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Evidence for coupling of resynthesis to hydrolysis in the phosphoinositide cycle

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Previous data suggest that agonist-induced hydrolysis of phosphatidylinositol bisphosphate is accompanied by resynthesis through phosphatidylinositol such that these metabolic events function in a cyclic manner. However, it is not known whether resynthesis depends on the presence of agonist or is a direct result of agonist-induced breakdown. In the present study we demonstrate that: (1) increasing the intracellular free inositol concentration will not stimulate phosphatidylinositol synthesis, as measured by assessing the amount of [^{32}P]P_i incorporation; (2) regeneration of free inositol is required for resynthesis; however, addition of exogenous inositol can sustain resynthesis under conditions which inhibit the regeneration of endogenous inositol; (3) resynthesis can take place in the absence of agonist provided that cells have been previously incubated under conditions which prevent resynthesis; and (4) the presence of agonist does not increase the rate of resynthesis. Thus the resynthetic phase of the phosphoinositide cycle is a compensatory event triggered either by the decrease in the level of phosphatidylinositol or by an increase in precursor substrates. The agonist itself appears to have no direct effect on the resynthesis process.

INTRODUCTION

The cyclic nature of the phosphoinositide effect has been deduced from the fact that rapid phospholipase C-induced breakdown of phosphatidylinositol bisphosphate is accompanied by an increase in the rate of incorporation of [^{32}P]P_i into phosphatidylinositol, as well as into the 1-phosphate of the polyphosphoinositides [1,2]. Failure to observe comparable increases in the rate of incorporation of labelled inositol under conditions in which the specific radioactivity of the free inositol pool is 18-fold higher than that in the lipid pool [3,4] has led to the suggestion that the inositol is reutilized during the resynthetic phase. The increase observed in synthesis of phosphatidylinositol has been assumed to be secondary to hormone-induced hydrolysis, but whether or not hormonal stimulation plays any more direct effect has not been addressed. In addition, it is not clear whether it is an increase in precursors or a decrease in phosphatidylinositol, or both, which trigger resynthesis. Imai & Gershengorn have suggested that it is the decrease in phosphatidylinositol [5].

Previous experiments from our laboratory have suggested the existence of vasopressin-sensitive and -insensitive pools within WRK-1 rat mammary tumour cells [2,3,6,7]. This conclusion was based on the differential labelling of these pools with [^{32}P]P_i. Similar experiments utilizing [^{3}H]inositol failed to differentiate between individual pools, possibly because the inositol was being incorporated via an exchange reaction rather than synthesis *de novo* [3,4]. In the same study, the inability of hormone to induce disappearance of [^{3}H]inositol-labelled phosphatidylinositol in the absence of Li^+ suggested reutilization of the labelled moiety, as did the inability of vasopressin to induce an increase in inositol incorporation into a hormone-sensitive pool. In addition, Li^+ partially inhibited the hormone-induced increase in incorporation of radioactive phosphate into phosphatidylinositol, although values never decreased to below basal levels, suggesting that the decrease in free inositol resulting from Li^+ treatment affected only cyclic resynthesis and not non-cyclic inositol incorporation [3]. Similar results concerning the ability of Li^+ to block cyclic resynthesis of phosphatidylinositol have been reported for GH_3 cells [8]), rat parotid cells [9], brain slices [10] and adrenal cells [11]. Together, these data suggested that resynthesis was linked to breakdown.

In the present study, we examine cyclic phosphoinositide metabolism in more detail, and demonstrate (1) that the inhibition of resynthesis seen in the presence of Li^+ can be overcome by addition of exogenous inositol, and (2) that resynthesis of phosphatidylinositol is not directly affected by hormone, but rather is regulated by the availability of either substrate or product.

MATERIALS AND METHODS

Materials

Tissue-culture media, trypsin, Dulbecco's phosphate-buffered saline, antibiotic–antimycotic and glutamine were purchased from GIBCO. Plasticware for tissue culture was from Costar. Anion-exchange resin (AG 1-X8, 200–400 mesh, formate form) was from Bio-Rad. Thin-layer chromatographs [silica gel (IB-2F)] were from Baker. [^{32}P]P_i (carrier-free) was purchased from ICN, and [^{3}H]inositol (15 Ci/mmol), [^{14}C]inositol (250 mCi/mmol) and [^{3}H]glycerol (20 Ci/mmol) were from American Radiolabelled Chemicals. Aquasol-2 was from DuPont–New England Nuclear. Vasopressin was purchased from Sigma.

