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Evidence that the inositol phospholipids are necessary for exocytosis

Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP

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We directly manipulated the levels of PtdIns, PtdIns*P* and PtdInsP_2 in digitonin-treated adrenal chromaffin cells with a bacterial phospholipase C (PLC) from *Bacillus thuringiensis* and by removal of ATP. The PtdIns-PLC acted intracellularly to cause a large decrease in [^{3}H]inositol- or [^{32}P]phosphate-labelled PtdIns, but did not directly hydrolyse PtdIns*P* or PtdInsP_2. [^{3}H]PtdIns*P* and[^{3}H]PtdInsP_2 levels declined markedly, probably because of the action of phosphatases in the absence of synthesis. Removal of ATP also caused marked decreases in [^{3}H]PtdIns*P* and [^{3}H]PtdInsP_2. The decrease in polyphosphoinositide levels by PtdIns-PLC treatment or ATP removal was reflected by the inhibition of the production of inositol phosphates upon subsequent activation of the endogenous PLC by Ca^{2+}. Ca^{2+}-dependent catecholamine secretion from permeabilized cells was strongly inhibited by PtdIns-PLC treatment and by ATP removal. Ca^{2+}-dependent secretion was similarly correlated with the sum of PtdIns*P* and PtdInsP_2 when the level of these lipids was changed by either manipulation. PtdIns-PLC inhibited only the ATP-dependent component of secretion and did not affect ATP-dependent secretion. Both PtdIns-PLC and ATP removal inhibited the late slow phase of secretion, but had little effect on the initial rapid phase. Although we found a tight correlation between polyphosphoinositide levels and secretion, endogenous phospholipase C activity (stimulated by Ca^{2+}, guanine nucleotides and related agents) was not correlated with secretion. Additional experiments indicated that neither the products of the PtdIns-PLC reaction (diacylglycerol and InsP_1) nor the inability to generate products by subsequent activation of the endogenous PLC is likely to account for the inhibition of secretion. Incubation of permeabilized cells with neomycin in the absence of ATP maintained the level of polyphosphoinositides and more than doubled subsequent Ca^{2+}-dependent secretion. The data suggest that: (1) Ca^{2+}-dependent secretion has a requirement for the presence of inositol phospholipids; (2) the enhancement of secretion by ATP results in part from increased polyphosphoinositide levels; and (3) the role for inositol phospholipids in secretion revealed in these experiments is independent of their being substrates for the generation of diacylglycerol and InsP_3.

INTRODUCTION

In many secretory cells, the coupling of the initial stimulus to secretion involves the activation of phospholipase C, which hydrolyses PtdInsP_2 to form InsP_3 and diacylglycerol (DAG). InsP_3 produces an increase in cytosolic Ca^{2+} by releasing Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C. Ca^{2+} and protein kinase C are then thought to act in concert to modulate secretion [for reviews see Nishizuka (1986) and Berridge (1987)]. Little is known, however, about the effects on secretion of varying the amounts of inositol phospholipids. In the present study we directly investigate the effects of changes in inositol phospholipid metabolism on Ca^{2+}-dependent catecholamine secretion in digitonin-permeabilized chromaffin cells. Because digitonin-treated chromaffin cells are permeable to proteins (Dunn & Holz, 1983; Lee *et al.*, 1987; Holz & Senter, 1988), we were able to examine the intracellular effects of a 34 kDa PtdIns-specific phospholipase C (PLC) from *Bacillus thuringiensis* on inositol phospholipids and on secretion. The enzyme decreased the inositol phospholipids and inhibited secretion. The relationship between the inhibition of secretion and changes in the inositol phospholipids caused by the enzyme and by removal of ATP suggests that the polyphosphoinositides are required for secretion, independent of their being substrates for endogenous PLC.

MATERIALS AND METHODS

Chromaffin cells were isolated by dissociation of bovine adrenal medullae, purified by differential plating (Waymire *et al.*, 1983), and cultured as monolayers in 6.4 mm-diameter collagen-coated wells, at 150000 cells/well as previously described (Holz *et al.*, 1982). The cultures consisted of at least 90% chromaffin cells and contained virtually no visually detectable fibroblasts or endothelial cells. Experiments were performed 4–12 days after culture preparation.

Cellular inositol-containing lipids were labelled by incubation for 36–60 h with *myo*-[2-^{3}H]inositol (20 μCi/ml) in Eagle's minimal essential medium (which contained 11 μM-*myo*-inositol) supplemented with 10% (v/v) dialysed fetal-bovine serum, glutamine, penicillin and streptomycin. Isotopic equilibrium was attained after about 48 h of labelling. Immediately before the

Abbreviations used: DAG, diacylglycerol; diC$_8$, 1,2-dioctanoyl-*sn*-glycerol; GTP[S], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]triphosphate; IPP, inositol polyphosphates; KGEP, buffer containing potassium glutamate, EGTA and Pipes; PLC, phospholipase C; PSS, physiological salt solution; PtdOH, phosphatidic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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start of an experiment, the labelling medium was removed and the cells were washed for 15 min in physiological salt solution (PSS) containing 145 mM-NaCl, 5.6 mM-KCl, 2.2 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 5.6 mM-glucose, 0.5 mM-ascorbic acid and 15 mM-Hepes, pH 7.4. Experiments were initiated by removing the PSS wash and permeabilizing the cells with 20 μM-digitonin in a solution (KGEP) containing 139 mM-potassium glutamate, 20 mM-Pipes (pH 6.6), 5 mM-EGTA and various amounts of Ca^{2+} to give free Ca^{2+} concentrations in the sub-micromolar and micromolar ranges. Ca^{2+} concentrations were calculated with the computer program of Chang *et al.* (1988), by using the constants of Portzehl *et al.* (1964). Other components of the KGEP solutions are detailed in the Figure legends; 50–100 μl of KGEP was used per well. Experiments were terminated by quantitatively transferring the KGEP to tubes containing 1.0 ml of ice-cold water. The samples were stored at −20 °C for up to 2 weeks before analysis of [^{3}H]inositol phosphates. Immediately after removal of KGEP from the cells, 100 μl of ice-cold methanol/conc. HCl (100:1, v/v) was added to the culture wells. The wells were scraped and the contents transferred to tubes on ice. The wells were washed with 100 μl of methanol, which was pooled with the first methanolic solution. Lipids were extracted from the samples on the same day as the experiment. Lipid extracts were stored at −20 °C for up to 1 week before separation by t.l.c. or anion-exchange chromatography of deacylation products.

Assay of [^{3}H]phosphoinositides

Lipids were extracted by mixing 400 μl of chloroform/methanol (2:1, v/v) with the methanolic samples, which were then washed with 250 μl of 10 mM-EDTA in 1 M-HCl. Samples were vortex-mixed and then centrifuged (1000 *g* for 5 min) to separate the phases. A 200 μl portion of the lower organic phase was transferred to another tube. The upper phase and interface were washed with 200 μl of chloroform, and 200 μl of the resulting lower phase was then pooled with the first organic sample. The organic samples (400 μl) were washed with 400 μl of 10 mM-EDTA in 1 M-HCl/methanol (1:1, v/v). The entire lower phase was transferred to a new tube for subsequent lipid analysis. If lipids were to be separated by t.l.c., the samples were dried under N_2 and redissolved in chloroform/methanol (2:1, v/v) before storage at −20 °C. After each wash in the extraction procedure, the total volumes of the organic phases were measured to determine recoveries.

[^{3}H]Phosphoinositides were separated either by t.l.c. or by deacylation followed by anion-exchange chromatography. The latter technique provided more reliable separations. Anion-exchange chromatography of lipid-deacylation products was performed essentially as described by Downes & Michell (1981). For separation of [^{3}H]phosphoinositides by t.l.c., ^{32}P-labelled lipids were added to the samples as internal standards. The samples were then either spotted on heat-activated oxalate-impregnated silica-gel 60 plates (Merck, Darmstadt, Germany) and developed in chloroform/acetone/methanol/acetic acid/water (40:15:15:12:8, by vol). (modified from Jolles *et al.*, 1981), or spotted on silica-gel HL plates (Analtech, Newark, DE, U.S.A.) and developed in chloroform/methanol/water/conc. NH_3 (sp.gr. 0.880) (44:44:7:5, by vol.) (adapted from Mitchell *et al.*, 1986). The former system provided good separations of PtdIns from phosphatidic acid (PtdOH), but poor resolution of lysoPtdIns from PtdIns*P*; the latter system separated lysoPtdIns and PtdIns*P* at the expense of the separation of PtdIns and PtdOH. The ^{32}P markers on the t.l.c. plates were detected by autoradiography, scraped off, sonicated in 250 μl of water, and counted for ^{3}H and ^{32}P radioactivities in scintillation cocktail. The ^{32}P-labelled lipids were prepared by incubating chromaffin cells for 30 min with [^{32}P]P_i in PSS, followed by lipid extraction; the short labelling period caused ^{32}P to be preferentially incorporated into the rapidly cycling phosphoinositides and PtdOH (Fisher *et al.*, 1981).

