion without inositol polyphosphates.

EGTA/10 mM 2-mercaptoethanol/1 μg/ml leupeptin/1 μg/ml aprotinin/0.5 mM phenylmethylsulfonyl fluoride) and sonicated. Enzyme activity was assayed in the presence of phospholipids (0.35 mg/ml phosphatidylserine and 0.035 mg/ml diacylglycerol) and either without (2.5 mM EGTA and 0.1 mM EDTA) or with Ca^{2+} (4.5 mM $CaCl_2$, 2.5 mM EGTA, and 0.1 mM EDTA) for 8 min at 30°C. Reactions were started by addition [γ-^{32}P]ATP and terminated by spotting on streptavi-

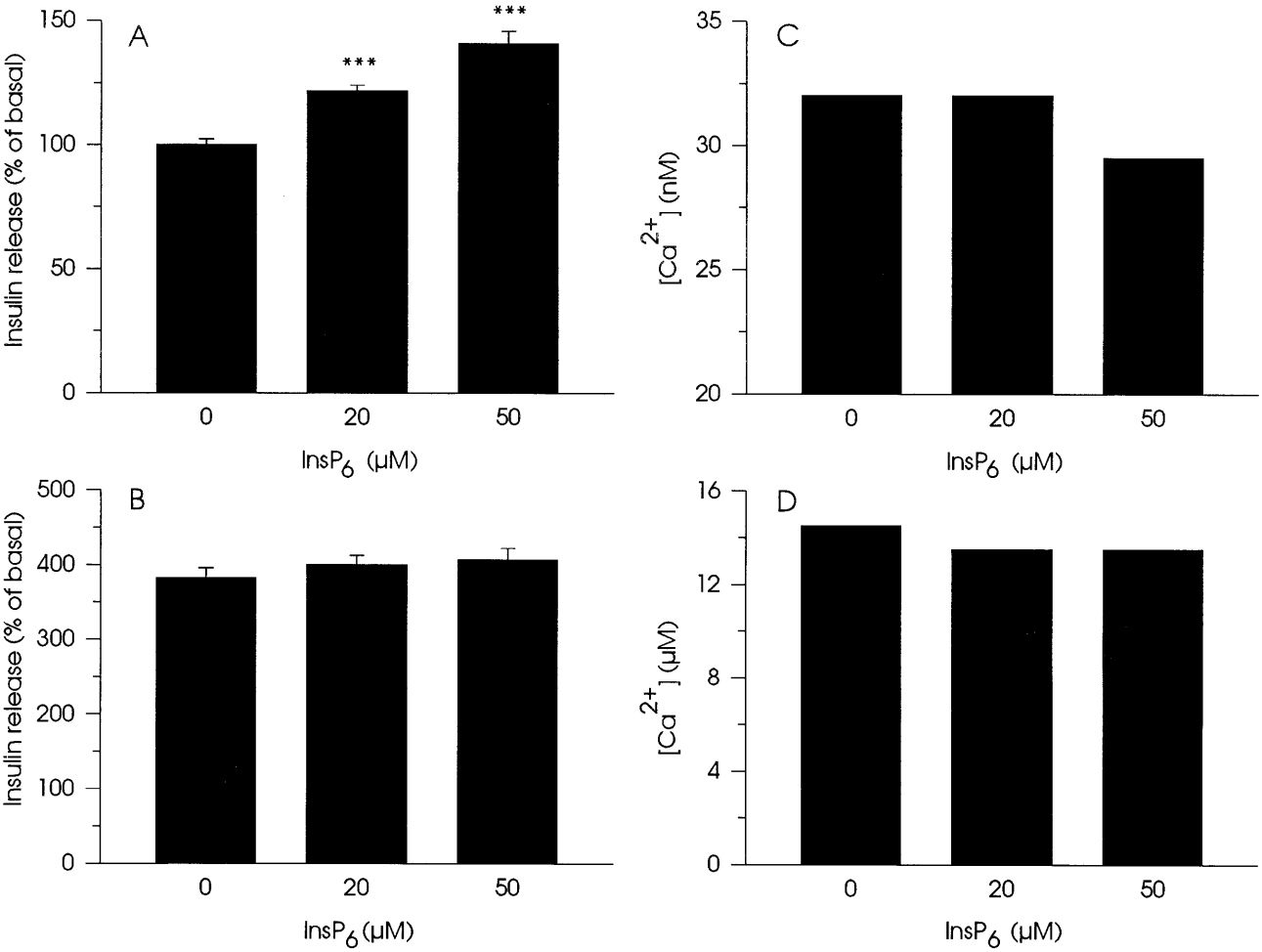

FIG. 2. Concentration-dependent effects of $InsP_6$ on insulin secretion in electropermeabilized cells in the presence of either 30 nM (*A*) or 10 μM (*B*) Ca^{2+} and on free Ca^{2+}-concentration in the permeabilization buffer containing either ≈30 nM (*C*) or ≈10 μM (*D*) Ca^{2+}. For measurements of insulin secretion, data represent mean ± SEM for 18 observations from three separate experiments. ***, $P < 0.001$, relative to insulin secretion without $InsP_6$. For measurements of Ca^{2+}, representative experiments of three are presented.

din-coated filters. Incorporation of ^{32}P into biotinylated substrate was assessed by liquid scintillation counting.

Presentation of Results. Data analysis was performed using the program SIGMA PLOT 1.02 for Windows (Jandel, San Rafael, CA). All results are expressed as means ± SEM for the indicated number of observations. Statistical significance of differences between means were assessed by Student's unpaired *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

