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Calcium Promotes the Accumulation of Polyphosphoinositides in Intact and Permeabilized Bovine Adrenal Chromaffin Cells

David A. Eberhard[1,2] and Ronald W. Holz[1,3]

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SUMMARY

1. Because cellular pools of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate turn over rapidly during phospholipase C stimulation, the continuing production of inositol phosphates requires continuing synthesis from phosphatidylinositol of the polyphosphoinositides. In the present study in adrenal chromaffin cells, we examined the effects of nicotinic stimulation and depolarization in intact cells and micromolar Ca^{2+} in permeabilized cells on the levels of labeled polyphosphoinositides. We compared the effects to muscarinic stimulation in intact cells and GTPγS in permeabilized cells.

2. Nicotinic stimulation, elevated K^+, and muscarinic stimulation cause similar production of inositol phosphates (D. A. Eberhard and R. W. Holz, *J. Neurochem.* **49**:1634–1643, 1987). Nicotinic stimulation and elevated K^+ but not muscarinic stimulation increased the levels of [^{3}H]inositol-labeled phosphatidylinositol phosphate by 30–60% and [^{3}H]phosphatidylinositol bisphosphate by 25–30%. The increase required Ca^{2+} in the medium, was maximal by 1–2 min, and was not preceded by an initial decrease in phosphatidylinositol phosphate and phosphatidylinositol bisphosphate.

3. In digitonin-permeabilized cells, Ca^{2+} caused as much as a twofold increase in [^{3}H]phosphatidylinositol phosphate and [^{3}H]phosphatidylinositol bis-

[1] Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109.
[2] Current address: Department of Pathology, University of Virginia Health Science Center, Charlottesville, Virginia 22908.
[3] To whom correspondence should be addressed at Department of Pharmacology, M6322 Medical Science I, University of Michigan Medical School, Ann Arbor, Michigan 48109-0626.

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phosphate. Similarly, Ca^{2+} enhanced the production of [^{32}P]phosphatidylinositol phosphate and [^{32}P]phosphatidylinositol bisphosphate in the presence of [γ-^{32}P]ATP. In contrast, GTPγS in permeabilized cells decreased polyphosphoinositides in the presence or absence of Ca^{2+}.

4. The ability of Ca^{2+} to increase the levels of the polyphosphoinositides decayed with time after permeabilization. The effect of Ca^{2+} was increased when phosphoesterase and phospholipase C activities were inhibited by neomycin.

5. These observations suggest that Ca^{2+} specifically enhances polyphosphoinositide synthesis at the same time that it activates phospholipase C.

INTRODUCTION

The inositol phospholipids play important roles in regulating cellular functions. The most well-defined function of these lipids is as substrates for receptor-activated phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate (PIP_2) to form the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1987). Although the production of inositol phosphates by phospholipase C has been intensively investigated, little is known about the mechanisms controlling the levels of the substrates, phosphatidylinositol phosphate (PIP) and PIP_2. Because the amounts of inositol phosphates produced can be many fold greater than changes in the levels of the polyphosphoinositides (Martin *et al.*, 1986) and is closely linked to the turnover of the polyphosphinositides (Agranoff *et al.*, 1983; Downes *et al.*, 1989), regulation of synthesis is crucial for the proper functioning of the phospholipase C signaling system. Phosphoinositides may also be involved in other cellular processes such as the regulation of protein function. For example, PIP or PIP_2 bind to and modulate the *in vitro* function of the g-actin binding protein profilin (Lassing and Lindberg, 1988; Goldschmidt-Clermont *et al.*, 1990) and the actin severing protein gelsolin (Janmey and Stossel, 1989). The findings that the type I PI kinase tightly associated with and is activated by PDGF receptor/PDGF (Coughlin *et al.*, 1989) and middle T antigen/pp60c-src complexes (Courtneidge and Heber, 1990) suggest that products of this PI kinase (phosphatidylinositol-3-phosphate and lipids derived from phosphatidylinositol-3-phosphate) may participate in a signal transduction mechanism.