Assay of [^{3}H]inositol phosphates

[^{3}H]Inositol phosphates released into the incubation media were separated by anion-exchange chromatography as described by Berridge *et al.* (1983). The InsP_3 fraction also contains InsP_4 (Batty *et al.*, 1985; Heslop *et al.*, 1985; Irvine *et al.*, 1985). Since the major source of InsP_4 is Ins(1,4,5)P_3, and therefore PtdInsP_2, for our purposes it was not necessary to separate these species, nor did the conclusions drawn from this study require that the individual isomers of the various inositol phosphates be separated and identified.

DAG assay

Cell cultures were incubated for 48 h with Eagle's minimal essential medium supplemented with 10% dialysed fetal-bovine serum and 5 mM-glutamine, and containing 40 μCi of [^{3}H]glycerol/ml. The cells were washed for 15 min with PSS without labelled glycerol before initiation of an experiment. Lipid extractions after termination of experiments were performed essentially as described for [^{3}H]inositol lipids, except that HCl was omitted from the extraction solutions. A sample of the lipid extract was dried under N_2 and the radioactivity measured to determine total lipid ^{3}H. Unlabelled standards of mono-olein, diolein and triolein were added to the remaining extracts, which were then spotted on silica-gel 60 plates and developed in hexanes/diethyl ether/ethanol/conc. NH_3 (150:40:10:1, by vol.). The standards were detected with I_2 vapour, scraped off, and vortex-mixed in 4 ml of ACS (Amersham Corp., Arlington Heights, IL, U.S.A.). The samples were left overnight before liquid-scintillation counting.

Secretion assay and cell permeabilization

Catecholamine secretion was measured by prelabelling catecholamine stores in chromaffin cells with [^{3}H]noradrenaline and measuring the release of [^{3}H]noradrenaline as previously described (Kilpatrick *et al.*, 1982; Dunn & Holz, 1983). Cells were permeabilized in KGEP containing 20 μM-digitonin. Ca^{2+} was buffered at various concentrations with EGTA. Mg^{2+} and MgATP were present as indicated.

Preparation of the bacterial phospholipases C

PtdIns-specific PLC (33–34 kDa) was purified from culture supernatants of *B. thuringiensis* by cation-exchange chromatography, gel filtration and anion-exchange chromatography (Low *et al.*, 1988). The enzyme preparation was > 90% pure as judged by SDS/PAGE and had no detectable ATPase activity. The PtdIns-PLC was stored in 50 mM-Tris acetate/glycerol (1:1, v/v), pH 7.4, of 1000 units/ml at 4 °C. PLC (type XIII) from *B. cereus* was obtained from Sigma, at 2500 units/ml in 3.2 M-$(NH_4)_2SO_4$. The enzymes were prepared for use by diluting the stocks 10–20-fold with KGEP, followed by concentration back to the original volume across a membrane permeable to molecules smaller than 10 kDa (Centricon-10 micro-concentrator; Amicon). The dilution/concentration series was repeated three times. The enzymes in KGEP were stored at −20 °C and used within 2 weeks.

Data analysis

Data are expressed as means ± S.E.M. with 3–4 samples per group. The standard error of the mean (S.E.M.) of the difference between equal-sized groups 1 and 2 was $(\text{S.E.M.}_1^2 + \text{S.E.M.}_2^2)^{\frac{1}{2}}$. Significance between groups was determined by Student's *t* test. Error bars smaller than symbols were omitted from Figures.

Materials

All reagents were obtained from standard commercial sources. *myo*-[2-^{3}H]Inositol (1 mCi/ml, 10–30 Ci/mmol) was from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). Polar contaminants were removed before use by mixing a few mg of Dowex AG1-X8 with aqueous [^{3}H]inositol. [^{3}H]Glycerol (2 Ci/mmol) was from ICN (Lisle, IL, U.S.A.). Guanine nucleotides (Li salts) were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.).

RESULTS

Effects of PtdIns-PLC on phosphoinositide metabolism

The specificity of PtdIns-PLC from *B. thuringiensis* for phospholipids in chromaffin cells prelabelled for 24 h with [^{32}P]P_i is shown in Fig. 1. Of the several lipid species which incorporate ^{32}P, only PtdIns was significantly decreased by PtdIns-PLC treatment (Fig. 1*a*). PtdOH was increased by PtdIns-PLC, an expected consequence of DAG phosphorylation with residual [^{32}P]ATP. Entry of the enzyme into the cell was required for PtdIns hydrolysis to occur, since treatment of [^{3}H]inositol-prelabelled cells with 50 units of PtdIns-PLC/ml for 15 min in the absence of digitonin had no effect on [^{3}H]PtdIns; treatment with the same amount of PtdIns-PLC in the presence of digitonin decreased [^{3}H]PtdIns by 80% (results not shown).

In the following experiments investigating the effects of PLC on PtdIns metabolism, we used an experimental protocol in which cells were permeabilized in the presence or absence of PtdIns-PLC. The permeabilizing medium was removed, and the cells were then subjected to a second incubation without PtdIns-PLC. This procedure allowed the direct effects of the enzyme during permeabilization to be distinguished from secondary effects occurring after the enzyme was removed. Fig. 2(*a*) shows that permeabilization of [^{3}H]inositol-prelabelled cells in the presence of PtdIns-PLC decreased [^{3}H]PtdIns and caused the release of [^{3}H]InsP_1, but not [^{3}H]inositol polyphosphates (IPP), into the permeabilizing medium. The result demonstrates the specificity of the enzyme for PtdIns over the polyphosphoinositides. After the PtdIns-PLC was removed, there was no further release of InsP_1 in the subsequent incubation. In [^{3}H]glycerol-prelabelled cells, PtdIns-PLC increased [^{3}H]DAG from a basal level of about 1% of total lipid ^{3}H to a maximum of 4–5%. In contrast with InsP_1, which was removed with the permeabilizing medium, cellular DAG remained elevated throughout the second incubation period.

Although PtdIns-PLC did not directly hydrolyse [^{3}H]PtdIns$P$ or [^{3}H]PtdInsP_2, the enzyme caused decreases in the levels of [^{3}H]PtdInsP_2 which occurred more slowly than the decrease in [^{3}H]PtdIns (Fig. 2*a*). The loss of PtdIns was maximal by the end of permeabilization. In contrast, PtdInsP_2 was partially decreased during the permeabilization with PtdIns-PLC, and was further decreased during the subsequent 10 min incubation period. In experiments with 10 units of PtdIns-PLC/ml, at least 75% of the decline occurred by 3 min after the removal of the PtdIns-PLC. PtdIns$P$ was also partially decreased by the incubation with PtdIns-PLC. The level of PtdIns$P$ in the absence of PtdIns-PLC increased somewhat during the second incubation. Prior incubation with PtdIns-PLC prevented the increase and caused a fall in the PtdIns$P$ level.

The inability of the cells to maintain polyphosphoinositide levels after PtdIns-PLC treatment was reflected by an inhibition of the Ca^{2+}-induced release of IPP. The depletion of the polyphosphoinositides is readily explained by depletion of the substrate (PtdIns) for polyphosphoinositide synthesis.