The effects of 20 μM $InsP_5$ and 20 μM $InsP_6$ on insulin secretion were tested at free Ca^{2+}-concentrations ranging from 30 nM to 10 μM in electropermeabilized HIT T15 cells (Fig. 1). Only at basal (30 nM) free Ca^{2+} concentration, both compounds stimulated exocytosis of insulin. The stimulatory effect could not be detected at higher Ca^{2+}-concentrations. Since $InsP_6$ is present at high concentrations in insulin-secreting cells (4), this inositol polyphosphate was chosen for the investigation of the mechanism underlying the stimulatory effects of inositol polyphosphates on insulin secretion.

Stimulation of non-Ca^{2+}-mediated insulin secretion by $InsP_6$ was concentration-dependent and at 50 μM $InsP_6$, insulin release reached ≈150% of secretion obtained in the absence of inositol polyphosphate (Fig. 2*A*). The stimulatory effects of $InsP_6$ on insulin secretion could not be detected when the Ca^{2+}-concentration was increased (Fig. 2*B*). To check if the observed effects of $InsP_6$ were due to possible changes in free Ca^{2+}-concentration induced by the compound, measurements of the ambient free Ca^{2+}-concentration in the permeabilization buffer were performed (Fig. 2 *C* and *D*). At a concentration of 20 μM, $InsP_6$ did not alter resting Ca^{2+}-concentration (30 nM) (Fig. 2*C*). Under this condition 50 μM of $InsP_6$ only reduced resting Ca^{2+}-concentration by 20%. $InsP_6$ (20 and 50 μM) decreased stimulating Ca^{2+}-concentration not more than 7–10% (Fig. 2*D*). Although $InsP_6$ has a well-documented ability to chelate Ca^{2+}-ions (16), the Ca^{2+}-buffers used in this study have enough capacity to prevent significant changes in free Ca^{2+}-concentration in the presence of $InsP_6$. Another Ca^{2+}-chelator, 100 μM EGTA, added to the incubation buffer produced analogous slight decrease in free Ca^{2+}-concentration but did not significantly affect insulin secretion measured at 30 nM Ca^{2+} (data not shown). Thus, the effects of $InsP_6$ on insulin secretion cannot be accounted for by changes in the free Ca^{2+}-concentration.