Cell culture

WRK-1 cells were established from a dimethylbenz-[*a*]anthracene-induced rat mammary tumour as previously described [12]. The variant used in these experiments (1B) arose spontaneously and has been previously described [3]. Monolayer cultures were maintained in Ham's F-12 medium supplemented with Earle's salts and fetal-calf serum (10%, v/v), rat serum (2%, v/v), penicillin (100 units/ml), Fungizone (0.25 μg/ml) and streptomycin (100 μg/ml). For experiments, cells were harvested with a solution of 0.05% trypsin/0.02% EDTA in 0.9% NaCl and replicately plated into 22 mm wells. Details for each experiment appear in the Figure legends.

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Extraction and analysis of phospholipids and inositol phosphates

Lipids and their breakdown products were extracted and analysed as previously described [6,7]. Diacylglycerol radioactivity was analysed by t.l.c. on silica gel with the solvent system benzene/diethyl ether/ammonia (400:100:1, by vol.). For analysis of CDP-diacylglycerol a number of systems were used: two-dimensional t.l.c. on silica gel: first dimension, chloroform/methanol/acetic acid/water (50:30:8:4, by vol.) and second dimension, chloroform/methanol/acetic acid/water (40:10:10:1, by vol.). Alternatively, in some experiments lipids were deacylated and the hydrophilic residue was run on Dowex as previously described [2], except that a batchwise elution was carried out with 0.06 M (glycerophosphoinositol), 0.12 M (glycerol phosphate) and 0.18 M (CDP-glycerol)-ammonium formate/0.005 M-sodium borate.

RESULTS

Effect of vasopressin and LiCl on levels of phosphoinositides, free inositol and inositol phosphates in WRK-1 cells

We have previously determined that, in the presence of Li^+, vasopressin causes a decrease in intracellular radiolabelled free inositol and phosphoinositide, as well as an increase in radiolabelled inositol phosphates [2,3]. In order to determine whether these values represented changes in the actual mass of these compounds, the following experiment was done. Cells were grown for several passages in medium containing 18 mg of unlabelled inositol/l and 100 μCi of [^{14}C]inositol/l. They were then plated out into 22 mm wells in the same medium. At 24–48 h after plating, cells were stimulated, without a change of medium, for 90 min, and the radioactivity in the various fractions was determined. Under these conditions, it was possible to determine the absolute amount of each moiety. Table 1 outlines the results. Although the presence of serum initially stimulates inositol phosphate accumulation, by 24 or 48 h after plating little stimulatory effect is seen, as evidenced by a comparison of the values for the control versus Li^+-treated cells. Vasopressin effects are identical with those previously observed when radioactivity was washed out before stimulation [2,3]. As shown in this experiment, vasopressin alone has no effect on the levels of free inositol; however, in the presence of Li^+ and vasopressin, the inositol levels are decreased by 88%. Phosphoinositide levels are decreased by 60%, and inositol phosphate levels are increased by 1067%. Thus two conclusions can be drawn about inositol levels: (1) free inositol levels are not increased upon stimulation with vasopressin alone; and (2) free inositol levels are drastically decreased in cells treated with a combination of vasopressin and LiCl.

Table 1. Levels of phosphoinositide-cycle intermediates

Cells were incubated for several passages in medium containing [^{14}C]inositol. Experiments were carried out in the same medium, as described in the text. After incubation for 90 min with the substances indicated [VP = vasopressin (0.1 μM); LiCl (10 mM)], the amount of radioactivity in each fraction was measured, and, based on the specific radioactivity of the inositol, absolute values were calculated. Values given are pmol/10^6 cells, and represent means ± 1 S.D. of sextuplicates. Similar values were obtained in several other experiments.