PtdIns-PLC inhibits secretion from permeabilized cells

The effects of PtdIns-PLC on catecholamine secretion were investigated (Fig. 2*b*) in the same experiment as that in Fig. 2(*a*). Pretreatment of permeabilized cells with PtdIns-PLC produced an inhibition of Ca^{2+}-dependent secretion which closely paralleled the inhibition of Ca^{2+}-stimulated release of inositol phosphates. Incubation of cells with PtdIns-PLC before permeabilization did not inhibit Ca^{2+}-dependent secretion from subsequently permeabilized cells. Thus the enzyme must enter the cells to inhibit

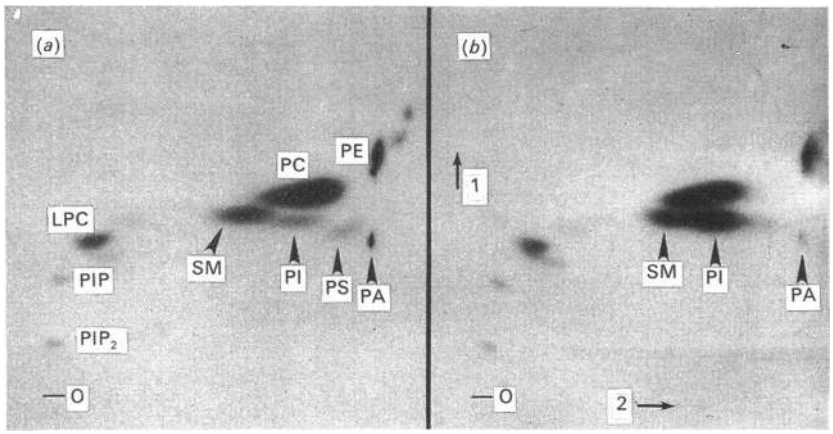
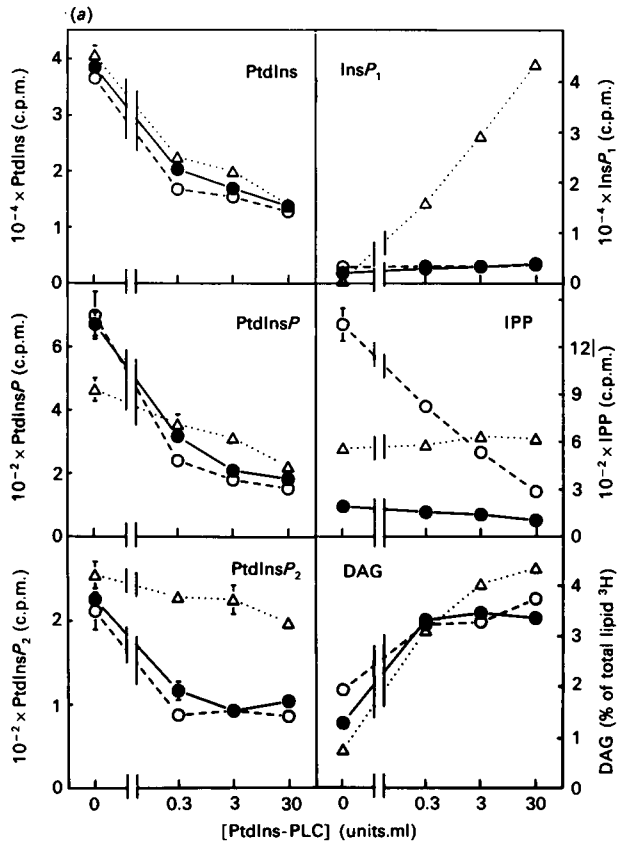


Fig. 1. Phospholipid specificity of PtdIns-PLC in permeabilized cells

Chromaffin cells prelabelled for 24 h with [^{32}P]P_i were permeabilized for 15 min with KGEP containing 20 μM-digitonin and 2 mM-MgATP in the presence (*a*) or absence (*b*) of 30 units of PtdIns-PLC/ml. Lipids were extracted from the cells, separated by two-dimensional t.l.c. on silica-gel HL-HPTLC plates (Analtech) as described by Mitchell *et al.* (1986), and observed by autoradiography. The arrows '1' and '2' indicate the directions of the first and second dimensions of separation. The developing solvents were chloroform/methanol/water/conc. NH_3 (48:40:7:5, by vol.) in the first dimension and chloroform/methanol/formic acid (11:5:1, by vol.) in the second dimension. The identities of PtdIns, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine were confirmed by detection with I_2 vapour of the authentic lipids added as internal standards. Other lipids were tentatively identified by comparison with previously published migration patterns (Mitchell *et al.*, 1986). Abbreviations: O, origin; SM, sphingomyelin; PC, phosphatidylcholine; LPC, lysophosphatidylchoine; PS, phosphatidylserine; PA, PtdOH; PE, phosphatidylethanolamine; PI, PtdIns; PIP, PtdIns$P$; PIP_2, PtdInsP_2.



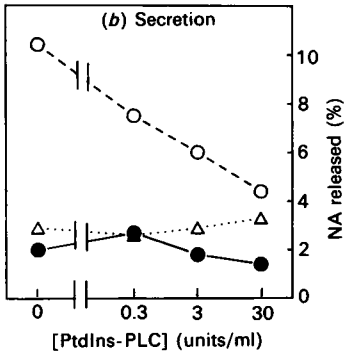


Fig. 2. Effect of PtdIns-PLC on inositol lipids, inositol phosphates, DAG, and catecholamine secretion in permeabilized cells

In parallel experiments, cells prelabelled with [^{3}H]inositol (panel *a*), [^{3}H]glycerol (panel *a*) or [^{3}H]noradrenaline (NA; panel *b*) were permeabilized for 7 min with KGEP containing 20 μM-digitonin, 2 mM-MgATP and various concentrations of PtdIns-PLC as indicated. The permeabilizing medium was replaced with KGEP (without PtdIns-PLC or digitonin) containing 2 mM-MgATP with or without 10 μM-Ca^{2+}, and the cells were incubated for an additional 10 min. [^{3}H]Inositol phosphates and [^{3}H]noradrenaline released into the medium and cellular levels of [^{3}H]inositol lipids and [^{3}H]DAG were determined at the end of the permeabilization period (△) and after the subsequent incubation with (○) or without (●) 10 μM-Ca^{2+}.

secretion. The inhibition of secretion by PtdIns-PLC required a preincubation period with permeabilized cells; when PtdIns-PLC was added together with Ca^{2+} during permeabilization, secretion was not affected.

Inhibition of secretion by PtdIns-PLC is not mediated by protein kinase C activation, DAG or InsP_1

Incubating chromaffin cells with 1,2-dioctanoylglycerol (diC_8) or phorbol esters enhances Ca^{2+}-induced secretion from permeabilized cells through activation of protein kinase C (Knight & Baker, 1983; Pocotte *et al.*, 1985; Brocklehurst & Pollard, 1985; Lee & Holz, 1986). However, Reisine & Zatz (1987) have reported an inhibition of corticotropin secretion by 1-oleoyl-2-acetyl-*sn*-3-glycerol in AtT20 cells, and Pandol & Schoeffield (1986) suggested that endogenous DAG may inhibit amylase secretion from pancreatic acini. We therefore performed experiments investigating whether DAG acting via protein kinase C or DAG alone was in some manner involved in the inhibition of secretion by PtdIns-PLC. In cells which were subjected to long-term treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) under conditions which resulted in the loss of 80–90% of total cellular protein kinase C (1 μM-TPA for 16 h) (Bittner & Holz, 1986), the effectiveness of PtdIns-PLC to inhibit secretion was unaltered. In cells which were pretreated with TPA or diC_8 at concentrations which maximally enhance secretion (100 nM and 100 μM respectively for 15–30 min), PtdIns-PLC inhibited secretion to the same degree as in cells that had not been treated with TPA or diC_8. Thus the effect of PtdIns-PLC to inhibit secretion was probably independent of protein kinase C activation.