Our interest in factors regulating the levels of the polyphosphoinositides stems from studies concerning the role of these lipids in chromaffin cells. Chromaffin cells of the adrenal medulla are specialized for the exocytotic secretion of catecholamine. Secretion from bovine chromaffin cells is triggered by Ca^{2+} influx following nicotinic stimulation or depolarization of intact cells or by the direct addition of micromolar Ca^{2+} to permeabilized cells (Knight and Baker, 1982; Dunn and Holz, 1983; Wilson and Kirshner, 1983). Ca^{2+} also activates phospholipase C in intact and permeabilized cells (Whitaker, 1985; Eberhard and Holz, 1987; Sasakawa *et al.*, 1989; Eberhard *et al.*, 1990). Muscarinic agonists stimulate phospholipase C in intact bovine chromaffin cells (Eberhard and Holz, 1987; Sasakawa *et al.*, 1989; Plevin and Boarder, 1988), presumably through a

GTP binding protein. However, muscarinic agonists stimulate little or no secretion, probably because the subsequent increase in cytosolic Ca^{2+} (Kao and Schneider, 1985) is not adjacent to the plasma membrane (Cheek *et al.*, 1989). We recently found that maintenance of the polyphosphoinositides in permeabilized cells is important for secretion independent of the lipids being substrates for phospholipase C (Eberhard *et al.*, 1990).

In the present study we examined the effects of nicotinic stimulation and depolarization in intact cells and micromolar Ca^{2+} in permeabilized cells on the levels of labeled polyphosphoinositides. We compared the effects to muscarinic stimulation in intact cells and guanosine 5′-[γ-thio]triphosphate (GTPγS) in permeabilized cells. We demonstrate a specific Ca^{2+}-dependent increase in the amounts of polyphosphoinositides in both intact and permeabilized cells. These observations suggest that Ca^{2+} plays an important role in regulating polyphosphoinositide synthesis that is distinct from its ability to activate phospholipase C.

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, 150,000 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 labeled by incubating for 36–60 hr with myo-[2-^{3}H]inositol (20 μCi/ml) in Eagle's minimal essential medium containing 11 μ*M* myoinositol (Whittaker M. A. Bioproducts, Walkersville, MD) supplemented with 10% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, NY), glutamine, penicillin, and streptomycin. [^{3}H]Inositol incorporation into lipid was maximal after about 48 hr of labeling. Immediately before starting an experiment, the labeling medium was removed and the cells were washed for 15 min in physiological salt solution (PSS) containing 145 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.6 m*M* glucose, 0.5 m*M* ascorbic acid, and 15 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4. Experiments were initiated by removing the PSS and adding fresh PSS or by permeabilizing the cells with 20 μ*M* digitonin in a solution (KGEP) containing 139 m*M* potassium glutamate, 20 m*M* 1,4-piperazinediethanesulfonic acid (Pipes, pH 6.6), 5 m*M* EGTA, and various amounts of Ca to achieve free Ca concentrations calculated (Chang *et al.*, 1988) using the constants of Portzehl *et al.* (1964). Other components of the KGEP and PSS solutions are detailed in the figure legends. Fifty to one hundred microliters of KGEP was used per well. Experiments with permeabilized cells were usually terminated by quantitatively transferring the incubation solution to tubes containing 1.0 ml ice-cold H_2O.

The samples were stored at −20°C for as long as 2 weeks prior to the analysis of the [^{3}H]inositol phosphates. The dilute samples were applied directly to

anion-exchange columns for the analysis of inositol phosphates as described below. Immediately after removing the incubation solution from the cells, 100 µl ice-cold methanol:conc HCl (100:1) 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 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 as long as 1 week prior to separation by thin-layer chromatography (TLC) or anion-exchange chromatography of deacylation products. In experiments with intact cells, trichloroacetic acid (TCA) was added to incubation solution (5% final TCA concentration; see tables and figures for details). The wells were scraped and TCA-insoluble phospholipids pelleted by centrifugation. Phospholipids in the precipitate were extracted as described below. The supernatent was extracted with 3 × 5 vol of diethyl ether. Residual ether was evaporated under a N_2 stream. The aqueous solutions were brought to pH 6.5–8.0 with NaOH and applied to anion-exchange columns for the analysis of inositol phosphates as described below.

Assay of [^{3}H]Phosphoinositides. Lipids were extracted by mixing 400 µl $CHCl_3$:methanol (2:1) with the methanolic samples or TCA precipitates, which were then washed with 250 µl 10 m*M* EDTA in 1*N* HCl (vigorous vortexing followed by centrifugation to separate phases). Two hundred microliters of the lower organic phase was transferred to another tube. The upper phase and interface were washed with 200 µl $CHCl_3$, 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 10 m*M* EDTA in 1*N* HCl:methanol (1:1). The entire lower phase was transferred to a new tube for subsequent lipid analysis. If lipids were to be separated by TLC, the samples were dried under N_2 and redissolved in $CHCl_3$:methanol (2:1) before storage at −20°C. After each wash in the extraction procedure, the total volumes of the organic phases were measured to determine recoveries.