$InsP_6$ is not the last member in the array of inositol polyphosphates. Higher phosphorylated inositol polyphosphate pyrophosphates, PP-$InsP_5$ and $(PP)_2$-$InsP_4$, formed from $InsP_6$ and $InsP_5$, have also been described (17). It has been proposed that inositol polyphosphate pyrophosphates are intracellular energy stores because of the high energy hydrolysis of pyrophosphoryl

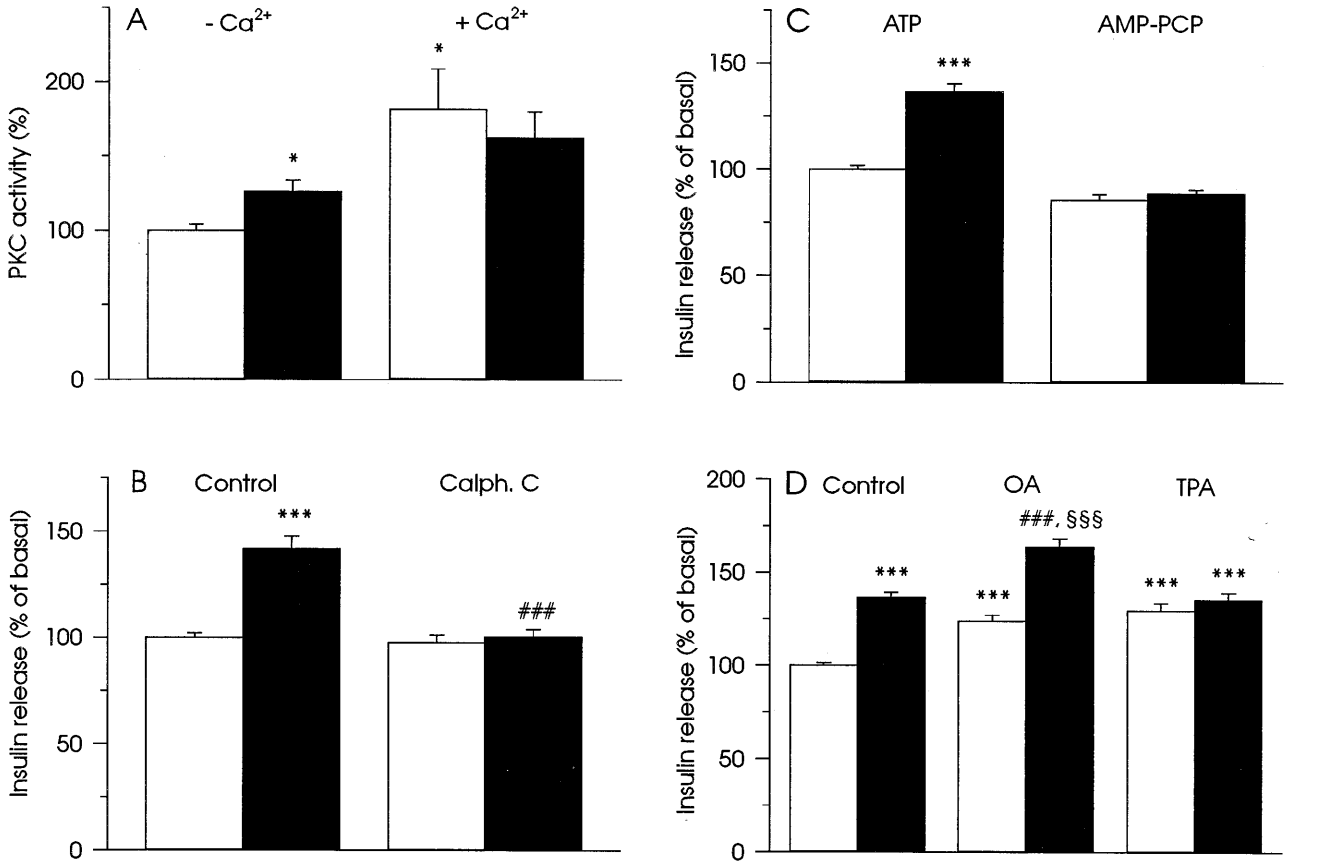

FIG. 3. Involvement of PKC activation in the stimulation of insulin secretion in HIT T15 cells by $InsP_6$. (*A*) Modulation of PKC activity by 50 μM $InsP_6$ (■) in the absence and the presence of free Ca^{2+} as described in *Materials and Methods*. (□) The enzyme activity without $InsP_6$. Data represent mean ± SEM of four experiments. *, $P < 0.05$, relative to PKC activity in the absence of free Ca^{2+} and $InsP_6$. (*B*) Inhibitor of PKC, 1.5 μM Calphostin C (Calph. C), blocked increase in insulin secretion induced by 50 μM $InsP_6$. (*C*) Requirement of ATP-hydrolysis for the development of the stimulatory effect of 50 μM $InsP_6$ on insulin secretion. ATP (2 mM) was substituted by 2 mM AMP-PCP, a nonhydrolyzable analog of ATP. (*D*) Additive effect of 50 μM $InsP_6$ and 1 μM okadaic acid (OA) on stimulation of insulin secretion and the absence of additive effect of 50 μM $InsP_6$ and 100 nM TPA. In *B–D* insulin secretion was measured in buffer with basal Ca^{2+} concentration (30 nM). (□) Insulin secretion without $InsP_6$ and black columns illustrate insulin secretion with 50 μM $InsP_6$. The presence of other compounds is indicated in the figure. For measurements of insulin secretion, data represent mean ± SEM for 17 (*B*), 18 (*C*), and 20 (*D*) observations from three separate experiments. ***, $P < 0.001$, relative to insulin secretion at 30 nM Ca^{2+}. ###, $P < 0.001$, relative to insulin secretion in the presence of 50 μM $InsP_6$. §§§, $P < 0.001$, relative to insulin secretion in the presence of 1 μM OA.

residues (18). Therefore, we tested the specificity of the effect of $InsP_6$ on exocytosis. The process of formation of pyrophosphates can be activated by NaF, through the inhibition of phosphatases responsible for pyrophosphate dephosphorylation (17). Addition of 5 mM NaF, this concentration maximally increased PP-$InsP_5$ concentration but did not significantly activate G-proteins (17), instead of increasing slightly decreased $InsP_6$-stimulated insulin release (139 ± 3% increase in secretion with 50 μM $InsP_6$ vs. 127 ± 2% with 50 μM $InsP_6$ and NaF, 15 observations from three separate experiments). This supports the suggestion that $InsP_6$ rather than PP-$InsP_5$ or $(PP)_2$-$InsP_4$ stimulated exocytosis.