We also considered the possibility that DAG could inhibit secretion through a mechanism not involving protein kinase C, and that this inhibition is normally masked by the simultaneous protein kinase C-induced enhancement of secretion. This does not appear to be the case, since in cells pretreated with 1 μM-TPA

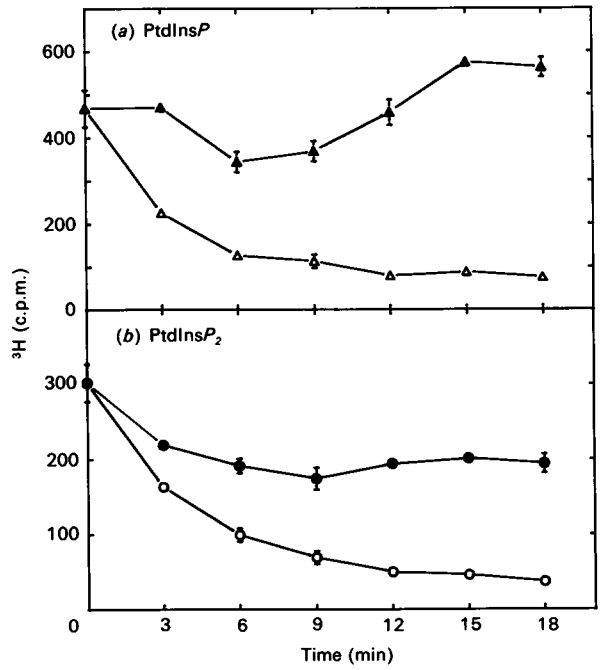


Fig. 3. Decrease of the polyphosphoinositides in the absence of MgATP

[^{3}H]Inositol-prelabelled cells were permeabilized for 3 min with KGEP containing 20 μM-digitonin in the presence (▲, ●) or absence (△, ○) of 2 mM-MgATP. The permeabilization solution was removed, and the cells were incubated for various times in digitonin-free KGEP in the continuing presence or absence of MgATP. The levels of PtdIns*P* and PtdInsP_2 that were measured in intact cells before permeabilization are plotted at 0 min. There were three wells/group.

for 24 h to down-regulate protein kinase C (Bittner & Holz, 1986) diC_8 (100 μM) had no effect on Ca^{2+}-stimulated secretion.

It is also unlikely that the inhibition resulted from $InsP_1$ production. Nearly all of the [^{3}H]$InsP_1$ produced was removed before adding Ca^{2+}. In addition, concentrations of $InsP_1$ (the $InsP_1$ isomer expected to be produced by PtdIns hydrolysis) as high as 3 mM added together with Ca^{2+} in cells not treated with PtdIns-PLC did not cause a significant inhibition of secretion.

Inhibition of secretion is not caused by inhibition of the production of $InsP_3$

Depletion of PtdIns and subsequently of the polyphosphoinositides resulted in decreased production of IPP upon incubation with Ca^{2+} (Fig. 2). To examine whether decreased production of Ins-(1,4,5)P_3 and its metabolites caused the inhibition of secretion, cells were permeabilized in the presence of 20 units of PtdIns-PLC/ml for 10 min and then stimulated to secrete with 10 μM-Ca^{2+} for 15 min in the absence or presence of 40 μM-Ins(1,4,5)P_3. The presence of Ins(1,4,5)P_3 did not reverse the inhibition induced by PtdIns-PLC.

ATP deprivation and incubation with PtdIns-PLC have similar effects on polyphosphoinositide metabolism and secretion

During the course of these studies it became apparent that the effects of PtdIns-PLC and ATP removal on phospholipid metabolism and secretion were similar. Permeabilization of the cells in the absence, but not in the presence, of MgATP caused a rapid fall in PtdIns$P$ and PtdInsP_2 levels (Fig. 3). The ability of MgATP to maintain or increase the polyphosphoinositides was reflected by the MgATP-dependent production of inositol

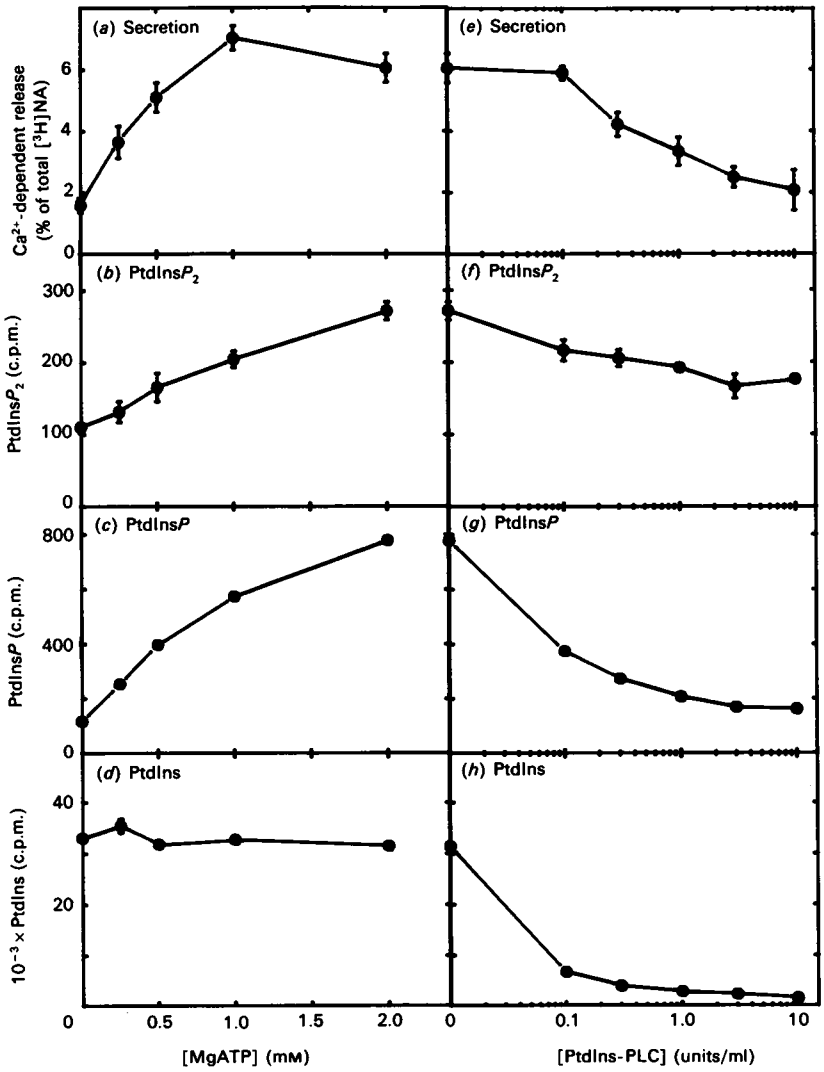


Fig. 4. Various concentrations of MgATP or PtdIns-PLC alter the inositol phospholipids and the secretory response

(*a*) [^{3}H]Noradrenaline (NA)- or (*b–d*) [^{3}H]inositol-labelled cells were permeabilized for 8 min in Ca^{2+}-free KGEP in the presence of various concentrations of MgATP and 1 mM-$MgCl_2$. Solutions were replaced with solutions containing the same concentration of MgATP with or without 10 μM-Ca^{2+}. Ca^{2+}-dependent secretion was determined after 6 min in [^{3}H]noradrenaline-labelled cells. The amounts of cellular [^{3}H]inositol phospholipids were determined in [^{3}H]inositol-labelled cells incubated for 3 min in 10 μM-Ca^{2+}. (*e*) [^{3}H]Noradrenaline- or (*f–h*) [^{3}H]inositol-labelled cells were permeabilized for 8 min in Ca^{2+}-free KGEP in the presence of 2 mM-MgATP and 1 mM-$MgCl_2$ with various concentrations of PtdIns-PLC. Solutions were replaced with PtdIns-PLC-free KGEP containing 2 mM-MgATP with or without 10 μM-Ca^{2+}. Ca^{2+}-dependent secretion was determined after 6 min in noradrenaline-labelled cells. The amounts of cellular [^{3}H]inositol phospholipids were determined in [^{3}H]inositol-labelled cells incubated for 3 min in 10 μM-Ca^{2+}. All panels are from one experiment. There were three wells/group.

phosphates induced by Ca^{2+} (results not shown). The effects of PtdIns-PLC pretreatment and ATP deprivation on polyphosphoinositide metabolism are thus qualitatively similar: each condition removes one of the substrates for PtdIns kinase, thereby eliminating PtdIns*P* (and subsequently, PtdInsP_2) synthesis. Inhibition of synthesis results in decreased polyphosphoinositide levels, probably because of the action of endogenous phosphatases or low levels of endogenous PLC activity.