Addition	Content		
	Phospho-inositides	Inositol	Inositol phosphates
Control	975.5 ± 22.7	341.2 ± 50.9	64.6 ± 12.7
VP	806.2 ± 55.5	352.1 ± 40.0	152.8 ± 20.9
LiCl	998.2 ± 57.3	201.0 ± 22.7	77.3 ± 10.0
VP + LiCl	387.6 ± 20.9	42.7 ± 5.2	747.1 ± 61.8

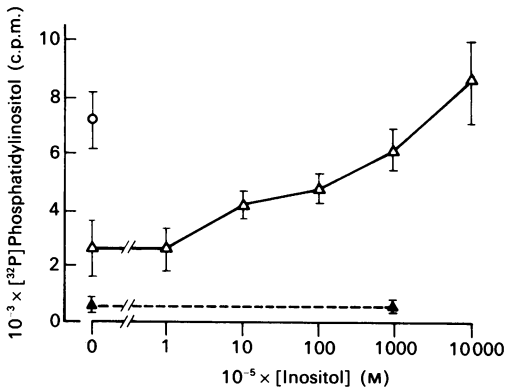

Fig. 1. Effect of exogenous inositol on Li^+-induced inhibition of vasopressin-stimulated [^{32}P]P_i incorporation into phosphatidylinositol

Cells were incubated for 2 h in serum-free inositol-free Minimal Essential Medium containing 25 mM-Tricine and 5 μCi of [^{32}P]P_i/ml. Exogenous inositol was added at the concentration indicated. Lipids were extracted and the phosphatidylinositol was quantified as described in the text. Values shown represent means ± 1 S.D. of triplicate determinations: ▲, no addition; ○, +vasopressin (0.1 μM); △, +vasopressin + LiCl (25 mM).

Effect of exogenous inositol on control and vasopressin-stimulated phosphate incorporation into phosphatidylinositol in the presence of LiCl

Li^+ blocks vasopressin-induced incorporation of radioactive phosphate into phosphatidylinositol, presumably by preventing regeneration of free inositol. In the experiment shown in Fig. 1, Li^+ treatment resulted in a 67% inhibition of the vasopressin effect in the absence of exogenous inositol. A concentration of inositol between 10 and 100 mM completely reversed the effect of Li^+. Basal incorporation was not significantly affected by the presence of exogenous inositol (10 mM). These data suggest that regeneration of free inositol is required for proper functioning of the resynthetic phase of the cycle, but that increasing the level of intracellular free inositol in the absence of a decrease in phosphatidylinositol will not increase the rate of synthesis of phosphatidylinositol.

Effect of exogenous inositol on levels of intracellular free inositol

In order to determine the relationship between a given level of extracellular inositol and the level of intracellular free inositol, cells were incubated with various levels of unlabelled inositol in the presence of 10 μCi of radiolabelled inositol/ml. Table 2 shows the amount of inositol taken up by the cells at the end of a 90 min incubation. Incubation with 1 mM-inositol results in uptake of 360.9 pmol/10^6 cells, an amount equivalent to that seen normally in cells. The uptake increases approximately linearly with the increase in exogenous concentration.

Effect of vasopressin/Li^+ pretreatment on phosphate incorporation into phosphatidylinositol

Others have demonstrated that treatment of cells with an agonist in the presence of Li^+ not only results in an increased accumulation of inositol phosphates, but also increases the amount of radioactive phosphate that accumulates in

Table 2. Uptake of exogenous inositol as a function of extracellular inositol concentration

Cells were incubated in inositol-free Minimal Essential Medium in the presence of 10 μCi of [^{3}H]inositol/ml and varying concentrations of unlabelled inositol for 90 min. Cells were then washed by dipping twice in ice-cold phosphate-buffered saline, followed by determination of the free inositol as described in the text. Values shown are means $\pm$ 1 S.D. of quadruplicate determinations.

[Inositol] (M)	Inositol uptake (pmol/10^6 cells)
1.2×10^{-6}	3.9 ± 0.75
3.0×10^{-5}	78.6 ± 8.38
1.0×10^{-4}	157.9 ± 3.75
1.0×10^{-3}	360.9 ± 21.7
1.0×10^{-2}	2233.7 ± 450.2
1.0×10^{-1}	20710.0 ± 6098.0

Table 3. Effect of vasopressin and Li^+ on the accumulation of labelled diacylglycerol

Cells were plated and allowed to grow for 48 h in the presence of 5 μCi of [^{3}H]glycerol/ml. The medium was then changed to serum-free Minimal Essential Medium, and the cells were incubated for a further 90 min with the additions listed (abbreviations as in Table 1). Labelled diacylglycerol was quantified as described in the text. Results shown are means $\pm$ 1 S.D. of triplicate determinations.