Anion-exchange chromatography of lipid deacylation products was performed essentially as described by Downes and Michell (1981). The volume of the lipid extract was brought to 500 µl with $CHCl_3$. One hundred microliters methanol and 100 µl 1.0*N* NaOH in methanol:H_2O (19:1) were added. After vortexing, the samples were allowed to stand 30 min at room temperature. The phases were then broken by adding 500 µl $CHCl_3$, 300 µl methanol, and 300 µl H_2O, followed by vigorous vortexing and centrifugation. Four hundred fifty microliters of the upper aqueous phase (total volume, 550 µl) was transferred to a new tube, blown briefly under N_2 to remove traces of $CHCl_3$, and then diluted to 3.0 ml with 5 m*M* Na tetraborate/60 m*M* Na formate. The samples were applied to 0.3-ml-bed volume Dowex AG1-X3 columns (formate form; Bio-Rad Laboratories, Richmond, CA). The void volume contained [^{3}H]glycerophosphoinositol (GPI). [^{3}H]GPI remaining on the columns was eluted with 3 × 1.0 ml 5 m*M* Na tetraborate/180 m*M* NH_4 formate. Each 3.0-ml fraction was mixed with 15 ml Universol ES scintillant (Dupont NEN, Wilmington, DE) for counting. Over 90% of total [^{3}H]GPI eluted in these fractions. The columns were then washed

with 4×3 ml of the same buffer; these washes were usually discarded. [^{3}H]Glycerophosphoinositol phosphate (GPIP) was eluted with 7×1.0 ml 0.3*M* NH_4 formate/0.1*M* formic acid, recovering >90% of the total [^{3}H]GPIP; 3.5-ml fractions were counted with 17 ml Universol ES. The columns were further washed with 4×3 ml of the same buffer; the washes were usually discarded. [^{3}H]Glycerophosphoinositol bisphosphate ($GPIP_2$) was eluted quantitatively with 4×1.0 ml 1.0*M* NH_4 formate/1.0*M* formic acid and counted as a single fraction with 15 ml Universol ES. The deacylation procedure quantitatively converted organic-soluble ^{3}H to aqueous ^{3}H. Aqueous contamination of the organic extracts was assessed by processing samples as described with the substitution of methanol for methanolic NaOH in the deacylation step. Contamination accounted for about 0.6% of [^{3}H]GPI and about 1.3% of both [^{3}H]GPIP and [^{3}H]$GPIP_2$.

Lipid Phosphorylation. Cells were permeabilized with KGEP containing 20 μ*M* digitonin, [γ-^{32}P]MgATP (50–100 Ci/mol), and other additions as noted. Incubations were terminated and lipids extracted as described above. Organic extracts were spotted on silica gel HL plates and developed in $CHCl_3$ (44):methanol (44):H_2O (7):30% NH_4OH (5). Bands were visualized by autoradiography, scraped, and counted in 4-ml Universol ES scintillant. The only lipids labeled under these conditions were the direct products of lipid kinases; PIP_2, PIP, and PA. PI was not labeled because small molecules required for PI synthesis from PA (inositol and CTP) were rapidly lost from the permeabilized cells (authors' unpublished observations).

Assays of [^{3}H]Inositol Phosphates. [^{3}H]Inositol phosphates were separated by anion-exchange chromatography according to Berridge *et al.* (1983). The samples were applied to Dowex AG1-X8 columns (formate form, 0.3-ml bed volume). Free inositol and GPI were eluted with 8×1.0-ml washes of 5 m*M* Na tetraborate/60 m*M* Na formate. These washes were usually discarded. [^{3}H]IP_1 was eluted with 3×1.0 ml 0.2*M* NH_4 formate/0.1*M* formic acid; the columns were then washed with 3×1.0 ml of the same buffer. [^{3}H]IP_2 was eluted with 7×1.0 ml 0.4*M* NH_4 formate/0.1*M* formic acid, followed by 3×3.0-ml washes with the same buffer. [^{3}H]IP_3 was eluted with 4×1.0 ml 1.0*M* NH_4 formate/0.1*M* formic acid. Radioactivity in the eluates was determined by scintillation counting as described above. The IP_3 fraction also contains IP_4 (Batty *et al.*, 1985).

Data Analysis. Unless otherwise indicated, the figures and the tables show data from individual experiments representative of similar experiments performed in two or more cell culture preparations. Data shown are average counts per minute (cpm) per well ± SE. There were four wells per group. Error bars smaller than the data point symbols were omitted. Differences between the means of groups were tested for significance with Student's *t* test. The standard error of the mean associated with the difference between the means of two groups was calculated by $(SE_1^2 + SE_2^2)^{1/2}$.