The relationship between the levels of the polyphosphoinositides and Ca^{2+}-dependent secretion was further investigated (Fig. 4). The levels of the polyphosphoinositides were altered by varying either the MgATP (Fig. 4, left panels) or the PtdIns-PLC (Fig. 4, right panels) concentrations. Secretion was determined after 6 min incubation with and without 10 μM-Ca^{2+}. PtdIns*P* and PtdInsP_2 levels were determined during the secretory period after a 3 min incubation in the presence of Ca^{2+}. The incubation with digitonin before the addition of Ca^{2+} was relatively long (8 min), to minimize ATP-independent secretion (Holz *et al.*, 1989). Over a range of MgATP concentrations that increased the polyphosphoinositides, there were increases in Ca^{2+}-dependent secretion. Conversely, over a range of PtdIns-PLC concentrations that decreased the polyphosphoinositides, there were decreases in Ca^{2+}-dependent secretion. Secretion was not well correlated with the levels of PtdIns.

If the effects of MgATP and PtdIns-PLC on secretion are both mediated by changes in the polyphosphoinositides, then the relationships between Ca^{2+}-dependent secretion and polyphosphoinositide levels should be similar for the two manipulations. The data from Fig. 4 are re-plotted in Fig. 5 to reveal these relationships. Indeed, Ca^{2+}-dependent secretion was similarly correlated with the sum of PtdIns*P* and PtdInsP_2 when the levels of these lipids were changed by either manipulation (Fig. 5*c*). This result was obtained in three experiments.

It should be noted that the levels of [^{3}H]PtdIns*P* were approximately twice those of [^{3}H]PtdInsP_2 over the entire range of MgATP concentrations. In contrast, PtdIns-PLC preferentially decreased [^{3}H]PtdIns*P*.

PtdIns-PLC selectively inhibits ATP-dependent secretion

Two components of Ca^{2+}-dependent secretion have been identified in digitonin-permeabilized chromaffin cells (Holz *et al.*, 1989). One is rapid, labile and MgATP-independent; the other is slow, stable and MgATP-dependent. Cells which exhibit ATP-independent as well as ATP-dependent secretion are permeable to nucleotides and proteins (Holz *et al.*, 1989). If one of the ways

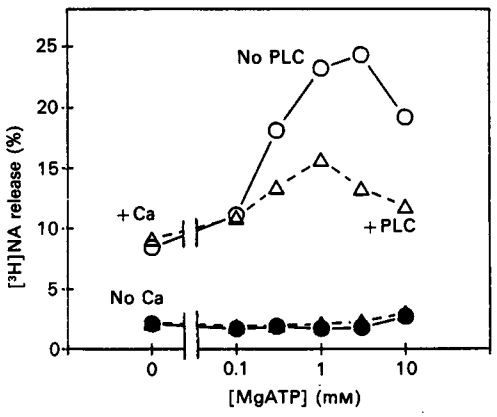


Fig. 6. Effect of PtdIns-PLC on the MgATP concentration/response relationship for secretion

[^{3}H]Noradrenaline-labelled cells were permeabilized for 5 min with Mg^{2+}- and ATP-free KGEP containing 20 μM-digitonin with (△, ▲) or without (○, ●) 10 units of PtdIns-PLC/ml. The permeabilizing medium was replaced with KGEP containing 1 mM-$MgCl_2$, various concentrations of MgATP as indicated, and either 0 Ca^{2+} (●, ▲) or 10 μM-Ca^{2+} (○, △). After 15 min the release of [^{3}H]noradrenaline (NA) into the medium was determined.

by which MgATP enhances secretion is by maintaining the polyphosphoinositides, then PtdIns-PLC, by decreasing PtdIns, should inhibit ATP-dependent, but not ATP-independent, secretion. Fig. 6 demonstrates that this is indeed the case. Cells were permeabilized in the absence of MgATP with or without PtdIns-PLC. Cells were subsequently incubated with and without 10 μM-Ca^{2+} in the presence of various concentrations of MgATP. PtdIns-PLC pretreatment inhibited only the ATP-dependent component of secretion; Ca^{2+}-dependent secretion in the absence of ATP was unaffected.

The effects of PtdIns-PLC and ATP deprivation on the concentration–response relationship for Ca^{2+} are shown in Fig. 7. In each case the maximal response to Ca^{2+} was decreased, with no change in the concentration of Ca^{2+} which half-maximally stimulated secretion. PtdIns-PLC pretreatment (Fig. 8*a*) and ATP removal (Fig. 8*b*) also had similar effects on the time course of secretion. Ca^{2+}-dependent secretion in the first 1 min was 5.4±0.2 % in the presence of ATP and 3.1±0.1 % in its absence (a 40 % decrease) [in a recent study in our laboratory using a different protocol (2 mM-EDTA in the preincubation and equi-

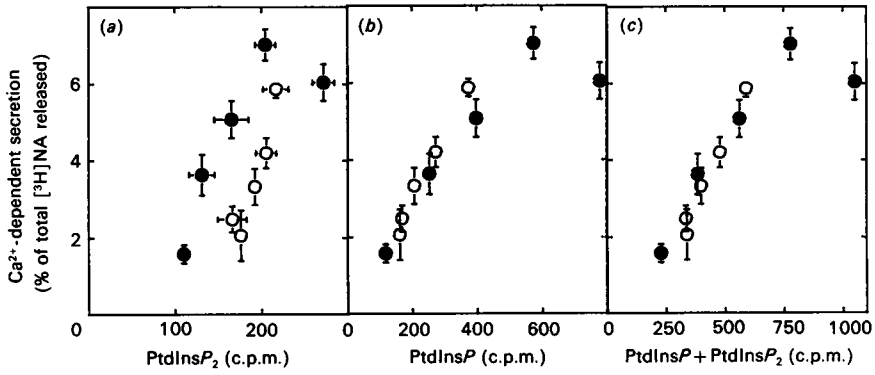


Fig. 5. Relationship between the levels of PtdIns*P* and PtdInsP_2 and Ca^{2+}-dependent secretion

The data are from Fig. 4. Dose/response data are shown for MgATP (●) and PtdIns-phospholipase C (○).

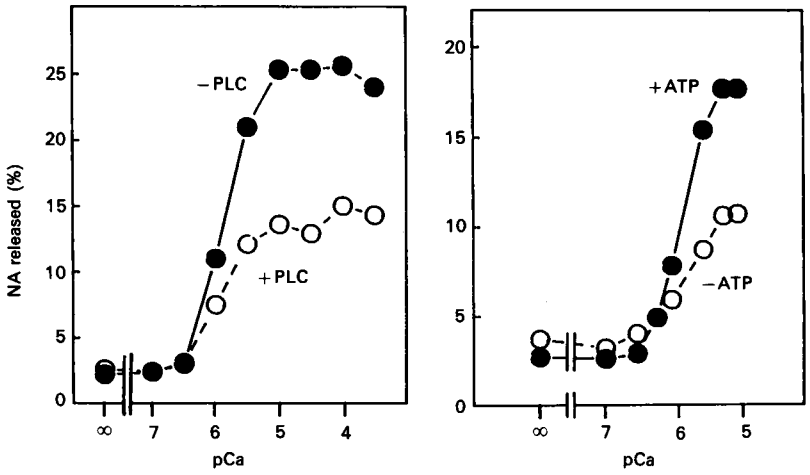


Fig. 7. Effects of PtdIns-PLC and ATP on the Ca^{2+} concentration/response relationship for secretion

(*a*) [^{3}H]Noradrenaline (NA)-labelled cells were permeabilized with KGEP containing 20 μM-digitonin and 2 mM-MgATP with or without 10 units of PtdIns-PLC/ml for 7 min. The permeabilizing media were replaced with KGEP containing various concentrations of free Ca^{2+}, as indicated, and 2 mM-MgATP without PtdIns-PLC (PLC). [^{3}H]Noradrenaline release into the medium was determined after 15 min. (*b*) [^{3}H]Noradrenaline-labelled cells were permeabilized with KGEP in the absence of Mg^{2+} and ATP. After 4 min the cells were incubated in the presence of various Ca^{2+} concentrations in the presence of 1 mM-$MgCl_2$ with (+ATP) or without (−ATP) 1 mM-MgATP. [^{3}H]Noradrenaline released into the medium was determined after 15 min.