Addition	[^{3}H]Diacylglycerol (c.p.m./well)
Control	353 ± 39
VP	394 ± 68
LiCl	288 ± 97
VP + LiCl	831 ± 255

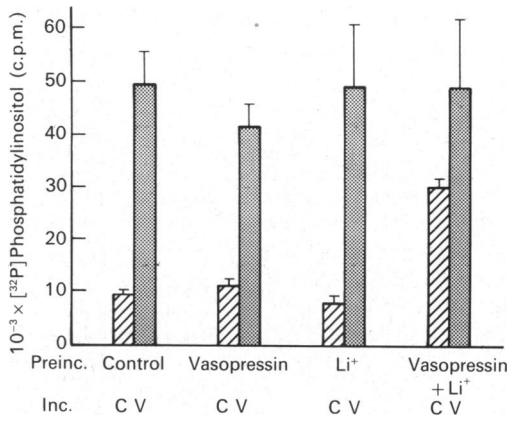

Fig. 2. Effect of preincubation with vasopressin and LiCl on incorporation of [^{32}P]P_i into phosphatidylinositol

Cells were preincubated (Preinc.) for 90 min under the conditions described in the text. This was followed by washing the cells and a further 30 min incubation (Inc.) with either no addition (C) or vasopressin (V). Concentrations were: LiCl (25 mM) and vasopressin (0.2 μM). Values represent means $\pm$ 1 S.D. of triplicate determinations.

Table 4. Effect of exogenous inositol on the ability of pretreatment with vasopressin and Li^+ to stimulate subsequent [^{32}P]P_i incorporation

Cells were pretreated in Minimal Essential Medium containing the compounds listed [LiCl (10 mM); VP = vasopressin (0.1 μM); inositol (0.1 M)]. Values represent means $\pm$ 1 S.D. of triplicate determinations.

Preincubation addition	[^{32}P]Phosphatidylinositol (c.p.m./well)
LiCl	167 ± 41
LiCl + VP	1598 ± 338
LiCl + inositol	221 ± 55
LiCl + VP + inositol	365 ± 88

phosphatidic acid and CDP-diacylglycerol [9,10] as well as the amount of radioactive glycerol which accumulates in diacylglycerol [8]. In WRK-1 cells we were able to see similar results with respect to phosphatidic acid (results not shown) and diacylglycerol (Table 3); however, we failed to see any effects on CDP-diacylglycerol levels. Even in cells labelled with 20 μCi of [^{3}H]cytidine/ml, we were unable to observe consistently incorporation into CDP-diacylglycerol, with or without agonist and Li^+ (results not shown). Furthermore, the results seen with respect to [^{32}P]P_i labelling of phosphatidic acid were also apparent for phosphatidylcholine and phosphatidylethanolamine (results not shown). We therefore decided to test whether or not pretreatment with vasopressin and Li^+ could subsequently increase phosphate incorporation in a post-labelling experiment, i.e. under conditions in which accumulation of phosphatidic acid and/or CDP-diacylglycerol would not contribute to any increase observed in subsequent labelling of phosphatidylinositol in cells. Thus cells were preincubated with nothing, vasopressin, Li^+ or a combination of the two, followed by a labelling period with radioactive phosphate in the presence or absence of vasopressin. Fig. 2 illustrates the results. Only those cells preincubated with vasopressin and Li^+ showed increased [^{32}P]P_i incorporation when the medium was exchanged for control medium. Although this increase was only 50% of that seen when vasopressin was added back to the incubation, one thing is clear: hormone is not required for resynthesis of phosphatidylinositol to occur. The hormone-independent resynthesis observed in this experiment could result from an increase in diacylglycerol accumulation, an increase in inositol phosphate accumulation, a decrease in the concentration of phosphatidylinositol, some combination thereof, or an as-yet unidentified mechanism. Table 4 illustrates that increasing the inositol phosphate concentration alone is not enough to stimulate synthesis, since in cells incubated with vasopressin and Li^+ in the presence of 100 mM exogenous inositol the secondary increase in phosphate incorporation is virtually abolished. Under these conditions, there is still an increased accumulation of inositol phosphates.