Materials. All reagents were obtained from standard commercial sources. Myo-[2-^{3}H]inositol (1 mCi/ml, 10–30 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Polar contaminants were removed before use by mixing a few milligrams of Dowex AG1-X8 with aqueous [^{3}H]inositol. [γ-

^{32}P]ATP was from Amersham Corp. (Arlington Heights, IL). GTPγS (Li salt) was from Boehringer Mannheim Biochemicals (Indianopolis, IN).

RESULTS

Effects of Stimulation of Intact Chromaffin Cells on the Levels of the Polyphosphoinositides. Stimulation of [^{3}H]inositol-prelabeled chromaffin cells with the nicotinic agonist DMPP increased the levels of [^{3}H]PIP and [^{3}H]PIP_2 within 1 min of stimulation (Fig. 1). The most prominent increase was in [^{3}H]PIP, which was maximal (approximately 50% increase) by 2 min after the addition of DMPP. The DMPP-stimulated increments in [^{3}H]PIP and [^{3}H]PIP_2 consisted entirely of the PI-4-P and P-4,5-P_2 isomers, respectively (D. A. Eberhard, R. W. Holz, and L. L. Cantley, unpublished observations). DMPP also increased [^{3}H]IP_2 and [^{3}H]IP_3 as has been described previously (Eberhard and Holz, 1987).

The increase in [^{3}H]polyphosphoinositide levels after cell stimulation probably resulted from Ca^{2+} influx. Table I shows that depolarization of the cells with elevated K^+ caused an increase in [^{3}H]PIP and [^{3}H]PIP_2 similar to that seen with

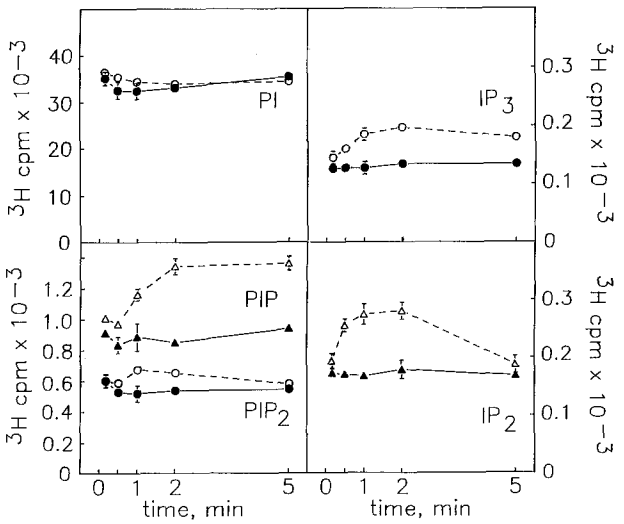

Fig. 1. Time course of DMPP effects on [^{3}H]inositol phospholipids and [^{3}H]inositol phosphates. Chromaffin cells prelabeled with [^{3}H]inositol were incubated with 50 μl PSS containing 2.2 m*M* $CaCl_2$ and 2 mg/ml BSA (CaPSS-BSA) for 30 min. CaPSS-BSA (100 μl) with 15 μ*M* DMPP (open symbols, dashed lines) or without DMPP (filled symbols, solid lines) was then added to the wells (final [DMPP] = 10 μ*M* when present. After continuing the incubations for 10 sec–5 min as indicated, 150 μl ice-cold 10% TCA was added to the wells (final [TCA] = 5%) and the cells were processed for determination of [^{3}H]inositol lipids and [^{3}H]inositol phosphates as described under Materials and Methods. The left ordinates correspond to the left panels; the right ordinates correspond to the right panels.

Table I. Ca^{2+} Dependence of the Effects of DMPP, Elevated K^+, and Methacholine on [^{3}H]PIP and [^{3}H]PIP_2 Levels[a]

Additions	% of total lipid ^{3}H	
	PIP	PIP_2
None	2.77 ± 0.33 (100)	1.11 ± 0.03 (100)
DMPP	3.75 ± 0.08* (135)	1.29 ± 0.04** (116)
39 m*M* KCl	3.57 ± 0.05* (129)	1.38 ± 0.04** (124)
MeCh	2.90 ± 0.08 (104)	1.17 ± 0.06 (105)
DMPP + MeCh	3.49 ± 0.06* (126)	1.26 ± 0.05 (114)
EGTA	2.83 ± 0.12 (102)	1.17 ± 0.03 (105)
DMPP + EGTA	2.94 ± 0.06 (106)	1.24 ± 0.08 (111)
39 m*M* KCl + EGTA	3.05 ± 0.14 (110)	1.23 ± 0.13 (111)