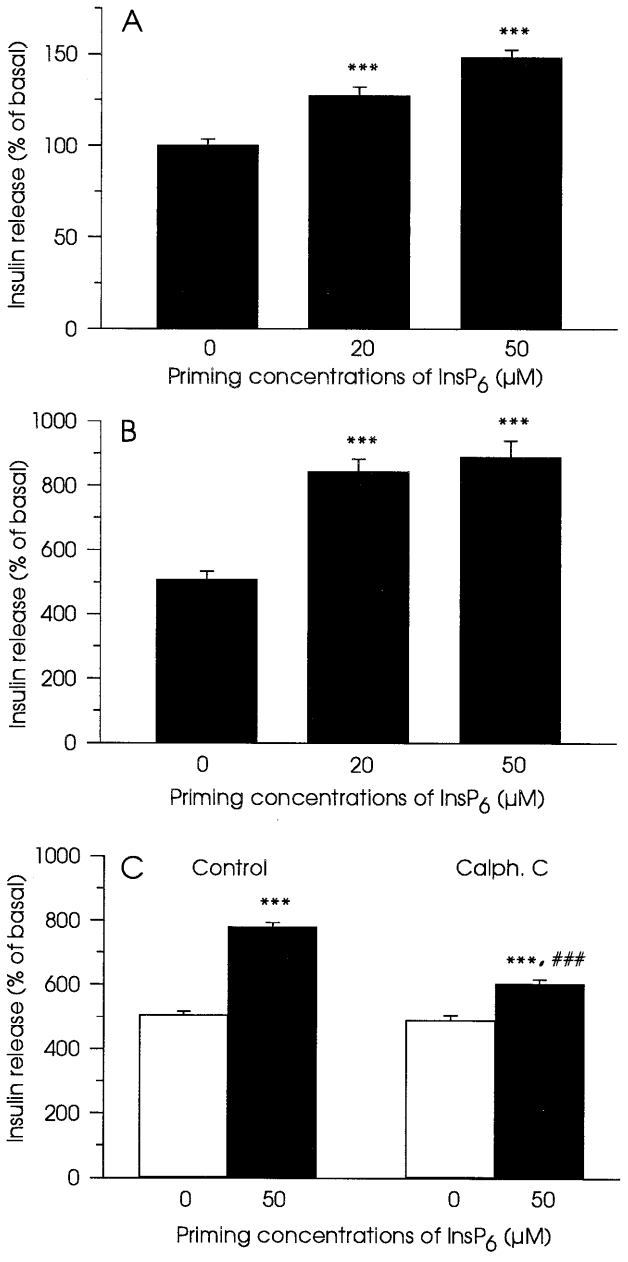

FIG. 4. Priming of insulin secretion by $InsP_6$ in streptolysin O-permeabilized cells. Cells were pretreated with or without different concentrations of $InsP_6$ for 15 min in ATP-containing buffer and then insulin secretion in ATP-free buffer was measured during 15 min at 30 nM (*A*) or at 10 μM Ca^{2+} in the absence of $InsP_6$ (*B* and *C*). In *C*, 1.5 μM Calphostin C (Calph. C) was introduced together with $InsP_6$. Data represent mean ± SEM for 15 observations from three separate experiments. ***, $P < 0.001$, relative to insulin secretion without $InsP_6$ and at appropriate Ca^{2+} concentration. ###, $P < 0.001$, relative to insulin secretion in the presence of $InsP_6$.

One of the reported effects of inositol polyphosphates, is activation of PKC isoenzymes as well as the PKC-related kinase, PRK1 (19). The kinase activation was observed only in a Ca^{2+}-free medium and at inositol polyphosphate concentrations similar to those that stimulated insulin secretion in our experiments. Therefore, we examined the possibility that stimulation of exocytosis by inositol polyphosphates can be explained by activation of PKC. In HIT T15 cells, 50 μM $InsP_6$ activated PKC in the absence of free Ca^{2+} and did not significantly affect Ca^{2+}-induced activation of the enzyme (Fig. 3*A*).

The selective inhibitor of PKC, Calphostin C (1.5 μM) abolished stimulation of insulin secretion by 50 μM $InsP_6$ (Fig. 3*B*). To confirm that protein kinase activation is involved in the stimulatory effect of $InsP_6$ on insulin secretion, experiments substituting ATP with its non-hydrolyzable analog β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), which cannot serve as a substrate for protein kinase-induced phosphorylation, were performed. AMP-PCP blocked $InsP_6$-induced exocytosis (Fig. 3*C*). Finally, 1 μM okadaic acid, an inhibitor of protein phosphatases type 1, 2A, and 3 (20), potentiated the effect of 50 μM $InsP_6$ on insulin release, whereas 100 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent activator of PKC, did not (Fig. 3*D*). This suggests that activation of PKC rather than inhibition of protein phosphatases is responsible for the stimulation of exocytosis by $InsP_6$.