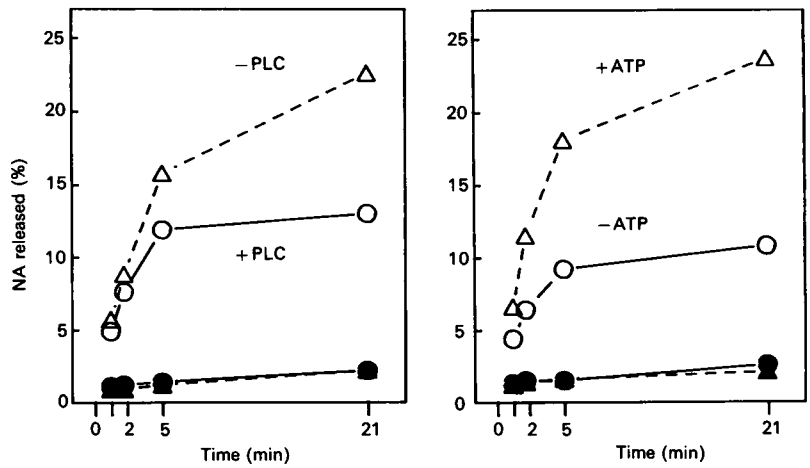


Fig. 8. Effects of PtdIns-PLC and ATP on the time course of secretion

(*a*) [^{3}H]Noradrenaline (NA)-labelled cells were permeabilized with KGEP containing 20 μM-digitonin and 2 mM-MgATP with (○, ●) or without (△, ▲) 10 units of PtdIns-PLC/ml for 7 min. The permeabilizing media were replaced with KGEP with (○, △) or without (●, ▲) 10 μM-free Ca^{2+} and 2 mM-MgATP without PtdIns-phospholipase C. (*b*) [^{3}H]Noradrenaline-labelled cells were permeabilized with KGEP containing 20 μM-digitonin in the absence of Mg^{2+} and ATP (○, ●) or in the presence of 1 mM-MgATP and 1 mM-$MgCl_2$ (△, ▲). After 4 min the permeabilizing medium was replaced with KGEP with (○, △) or without (●, ▲) 10 μM-Ca^{2+} and 1 mM-$MgCl_2$ with (△, ▲) or without (○, ●) 1 mM-MgATP. At the indicated times, the medium was removed for determination of [^{3}H]noradrenaline release.

molar concentrations of Mg^{2+} and ATP when present), ATP was found to have little effect on the secretory rate in the first 2 min (Holz *et al.*, 1989)]. The rate of secretion between 5 and 21 min was 0.319 ± 0.042 %/min in the presence of ATP and 0.031 ± 0.0013 %/min in its absence, a 90% inhibition. PtdIns-PLC caused no change in secretion in the first 1 min, but decreased the secretion rate between 5 and 21 min by 95% from the control value of 0.363 ± 0.044 %/min to 0.019 ± 0.037 %/min in the presence of PtdIns-PLC. Thus Ca^{2+}-dependent secretion can be separated into two phases: an initial phase little affected by PtdIns depletion or ATP removal, and a later phase which strongly requires PtdIns and added ATP.

Preincubation with neomycin increases the polyphosphoinositides and increases secretion

In order to test further the hypothesis that the polyphosphoinositides are required for secretion, a pharmacological method was devised to increase the polyphosphoinositides in the absence of ATP. Neomycin binds to the polyphosphoinositides (Schacht, 1978) and inhibits their breakdown by phospholipase C (Downes & Michell, 1981) and perhaps by lipid phosphatases (Schacht, 1976). We found that neomycin maintained the polyphosphoinositides in cells permeabilized in the absence of ATP (Fig. 9). Removal of the neomycin caused a decrease in the polyphosphoinositides, indicating that the effect of neomycin on

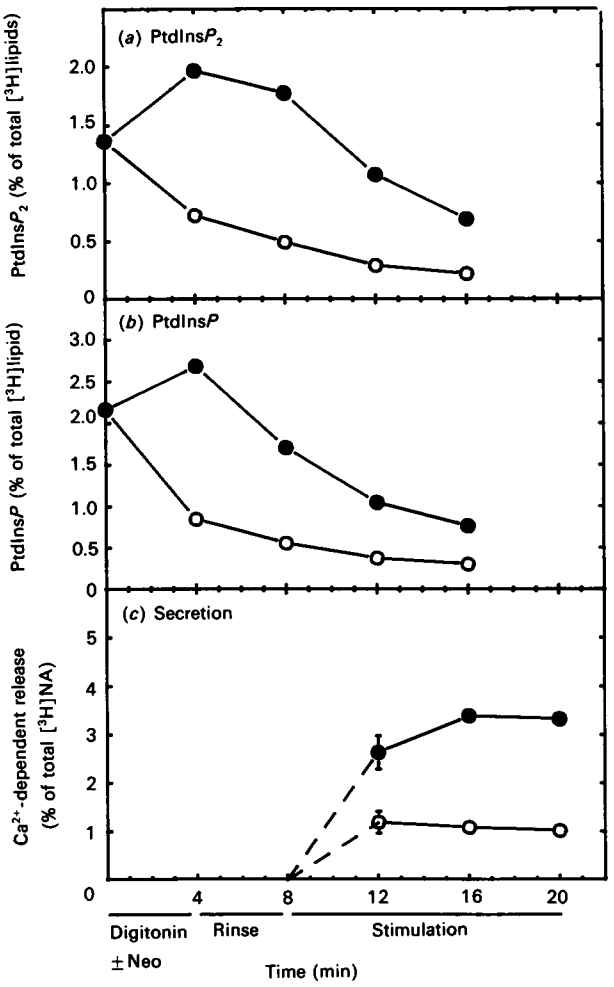


Fig. 9. Preincubation with neomycin in the absence of ATP maintains the polyphosphoinositides and enhances secretion

Chromaffin cells prelabelled with either [^{3}H]inositol or [^{3}H]noradrenaline (NA) were permeabilized for 4 min in the presence (●) or absence (○) of 200 μM-neomycin in ATP-free KGEP containing 20 μM-digitonin without Ca^{2+}. Media from all wells were replaced with KGEP without neomycin, ATP and Ca^{2+}. After an additional 4 min, media were replaced with ATP-free KGEP with or without 10 μM-Ca^{2+} (without neomycin). All solutions contained 0.1 mM-$MgCl_2$. The polyphosphoinositides were measured in intact cells at 0 min and at subsequent times in permeabilized cells as indicated. Values for lipids from cells incubated in the presence of Ca^{2+} are presented after 8 min. Ca^{2+}-dependent secretion was determined after 8 min. There were three wells/group.

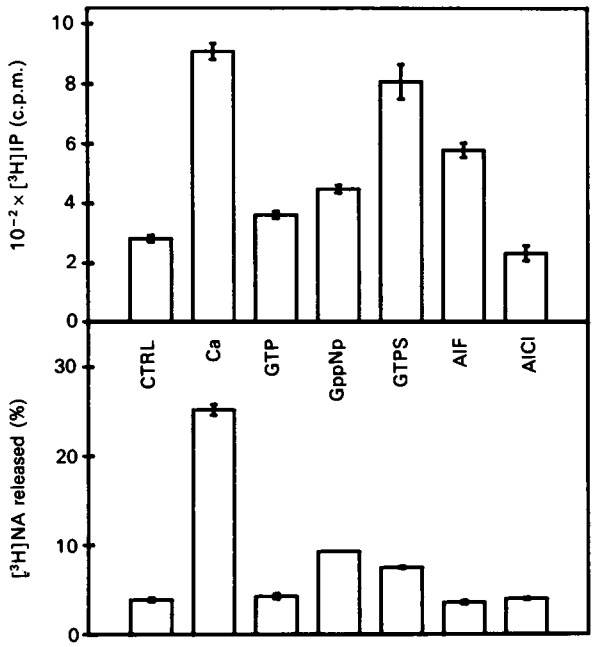


Fig. 10. Effects of guanine nucleotides and AlF_4^- on secretion and release of inositol phosphates

Chromaffin cells prelabelled with either [^{3}H]inositol (top) or [^{3}H]noradrenaline (bottom) were permeabilized with KGEP containing 20 μM-digitonin, 2 mM-MgATP, and no further additions (CTRL), or 10 μM-Ca^{2+} (Ca), or 1 mM-GTP (GTP), or 100 μM-p[NH]ppG (GppNp), or 100 μM-GTP[S], or 10 μM-$AlCl_3$ + 1 mM-NaF (AlF), or 10 μM-$AlCl_3$ + 1 mM-NaCl (AlCl). After 15 min the release of total [^{3}H]inositol phosphates (IP) and [^{3}H]noradrenaline (NA) into the medium was determined.

the lipids was reversible; however, the polyphosphoinositides were still increased compared with control groups for at least 12 min after the removal of the drug. Neomycin over a concentration range which protected the lipids also inhibits secretion when present together with Ca^{2+} in permeabilized chromaffin cells (Bittner *et al.*, 1986). Neomycin also inhibits secretion in mast cells (Cockcroft *et al.*, 1987). However, when permeabilized chromaffin cells were preincubated with neomycin, rinsed for 4 min and then stimulated to secrete in the absence of the drug, neomycin more than doubled the secretory response (Fig. 9). Thus, in spite of direct inhibitory effects of neomycin on secretion, maintenance of the polyphosphoinositides by preincubation with neomycin is associated with an increased secretory response.