[a] [^{3}H]Inositol-prelabeled cells were incubated 30 min with 50 µl PSS (pH 7.40) containing 2 mg/ml BSA and 2.2 m*M* $CaCl_2$. PSS-BSA (100 µl) containing various additions as indicated was then added to the culture wells. The final concentrations of the additions in the wells were 10 µ*M* DMPP, 39 m*M* KCl replacing NaCl, 333 µ*M* methacholine (MeCh), 3.33 m*M* EGTA. The Ca^{2+} concentration in wells without EGTA was 2.2 m*M*; the total Ca^{2+} in wells with EGTA was 0.73 m*M* (free $Ca^{2+} < 0.1$ µ*M*). After 3 additional min of incubation, 10% TGA (150 µl) was added to the wells (final [TCA] = 5%) and the cells were processed for determination of [^{3}H]inositol lipids as described under Materials and Methods. Data are expressed as percentage of total lipid radioactivity ± SE. There were four wells per group.

* $P < 0.01$ vs no additions (Bonferroni method).

** $P < 0.05$ vs no additions (Bonferroni method).

DMPP. The effects of DMPP or elevated K^+ on [^{3}H]PIP were inhibited when EGTA was included in the incubation medium. The muscarinic agonist methacholine (300 µ*M*), which does not cause Ca^{2+} influx, had little effect on [^{3}H]PIP or [^{3}H]PIP_2 levels. The effects of various treatments on [^{3}H]PIP_2 levels were smaller than on [^{3}H]PIP and significant differences in [^{3}H]PIP_2 levels between groups were difficult to discern.

Effects of Ca^{2+} and GTPγS on Polyphosphoinositide Levels in Permeabilized Chromaffin Cells. Permeabilization of [^{3}H]inositol-prelabeled chromaffin cells with digitonin in the presence of Ca^{2+} caused increases in the levels of [^{3}H]PIP and [^{3}H]PIP_2 and stimulated the formation of [^{3}H]inositol phosphates (Fig. 2). The Ca^{2+}-induced increases in both [^{3}H]PIP and [^{3}H]PIP_2 were half-maximal at about 1 µ*M* Ca^{2+}, peaked at 3–10 µ*M* Ca^{2+}, and declined at higher Ca^{2+} concentrations. Formation of [^{3}H]inositol phosphates was half-maximal at 3–10 µ*M* Ca^{2+} and maximal at 100–300 µ*M* Ca^{2+}. The amount [^{3}H]inositol phosphates produced was several fold greater than the total amount of the polyphosphoinositides and was approximately equal to the loss of [^{3}H]PI. Thus, the continued production of inositol polyphosphates requires continued synthesis from PI. Micromolar Ca^{2+} increased the levels of the polyphosphoinositides despite the stimulation of phosphoinositide hydrolysis by Ca^{2+}-induced activation of phospholipase C.

The effects of Ca^{2+} on the inositol lipids were also observed by measurement of the phosphorylation of the inositol phospholipids with [γ-^{32}P]ATP. Ca^{2+} (10 µ*M*) in the presence of [γ-^{32}P]ATP stimulated ^{32}P incorporation into PIP and

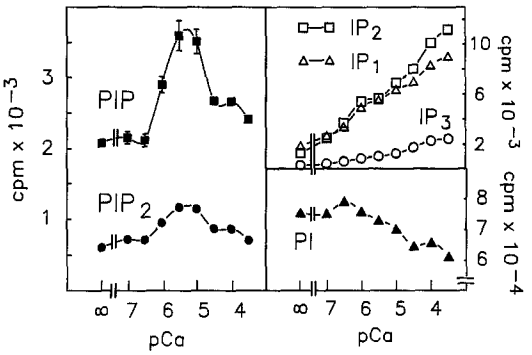

Fig. 2. Concentration–response relationships for Ca^{2+} on phosphoinositide levels and release of inositol phosphates. [^{3}H]Inositol-prelabeled cells were incubated 15 min with KGEP containing 20 μ*M* digitonin, 2 m*M* MgATP, and various concentrations of free Ca^{2+} as indicated. The incubation medium was then removed and methanol:HCl (50:1) was added to the cells. [^{3}H]Inositol phosphates released into the medium and cellular [^{3}H]lipids were assayed (see Materials and Methods). *p*Ca is $-\log[Ca^{2+}]$. The left ordinate corresponds to the left panel; the right ordinates correspond to the right panels.