Experiments presented so far described acute effects of $InsP_6$ on non-Ca^{2+}-mediated insulin exocytosis. To evaluate possible effects of $InsP_6$ on Ca^{2+}-mediated exocytosis, cells were preincubated with the inositol polyphosphate in the absence of Ca^{2+} and subsequently stimulated with high concentrations of the ion. We have employed streptolysin O-permeabilized cells for this type of experiments, since the stability of the formed holes in the plasma membrane during prolonged time of incubation was essential. Permeabilized cells were first incubated in a buffer containing ATP, an ATP-regenerating system, 30 nM Ca^{2+}, and different concentrations of $InsP_6$. This buffer was replaced by a buffer containing 30 nM or 10 μM Ca^{2+} without $InsP_6$ and ATP. Insulin content in the latter buffer was measured. Changing of permeabilization procedure did not perturb the ability of $InsP_6$ to affect insulin exocytosis. $InsP_6$ (20 and 50 μM) also primed non-Ca^{2+}-mediated exocytosis (Fig. 4*A*). The effect of $InsP_6$ on Ca^{2+}-mediated exocytosis was pronounced. In this case, both 20 μM and 50 μM of the compound stimulated insulin secretion by 45% (Fig. 4*B*). Calphostin C (1.5 μM) suppressed insulin secretion induced by 50 μM $InsP_6$ (Fig. 4*C*).

DISCUSSION

Permeabilized insulin-secreting cells were used to investigate a possible role for $InsP_6$ in regulated exocytosis. $InsP_6$ stimulated non-Ca^{2+}-mediated and primed Ca^{2+}-mediated exocytosis of insulin, effects mediated through the activation of PKC. The involvement of PKC was verified by experiments showing that the $InsP_6$ effect was blocked with Calphostin C and substitution of ATP with the non-hydrolyzable analog AMP-PCP. The potentiation of insulin secretion by $InsP_6$ in the presence of 1 μM okadaic acid, a concentration known to inhibit protein phosphatases type 1, 2A, and 3 (20), suggests that the observed stimulatory activity of $InsP_6$ on insulin release cannot be explained by an inhibitory activity of the compound on these protein phosphatases. Activation of PKC by $InsP_6$ in insulin-secreting cells, in the absence of Ca^{2+}, is consistent with the data that $InsP_6$, at concentrations higher than 20 μM, activates several isoenzymes of PKC as well as PKC-related kinase (19).

Processes of protein phosphorylation play a central role in the regulation of exocytosis in the pancreatic β-cell (21, 22). Activation of PKC by $InsP_6$ may lead to increased availability of releasable secretory granules, by promoting the recruitment and transport of granules to the site of exocytosis. The increasing amount of granules associated with the plasma membrane and

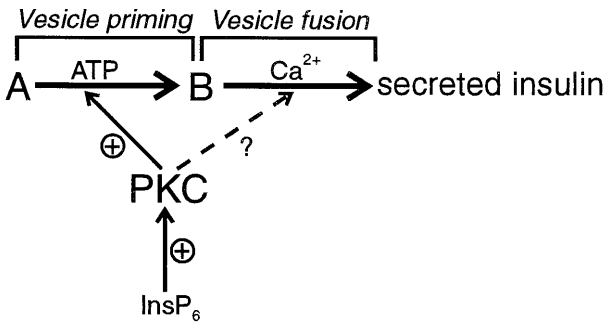

FIG. 5. Scheme illustrating the role of $InsP_6$ in insulin exocytosis. *A* denotes an intracellular pool of insulin containing granules that are not activated for fusion, and *B* denotes a pool of granules close to the plasma membrane that undergoes Ca^{2+}-activated fusion. The exocytotic process of insulin can thus be separated into ATP-dependent vesicle priming and ATP-independent Ca^{2+}-activated fusion. According to the scheme the rate of insulin secretion is determined by the amount of primed vesicles and the probability of the fusion event for each granule. Through activation of PKC, $InsP_6$ may influence both of these steps of insulin secretion. Hence, the processes discussed in this paper may be presented in the following way. (*i*) Insulin secretion at resting Ca^{2+} concentration and in the absence of $InsP_6$. Insulin release is restricted by the amount of primed vesicles and a low probability of vesicle fusion at resting Ca^{2+} concentration. (*ii*) Activation of insulin secretion at resting Ca^{2+} concentration by $InsP_6$. $InsP_6$ activates PKC, which leads to an increased priming of secretory vesicles and maybe in addition direct activation of the fusion process. Modest stimulation of insulin release is observed relative to *i*. (*iii*) Ca^{2+}-induced exocytosis in the absence of $InsP_6$. Probability of vesicle fusion is high. It gives rise to a pronounced stimulation of insulin secretion, but the stimulation is still not maximal and limited by the availability of primed vesicles. (*iv*) Priming of Ca^{2+}-induced exocytosis by $InsP_6$. Similar to *ii*, $InsP_6$ increased priming of secretory vesicles, an effect mediated by PKC activation. A high probability of vesicle fusion together with an increased amount of vesicles at the site of exocytosis lead to pronounced insulin secretion.