Effects of guanine nucleotides on secretion

The proposed requirement for polyphosphoinositides in secretion led us to consider further whether the endogenous polyphosphoinositide-PLC plays a direct role in secretion. GTP[S] activates PLC and enhances the Ca^{2+}-stimulated release of inositol phosphates in digitonin-permeabilized chromaffin cells (D. A. Eberhard & R. W. Holz, unpublished work). We therefore compared catecholamine secretion and the release of inositol phosphates stimulated by Ca^{2+}, guanine nucleotides and related agents (Fig. 10). In other experiments sodium vanadate (3 mM), which, like fluoroaluminate, has been proposed to activate PLC through a GTP-binding protein (Paris & Pouyssegur, 1987), produced a large release of inositol phosphates, comparable with that with GTP[S], but did not stimulate secretion. The rank order of these agents for the stimulation of the release of inositol phosphates was Ca^{2+} = GTP[S] = vanadate > fluoroaluminate > p[NH]ppG > GTP = $AlCl_3$. The rank order of the ability of these agents to stimulate secretion was different: $Ca^{2+} \gg$ p[NH]ppG = GTP[S] > GTP = fluoroaluminate = $AlCl_3$ = vanadate. The different rank orders of the guanine nucleotides, fluoraluminate and vanadate for stimulation of secretion and PLC activity suggest that these processes are regulated by different GTP-dependent mechanisms. They further indicate that activation of the endogenous PLC does not directly lead to secretion. This conclusion is consistent with previous findings (Bittner *et al.*, 1986; Barrowman *et al.*, 1986; Vallar *et al.*, 1987; Cockcroft *et al.*, 1987).

Effects of a PLC from *B. cereus* on catecholamine release

To determine whether the effects of PLC on secretion required specificity of the enzyme for PtdIns, we examined the effects of a PLC from *B. cereus* which hydrolyses phosphatidylcholine and to a lesser extent phosphatidylserine and phospha-

tidylethanolamine but not PtdIns (Otnaess *et al.*, 1977). The enzyme did not hydrolyse [^{3}H]inositol-containing lipids in permeabilized chromaffin cells (results not shown). In contrast with the effects of PtdIns-PLC to inhibit Ca^{2+}-dependent secretion, the *B. cereus* PLC increased the release of catecholamine in both the absence and the presence of Ca^{2+}; the increases in catecholamine release closely paralleled the increases in DAG levels. Release of 5–10% of the catecholamine occurred when DAG was increased from 1% to 5% of the total [^{3}H]glycerol lipid. The *B. cereus* PLC did not inhibit Ca^{2+}-dependent secretion. Thus the inhibition of secretion by the PtdIns-PLC is a specific effect of the enzyme. Because the *B. cereus* PLC caused catecholamine release from granules in homogenates from chromaffin cells over a similar concentration range of PLC to that used in the experiments with permeabilized cells, it is likely that in permeabilized cells the enzyme directly released [^{3}H]noradrenaline in a non-exocytotic manner.

DISCUSSION

Activation of many cell types results in the breakdown of inositol phospholipids by an endogenous PLC with the production of two second messengers, InsP_3 and DAG. InsP_3 releases Ca^{2+} from intracellular stores, and DAG activates protein kinase C. These messengers can trigger secretion in a variety of cells (Nishizuka, 1986). In the present study we utilized digitonin-permeabilized chromaffin cells, which are permeable to proteins, to study the relationship between inositol phospholipids and catecholamine secretion. In these experiments, the primary stimulus for secretion, cytosolic Ca^{2+}, was controlled with strongly buffered solutions containing EGTA, so that the Ca^{2+}-releasing effects of inositol phosphates would not influence the secretory response. An important aspect of this study was the use of a bacterial phospholipase C which is highly specific for PtdIns to manipulate directly the level of intracellular PtdIns and, consequently, the levels of the polyphosphoinositides.

Secretion requires inositol phospholipids

An important conclusion of this study, based on the action of the PtdIns-PLC, is that secretion requires inositol phospholipids. The specificity of the PtdIns-PLC from *B. thuringiensis* for PtdIns was confirmed by its effects on lipids in digitonin-permeabilized chromaffin cells. The enzyme decreased the level of PtdIns and simultaneously produced the expected products InsP_1 and DAG. Neither InsP_2 nor InsP_3 was produced. The enzyme had no effects on phosphatidylcholine or phosphatidylethanolamine. Because it had effects when added to permeabilized cells, but not to intact cells, the enzyme must have acted intracellularly to decrease PtdIns facing the cytosolic compartment. Although the PtdIns-PLC did not directly hydrolyse the polyphosphoinositides, there was a decrease in PtdIns$P$ and PtdInsP_2 after the decrease in PtdIns, probably because breakdown of the polyphosphoinositides by phosphatases or endogenous PLC was not balanced by synthesis.

Closely correlated with the PtdIns-PLC-induced loss of the inositol phospholipids was the inhibition of Ca^{2+}-dependent secretion (Figs. 2, 4 and 5). Secretion was best correlated not with the level of PtdIns but with that of the polyphosphoinositides which were present during the secretory response (Figs. 4 and 5). In addition, the delay in the inhibition of secretion after PtdIns was decreased coincided with the latency required for the decrease of the polyphosphoinositides (see Fig. 2 and the text).

It is unlikely that secretion was inhibited by one of the products of the PtdIns-PLC reaction. Increases in DAG, by activation of protein kinase C, would be expected to enhance rather than inhibit secretion. Furthermore, an exogenous DAG, diC_8, did not inhibit secretion in a variety of experiments, including those in which protein kinase C had been down-regulated by prior incubation with phorbol ester. Thus, even in experiments in which an inhibitory effect of diC_8 would not be masked by activation of protein kinase C, secretion was not inhibited. Similarly, millimolar concentrations of InsP_1, the other product of PtdIns-PLC, had no effect on secretion from permeabilized cells. PtdOH (100 μg/ml), a metabolite of DAG, also had no effect on secretion (results not shown).

ATP supports secretion in part by maintaining the polyphosphoinositides

If the PtdIns-PLC inhibits secretion by inhibiting the synthesis of PtdIns$P$ by PtdIns kinase because of removal of PtdIns, then its effects should be mimicked by removal of ATP, the other substrate of PtdIns kinase. Indeed, strong support for the hypothesis that the polyphosphoinositides are necessary for secretion comes from an analysis of the effects of ATP.

In a recent study, we demonstrated that there is a rapid and labile ATP-independent component of secretion and a slower, more stable, MgATP-dependent component of secretion in digitonin-permeabilized cells (Holz *et al.*, 1989). That study also indicated that ATP acts before Ca^{2+} in the secretory pathway, a conclusion also reached by Howell *et al.* (1989) in mast cells. In the present paper we found that removal of ATP causes a rapid decrease in the levels of the polyphosphoinositides (Fig. 3). The concentration range over which ATP enhances secretion (0.1–1 mM) is also that over which it maintains or increases the levels of the polyphosphoinositides (Figs. 3 and 4*a*). Most importantly, the relationship between the sum of the polyphosphoinositides and the secretory response was almost identical when the polyphosphoinositides were varied by various concentrations of ATP or PtdIns-PLC (Fig. 5*c*). Thus the ability of ATP deprivation to mimic, in a manner predictable by the effects on the polyphosphoinositides, the effects of the PtdIns-PLC, supports the notion that the presence of the polyphosphoinositides is necessary for secretion. This conclusion is also supported by the ability of PtdIns-PLC to inhibit specifically the ATP-dependent component of secretion (Fig. 6). In addition, when the polyphosphoinositides were maintained by pre-incubation with neomycin instead of ATP, the secretory response was also increased. ATP-independent secretion may reflect the presence of the polyphosphoinositides initially present in permeabilized cells before they are decreased by metabolism.