PIP_2 in permeabilized cells (Table II). [^{32}P]PA was also increased, presumably reflecting the phosphorylation of diacylglycerol produced by phospholipase C activity. GTP-γ-S (100 μ*M*), which stimulates approximately the same production of inositol phosphates production as 10 μ*M* Ca^{2+} (Eberhard *et al.*, 1990), caused an increase in phosphorylated diacylglycerol similar to that produced by 10 μ*M* Ca^{2+} but decreased the amounts of [^{32}P]PIP and [^{32}P]PIP_2. The addition of GTP-γ-S together with Ca^{2+} prevented the increase in PIP and PIP_2 by Ca^{2+} and further increased [^{32}P]PA formation, suggesting that polyphosphoinositides which would have accumulated in the presence of Ca^{2+} alone were hydrolyzed by GTP-γ-S-activated phospholipase C. The data demonstrate that the ability of

Table II. Effect of Ca^{2+} and GTPγS on Lipid Phosphorylation[a]

	cpm/well		
	PIP_2	PIP	PA
No additions	195 ± 12 (100)	255 ± 10 (100)	320 ± 7 (100)
Ca^{2+}	400 ± 17* (205)	390 ± 16* (153)	826 ± 25* (258)
GTPγS	141 ± 15 (72)	123 ± 6* (48)	873 ± 43* (254)
Ca^{2+} + GTPγS	122 ± 8* (63)	115 ± 10* (45)	1412 ± 31* (441)

[a] Cells were permeabilized 5 min in the presence of 5 m*M* Mg[γ-^{32}P]ATP with either no additions, 10 μ*M* Ca^{2+}, 100 μ*M* GTPγS, or Ca^{2+} + GTPγS. Incubations were terminated and [^{32}P]lipids quantitated by TLC as described under Materials and Methods. The cpm/well of the groups in each column expressed as the percentage of *no additions* is shown in parentheses. There were four wells per group.

* $P < 0.01$ compared to no additions.

Ca^{2+} to promote increases in labeled polyphosphoinositides is specific and does not reflect a generalized secondary response to phospholipase C activation.

Effect of Neomycin on Ca^{2+}-Induced Increases in the Polyphosphoinositides. Ca^{2+} could increase levels of polyphosphoinositides by activating PI or PIP kinase or by inhibiting phosphomonoesterases. The polycation neomycin binds through ionic interactions with the highly charged anionic phosphoinositides, protecting the headgroups from phosphatase and phospholipase C activities (Schacht, 1976; Schacht, 1978; Downes and Michell, 1981). We recently demonstrated in digitonin permeabilized cells that neomycin protects the polyphosphoinositides from breakdown in the absence of MgATP (Eberhard *et al.*, 1990). We also found that neomycin enhances the levels of the polyphosphoinositides in the presence of MgATP (C. L. Cooper and R. W. Holz, manuscript in preparation). The latter effect probably results from inhibited polyphosphoinositide phosphatase activity and continuing polyphosphoinositide synthesis. If Ca^{2+} stimulates PI or PIP kinase, the Ca^{2+}-induced increase in polyphosphoinsoitides should be enhanced by phosphatase and phospholipase C inhibition caused by neomycin. Conversely, if the Ca^{2+}-induced increases in PIP and PIP_2 levels result from inhibition of PIP or PIP_2 phosphatases (or activation of phospholipase C), the effect of Ca^{2+} should be reduced when these enzymes are simultaneously inhibited by neomycin. Table III shows the result of such an experiment. Permeabilizing [^{3}H]inositol-prelabeled cells in the presence of neomycin (250 μM) increased the basal levels of [^{3}H]PIP and [^{3}H]PIP_2. Neomycin did not inhibit the effects of Ca^{2+} on the polyphosphoinositides. Instead, neomycin increased the Ca^{2+}-induced increments in [^{3}H]PIP_2 and [^{3}H]PIP 2.12-

Table III. Effect of Ca^{2+} on [^{3}H]PIP and [^{3}H]PIP_2 Levels in the Absence and Presence of Neomycin[a]

Addition	% of total ^{3}H	
	PIP	PIP_2
− neomycin		
$-Ca^{2+}$	1.86 ± 0.06	0.600 ± 0.017
$+Ca^{2+}$	3.40 ± 0.11*	1.116 ± 0.045*
Ca^{2+} dependent	1.54 ± 0.12	0.516 ± 0.048
+ neomycin		
$-Ca^{2+}$	3.31 ± 0.07	1.93 ± 0.05
$+Ca^{2+}$	5.43 ± 0.05*	3.03 ± 0.07*
Ca^{2+} dependent	2.12 ± 0.09	1.10 ± 0.09

[a] [^{3}H]Inositol-prelabeled cells were permeabilized for 15 min with KGEP containing 20 μM digitonin, 3 mM MgATP, with or without neomycin (250 μM) as indicated and with or without added Ca^{2+} (10 μM) as indicated. The incubations were terminated by removing the permeabilization medium and adding 5% TCA to the wells. [^{3}H]Inositol phospholipids were quantitated by anion-exchange chromatography of deacylation products as described under Materials and Methods. Data are expressed as the percentage of total radioactivity present in lipids and inositol phosphates ± SE. The Ca^{2+}-dependent accumulations were calculated as the difference between the "$+Ca^{2+}$" and the "$-Ca^{2+}$" groups. There were four wells per group.