ready-to-fuse would lead to the subsequent increase in insulin exocytosis. In addition, $InsP_6$ may directly affect granule fusion since the conformation of proteins responsible for fusion events in exocytosis could be controlled by PKC activity (23, 24).

The fact that the stimulatory effect of $InsP_6$ on insulin exocytosis disappeared at elevated Ca^{2+}-concentrations, may be explained by the conversion of $InsP_6$ into the inactive Ca^{2+}-bound form, each molecule of $InsP_6$ binding two or three Ca^{2+}-ions (16). Such a conversion is in agreement with what has previously been suggested in the context of the disappearance of the stimulatory effect of inositol hexakisphosphate on PKC activity at high concentrations of Ca^{2+} (19). In the latter study, the authors suggested that the negative charge of phosphate groups plays a significant role in the effects of $InsP_6$.

As discussed above, $InsP_6$ readily chelates Ca^{2+}-ions. This complicates any investigation of the role of the compound in Ca^{2+}-mediated exocytosis. We have separated in time the preincubation of cells with $InsP_6$ in ATP-containing medium and subsequent incubation of cells in the buffer containing high Ca^{2+}-concentration in the absence of ATP and $InsP_6$. The results obtained show that in HIT T15 cells Ca^{2+} triggers exocytosis of insulin in an ATP-independent manner, as described before for exocytosis of biogenic amines in PC12 cells (25–27). By applying this type of incubation procedure, we found that preincubation with $InsP_6$ led to a markedly pronounced stimulatory effect on Ca^{2+}-induced insulin exocytosis. It is likely that $InsP_6$ under these conditions potentiated insulin release by priming Ca^{2+}-mediated fusion of secretory granules with the plasma membrane. Hence, inositol polyphosphates may keep granule transport active under basal conditions and thereby increasing the number of granules available at the site of exocytosis, explaining both the stimulatory effect of $InsP_6$ on non-Ca^{2+}-mediated- and the priming of Ca^{2+}-mediated insulin release (Fig. 5).

Intracellular levels of inositol polyphosphates vary, being particularly high in several cell types like the insulin-secreting cell. By influencing the rates of $InsP_6$ synthesis and conversion, $InsP_6$ concentration may be rapidly changed, which should in turn give rise to modulation of insulin exocytosis. We have shown that $InsP_6$, in a physiological concentration range, is able to modulate exocytosis. At 20–50 μM concentrations, this compound stimulated insulin secretion at basal Ca^{2+}-concentration and primed Ca^{2+}-mediated exocytosis. Both effects of $InsP_6$ were dependent on the activation of PKC. This suggests that inositol polyphosphates can function as signaling molecules in stimulus-secretion coupling in pancreatic β-cells.

We are grateful to Dr. C. J. Barker for fruitful discussions. This work was supported by the Swedish Medical Research Council (03X-09890 and 19X-00034), the Royal Swedish Academy of Sciences, the Swedish Foundation for International Cooperation in Research and Higher Education, the Swedish Diabetes Association, the Nordic Insulin Foundation Committee, Juvenile Diabetes Foundation International, and funds from the Karolinska Institute.