There are at least three forms of PtdIns kinase (Whitman *et al.*, 1988; Endemann *et al.*, 1987): types 1 and 2 have K_m values for ATP of 10 μM and 54 μM respectively; type 3 has a K_m of 0.7 mM. ATP increased the polyphosphoinositides at concentrations greater than 0.1 mM and half-maximally stimulated secretion at approx. 0.5 mM. Thus the type 3 enzyme may be responsible for the synthesis of PtdIns$P$ and the ATP-dependence of secretion in digitonin-permeabilized chromaffin cells.

It is possible that the ATP requirement for secretion reflects the need for protein phosphorylation in the secretory pathway as well as for maintenance of the polyphosphoinositides. There is abundant evidence that protein phosphorylation modulates exocytosis. For example, protein kinase C enhances secretion from many cells (Kikkawa & Nishizuka, 1986), including chromaffin cells (Knight & Baker, 1983; Pocotte *et al.*, 1985; Brocklehurst & Pollard, 1985; TerBush *et al.*, 1988). However, it is unlikely that ATP-dependent secretion is solely a reflection of protein kinase C activity, since the K_m of the kinase for ATP is 6 μM (Kikkawa *et al.*, 1982), whereas the concentration of ATP necessary for half-maximal stimulation of secretion is approx. 0.5 mM. Furthermore, there is direct evidence that protein kinase C plays a modulatory, but not an obligatory, role in secretion in

chromaffin cells (Holz & Senter, 1988) and PC12 cells (Matthies *et al.*, 1987). The K_m values for ATP of a large number of other serine/threonine-specific protein kinases, including Ca^{2+}/calmodulin kinase II and myosin light-chain kinase are also less than 100 μM (see Edelman *et al.*, 1987, for a review). [Not all secretory cells require 0.1–1 mM-ATP to maintain secretion. In permeabilized mast cells, the concentration of ATP that maintains half-maximal secretion is 30 μM (Howell *et al.*, 1989).] In paramecia, it is suggested that Ca^{2+}-activated exocytosis of trichocysts requires a prior ATP-requiring step that may be protein phosphorylation (Vilmart-Seuwen *et al.*, 1986). A Ca^{2+}-activated phosphatase can subsequently trigger trichocyst release (Momayezi *et al.*, 1987). In digitonin-permeabilized chromaffin cells, addition of as much as 230 μg of calcineurin/ml in the presence of MgATP did not enhance Ca^{2+}-dependent secretion (P. Wick & R. W. Holz, unpublished work).

Inositol phospholipids may play a role in secretion independent of their being substrates for PLC

The PtdIns-PLC, by decreasing the polyphosphoinositides, decreases the activity of the endogenous Ca^{2+}-activated PLC. It is unlikely that the PtdIns-PLC inhibited secretion as a result of the cells being unable to generate products through activation of the endogenous Ca^{2+}-activated PLC. Preincubation with PtdIns-PLC produced more DAG than with the endogenous Ca^{2+}-activated PLC. This DAG persisted in the membrane during the incubation with Ca^{2+}. Furthermore, the DAG resulting from the PtdIns-PLC probably originated from the same pool of phospholipid as the DAG resulting from activation of the endogenous PLC, since the polyphosphoinositides are derived from PtdIns. The addition of Ins(1,4,5)P_3, the initial product of the endogenous Ca^{2+}-activated PLC, did not reverse the inhibition induced by PtdIns-PLC when added together with Ca^{2+} after preincubation with the enzyme. Ins(1,4,5)P_3 also had no effect on Ca^{2+}-dependent secretion from permeabilized chromaffin cells in the absence of the PtdIns-PLC (Bittner *et al.*, 1986). (In these experiments Ca^{2+} was strongly buffered with EGTA, so that released Ca^{2+} would not significantly alter the free Ca^{2+} concentration.)

There is additional evidence that PLC activity is not required for secretion. In intact chromaffin cells, Ba^{2+} strongly stimulates exocytosis, but does not activate PLC (Eberhard & Holz, 1987). In the present study there was no obvious correlation between the activation of PLC by various agents and secretion (Fig. 9). Furthermore, we recently found that GTP[S] and Ca^{2+} have synergistic effects to stimulate phospholipase C activity in permeabilized cells (D. A. Eberhard & R. W. Holz, unpublished work), although GTP[S] and Ca^{2+} have sub-additive effects on secretion (Bittner *et al.*, 1986).

Some proteins are attached to the extracellular surface of the plasma membrane by a PtdIns–glycoconjugate linkage which is cleaved by the bacterial PtdIns-PLC (Low & Saltiel, 1988). It is unlikely that the inhibition of secretion by the PtdIns-PLC was a consequence of the loss of such a protein, since the inhibition required entry of the enzyme into the cell. The PtdIns-PLC may also cleave a glycoconjugate attached to PtdIns that mediates some of the intracellular effects of insulin (Saltiel & Cuatrecasas, 1986). It is unlikely that such a species is responsible for the inhibition of secretion, since the cleaved product should exit from the permeabilized cell before the introduction of Ca^{2+}. We cannot rule out that a glycoconjugate linked to PtdIns is required for secretion and is cleaved within the cell by the PtdIns-PLC. However, the similarity of the effects on secretion of removing either of the substrates for PtdIns kinase (PtdIns or ATP) is compelling evidence for a role in secretion of PtdIns$P$ or PtdInsP_2 rather than of a hypothetical PtdIns glycoconjugate.

Possible mechanisms by which the polyphosphoinositides play a role in secretion

Polyphosphoinositides are synthesized both on the plasma membrane and on the chromaffin-granule membrane (Buckley *et al.*, 1971; Trifaró & Dworkind, 1972; Phillips, 1973; Muller & Kirshner, 1976; Husebye & Flatmark, 1988). The binding of a protein to PtdInsP_2 (and/or PtdIns$P$) on either membrane may regulate its function in secretion. Myosin I has been recently demonstrated to bind to PtdInsP_2 (Adams & Pollard, 1989). In addition, there are at least three proteins which interact with the polyphosphoinositides and also with f-actin, i.e. gelsolin, which severs f-actin (Janmey *et al.*, 1987; for review see Yin, 1987), profilin (Lassing & Lindberg, 1985) and calpactin, which binds to chromaffin granules (Drust & Creutz, 1988; for review see Glenney, 1987). Because chromaffin granules also interact with f-actin (Burridge & Phillips, 1975; Wilkins & Lin, 1981; Fowler & Pollard, 1982), it is possible that the PtdInsP_2 or PtdIns$P$ localizes these proteins to secretory sites and controls the interaction of chromaffin granules with the cortical cytoskeleton and the plasma membrane. Indeed, a recent report suggests that calpactin can maintain secretion in digitonin-permeabilized chromaffin cells (Ali *et al.*, 1989).

It is unlikely that Ca^{2+} specifically interacts with the polyphosphoinositides to trigger exocytosis by altering local surface charge. The affinity of Ca^{2+} for PtdInsP_2 is only $2\frac{1}{2}$ times that of Mg^{2+} (Toner *et al.*, 1988), whereas secretion is stimulated by submicromolar Ca^{2+} and not by millimolar Mg^{2+} (in the absence of Ca^{2+}). [The intrinsic K_m values for Ca^{2+} and Mg^{2+} binding to PtdInsP_2 are 2 mM and 5 mM respectively (Toner *et al.*, 1988). It should be noted that the concentration of Ca^{2+} or Mg^{2+} in the medium that results in half-maximal binding to PtdInsP_2 may be orders of magnitude lower because of negative surface potentials of the membranes.] Furthermore, the concentration of Ca^{2+} which is necessary to stimulate secretion half-maximally (1–2 μM) was unaltered by PtdIns-PLC (or the absence of ATP).

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