* $P < 0.001$ vs $-Ca^{2+}$.

and 1.38-fold, respectively. In another experiment the increases were 2.13- and 1.01-fold, respectively. Under these conditions, phospholipase C activity was inhibited approximately 50% (data not shown). Thus, neomycin, which inhibits the degradation of the polyphosphoinositides, augmented the Ca^{2+}-dependent increase in the polyphosphoinositides. The predominant increase in [^{3}H]PIP_2 rather than [^{3}H]PIP may reflect Ca^{2+}-induced stimulation of PIP as well as PIP_2 synthesis since PIP is a precursor of PIP_2. Because neomycin inhibits Ca^{2+}-dependent secretion when present together with Ca^{2+} in permeabilized cells (Bittner *et al.*, 1986), the experiment rules out the possibility that the effects of Ca^{2+} on the polyphosphoinositides are caused by exocytosis.

The Effects of Ca^{2+} on PI Metabolism May Be Mediated by Soluble Factors. The ability of Ca^{2+} to increase the polyphosphoinositides became progressively smaller as the time between the initial permeabilization with digitonin and the subsequent Ca^{2+} challenge increased (Fig. 3). The maximal increase in polyphosphoinositides occurred when Ca^{2+} was added together with digitonin. The ability of Ca^{2+} to stimulate phosphoinositide [^{32}P]phosphorylation was also lost with increasing permeabilization time (data not shown). It is unlikely that phosphoinositide kinase or phosphatase activities are lost after permeabilization, since the basal levels of PIP_2 and PIP were maintained (in the presence of millimolar MgATP) for at least 18 min in permeabilized cells (Eberhard *et al.*, 1990). Indeed, permeabilization with digitonin for up to 10 min did not reduce cellular PI kinase activity, assayed with exogenous PI in Triton X-100 lysates (data not shown). The loss of responses to Ca^{2+} with increasing permeabilization time suggests that the responses may involve a soluble factor which is lost from the cells.

PI kinase activity was not increased by Ca^{2+} (10 μM) in chromaffin cell homogenates or in homogenates prepared from Ca^{2+}-stimulated cells (data not shown). It is unlikely that the Ca^{2+} effects in cells are mediated by calmodulin or protein kinase C. The calmodulin binding peptide Ca^{2+}/calmodulin kinase II(291-317) and the protein kinase C inhibitory peptide PKC(19-31) (House and Kemp, 1987) at concentrations which selectively inhibit Ca^{2+}- or TPA-induced protein phosphorylation in permeabilized chromaffin cells [3 and 10 μM,

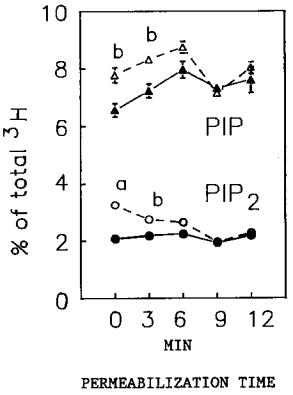

Fig. 3. Rundown of Ca^{2+} effects in permeabilized cells. [^{3}H]Inositol-prelabeled cells were permeabilized in KGEP containing 20 μM digitonin and 2 mM MgATP for the indicated times (permeabilization time). The permeabilizing medium was then removed, and fresh medium, containing digitonin, MgATP, and either 0 Ca (filled symbols) or 10 μM free Ca (open symbols), was added. After 15 min the medium was removed and assayed for [^{3}H]IP_1 and [^{3}H]inositol polyphosphates (IPP) as described under Materials and Methods. [^{3}H]Inositol phospholipids remaining in the cells were assayed by anion-exchange chromatography of the deacylation products. The data were normalized as the percentage of the sum of [^{3}H]inositol phosphates + [^{3}H]inositol phospholipids (% of total ^{3}H). Significant differences between Ca-treated and untreated groups in PIP_2 and PIP are (a) $P < 0.001$ and (b) $P < 0.01$.

respectively (TerBush and Holz, 1990)] did not alter [^{3}H]phosphoinositide levels or [^{3}H]inositol phosphates released in the presence or absence of Ca^{2+} in permeabilized cells.