1. Sasakawa, N., Sharif, M. & Hanley, M. R. (1995) *Biochem. Pharmacol.* **50,** 137–146.
2. French, P. J., Bunce, C. M., Stephence, L. R., Lord, J. M., McConnell, F. M., Brown, G., Creba, J. A. & Michell, R. H. (1991) *Proc. R. Soc. London B* **245,** 193–201.
3. Jackson, T. R., Hallam, T. J., Downes, C. P. & Hanley, M. R. (1987) *EMBO J.* **6,** 49–54.
4. Li, G., Pralong, W. F., Pittet, D., Mayr, G. W., Schlegel, W. & Wollheim, C. B. (1992) *J. Biol. Chem.* **267,** 4349–4356.
5. Pittet, D., Schlegel, W., Lew, D. P., Monod, A. & Mayr, G. W. (1989) *J. Biol. Chem.* **264,** 18489–18493.
6. Sasakawa, N., Nakaki, T., Kakinuma, E. & Kato, R. (1993) *Brain Res.* **623,** 155–160.
7. Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. & Mikoshiba, K. (1994) *J. Biol. Chem.* **269,** 29206–29211.
8. Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M., Mikoshiba, K. & Llinas, R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10708–10712.
9. Beck, K. A. & Keen, J. H. J. (1991) *J. Biol. Chem.* **266,** 4442–4447.
10. Ye, W., Ali, N., Bembenek, M. E., Shears, S. B. & Lafer, E. M. (1995) *J. Biol. Chem.* **270,** 1564–1568.
11. Prentki, M., Glennon, M. C., Geschwind, J. F., Matschinsky, F. M. & Corkey, B. E. (1987) *FEBS Lett.* **220,** 103–107.
12. Vallar, L., Biden, T. J. & Wollheim, C. B. (1987) *J. Biol. Chem.* **262,** 5049–5056.
13. Li, G., Regazzi, R., Balch, W. E. & Wollheim, C. B. (1993) *FEBS Lett.* **327,** 145–149.
14. Knight, D. E. & Scrutton, M. C. (1986) *Biochem J.* **234,** 497–506.
15. Ahnert-Hilger, G., Stecher, B., Beyer, C. & Gratzl, M. (1993) *Methods Enzymol.* **221,** 139–149.
16. Luttrell, B. M. (1993) *J. Biol. Chem.* **268,** 1521–1524.
17. Menniti, F. S., Miller, R. N., Putney, J. W. & Shears, S. B. (1993) *J. Biol. Chem.* **268,** 2850–3856.
18. Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dorman, G., Olszewski, J. D., Prestwich, G. D. & Snyder, S. H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4305–4310.
19. Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, J. A. L., Gigg, R. & Parker, P. J. (1995) *J. Biol. Chem.* **270,** 22412–22416.
20. Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256,** 283–290.
21. Ämmälä, C., Eliasson, L., Bokvist, K., Berggren, P.-O., Honkanen, R. E., Sjöholm, Å. & Rorsman, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4343–4347.
22. Zaitsev, S. V., Efendić, S., Arkhammar, P., Bertorello, A. M. & Berggren, P.-O. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9712–9716.
23. Fujita, Y., Sasaki, T., Fukui, K., Kotani, H., Kimura, T., Hata, Y., Sudhof, T. C., Scheller, R. H. & Takai, Y. (1996) *J. Biol. Chem.* **271,** 7265–7268.
24. Shimazaki, Y., Nishiki, T., Omori, A., Sekiguchi, M., Kamata, Y., Kozaki, S. & Takahashi, M. (1996) *J. Biol. Chem.* **271,** 14548–14553.
25. Hay, J. C. & Martin, T. F. J. (1992) *J. Cell Biol.* **119,** 139–151.
26. Bittner, M. A. & Holz, R. W. (1992) *J. Biol. Chem.* **267,** 16219–16225.
27. Thomas, P., Wong, J. G., Lee, A. K. & Almers, W. (1993) *Neuron* **11,** 93–104.