DISCUSSION

Hormone or neurotransmitter receptors acting through a GTP binding protein or Ca^{2+} entry into cells can stimulate phospholipase C activity and produce inositol phosphates (Berridge, 1987; Eberhard and Holz, 1987; Eberhard and Holz, 1988). Because the amount of inositol phosphates produced can be manyfold greater than the changes in the polyphosphoinositide levels, the continuing production of inositol phosphates requires continuing synthesis of the polyphosphoinositides from PI via PI and PIP kinases. An initial loss of polyphosphoinositides upon phospholipase C activation followed by a compensatory increase in synthesis has been observed in thrombin-stimulated platelets (Agranoff *et al.*, 1983), vasopressin-treated hepatocytes (Creba *et al.*, 1983), pituitary cells stimulated with TRH (Rebecchi and Gershengorn, 1983), and fibroblast homogenates stimulated with GTPγS (Chahwala *et al.*, 1987).

In the present study, the amounts of the inositol phosphates produced in permeabilized cells were manyfold greater than the *total* polyphosphoinositides (Fig. 2). One possible mechanism for regulating the synthesis of the polyphosphoinositides is through product inhibition which is relieved by activation of phospholipase C (Van Rooijen *et al.*, 1985). In this case, activation of phospholipase C by different mechanisms should have similar effects on the levels of the polyphosphoinositides. We have investigated in intact and permeabilized adrenal chromaffin cells the effects of different phospholipase C activators on polyphosphoinositide levels. We found that nicotinic agonist in the presence of Ca^{2+} in intact cells or micromolar Ca^{2+} in permeabilized cells, in addition to strongly activating phospholipase C, *increased* the polyphosphoinositides. In contrast, muscarinic receptor activation in intact cells or GTPγS in permeabilized cells, which also strongly activates phospholipase C, had no effect (intact cells) or decreased (permeabilized cells) the levels of the polyphosphoinositides. Neomycin, which inhibits polyphosphoinositide hydrolysis by phospholipase C, enhanced the effects of Ca^{2+}. These experiments indicate for the first time that micromolar Ca^{2+} regulates the cellular levels of the polyphosphoinositides through a specific mechanism that is not simply coupled to phospholipase C activation.

Micromolar Ca^{2+} could specifically increase the polyphosphoinositides by increasing synthesis and/or decreasing metabolism. Neomycin, which inhibits polyphosphoinositide phosphatases and phospholipase C (Schacht, 1976; Eberhard *et al.*, 1990), augmented the increases caused by Ca^{2+}, which suggests that Ca^{2+} increased polyphosphoinositide synthesis. Experiments with sea urchin cortices (Oberdorf *et al.*, 1989) and rat liver membranes (Whipps *et al.*, 1987) are consistent with Ca^{2+} being able to activate polyphosphoinositide synthesis. Pulse-chase experiments with [γ-^{32}P]ATP in permeabilized cells were inconclusive in determining whether PIP or PIP_2 phosphatase activities were decreased by

Ca^{2+} (data not shown). Because the ability of Ca^{2+} to increase the polyphosphoinositides was lost with time after permeabilization (Fig. 3), it is possible that the Ca^{2+}-dependent regulation of polyphosphoinositide levels is mediated through a soluble factor that is lost from permeabilized cells.

It has been reported that micromolar Ca^{2+} inhibits the PI kinase activity of purified chromaffin granule membranes (Husebye and Flatmark, 1988). The present study indicates that in intact and permeabilized cells, micromolar Ca^{2+} increases rather than decreases polyphosphoinositide synthesis. We were unable to detect an effect of micromolar Ca^{2+} on PI kinase activity in chromaffin cell homogenates (data not shown). It is possible that PI kinase in the plasma membrane is the predominant PI kinase activity in chromaffin cells and differs from PI kinase in chromaffin granule membranes.

We have previously suggested that an increase in intracellular Ca^{2+} acting at the level of phospholipase C promotes hormone-induced generation of IP_3 and diacylglycerol (Eberhard and Holz, 1988). The present study indicates that Ca^{2+} could also enhance the production of these second messengers by increasing the synthesis of the phospholipase C substrates.

In addition to the importance of polyphosphoinositides in the generation of IP_3 and DAG via phospholipase C, the polyphosphoinositides may play a more direct role in secretion. We have observed that secretion in permeabilized cells is closely correlated with the levels of the polyphosphoinositides but not with phospholipase C activity (Eberhard *et al.*, 1990). The experiments suggested that the polyphosphoinositides play a role in secretion in addition to their being substrates for the generation of IP_3 and DAG. Thus, the effects of Ca^{2+} on the levels of the polyphosphoinositides may not only affect the production of IP_3 and DAG but also secretion through a separate, as yet unknown mechanism.

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