Effect of vasopressin on reincorporation of labelled inositol

Although the previous experiment demonstrated that stimulated resynthesis could take place in the absence of hormone, it was still not clear whether the presence of hormone increased this rate even further. To determine whether or not vasopressin had any direct effect on the rate of resynthesis, experiments were carried out in which [^{3}H]inositol-prelabelled cells were preincubated for 90 min with vasopressin and LiCl to induce maximal accumulation of inositol phosphates and a maximal decrease in phosphatidylinositol, and subsequently reincubated in the absence of Li^+ with or without vasopressin, and the rate of reincorporation of radioactive inositol back into phosphoinositides was determined. Fig. 3 details the results of such an experiment. In control cells containing both vasopressin

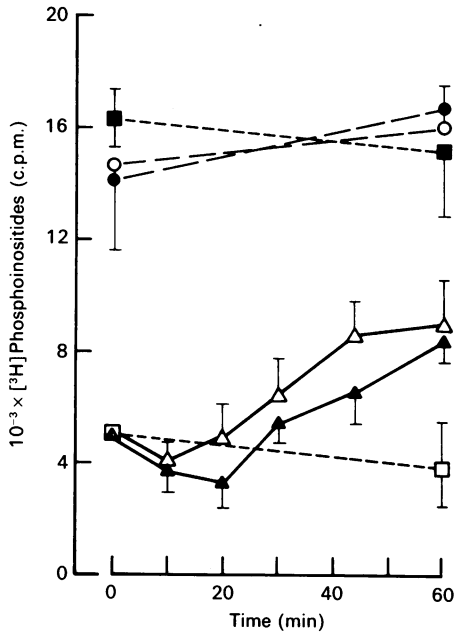

Fig. 3. Effect of vasopressin on reincorporation of labelled inositol into phosphoinositides

Cells were prelabelled with radioactive inositol (5 μCi/ml) for 48 h. They were then incubated for 90 min in serum-free Minimal Essential Medium containing the following: ○, no addition; ■, LiCl (25 mM); ●, vasopressin (0.2 μM); □, LiCl + vasopressin. Subsequently, the cells were incubated for a further 60 min under identical conditions, or cells that had been incubated with LiCl and vasopressin were washed and the incubation was continued without LiCl but with (▲) or without (△) vasopressin. The labelled phosphoinositides were quantified as described in the text. Values represent means ± 1 S.D. of triplicate determinations. Similar results were obtained in two separate experiments.

and Li^+, there was no reincorporation of radioactivity. Removal of the Li^+, however, resulted in reincorporation of approx. 40% of the radioactivity. The presence of vasopressin had no effect on the rate of reincorporation, except perhaps to inhibit it at earlier times. This may reflect the time required for Li^+ to leave the cell. However, once reincorporation begins, the slope of the two lines is similar (141 c.p.m./min for controls and 122 c.p.m./min for vasopressin-treated). In a separate experiment, the rate at which labelled phosphoinositides reappeared was compared with the rate at which free inositol reappeared and inositol phosphates disappeared (Table 5). Reincorporation of radioactivity into phosphoinositides begins by 15 min, causing a further decrease in inositol levels; however, replenishment of the free inositol pool, which begins at 30 min and reaches control levels by 45 min, does not influence the rate of reincorporation. In this experiment, the rate of reincorporation in the presence of vasopressin is again slightly slower than in control cells, although the rate at which inositol is regenerated is slightly faster. If one examines the rate at which the radioactivity in the inositol phosphate fraction disappears, then one sees that vasopressin retards this process, although it is impossible to determine if this is due to a decrease in the rate of hydrolysis of the inositol phosphates, or due to continued generation in the presence of vasopressin. In conclusion, it is possible that inositol regeneration is rate-limiting in terms of resynthesis under these conditions; however, at no level of free inositol is vasopressin able to increase the rate of reincorporation, and the apparent decrease in the rate of reincorporation could be the result of simultaneous phospholipase C-stimulated hydrolysis.

Table 5. Regeneration of free inositol after treatment of cells with vasopressin and Li^+

Cells were grown for 24 h in medium containing 5 μCi of [^{3}H]inositol/ml and subsequently preincubated for 90 min with or without vasopressin (VP, 0.1 μM) in the presence of LiCl (10 mM). After the preincubation, some cells were harvested and the labelled compounds quantified (zero time). Cells which had been preincubated with vasopressin were washed with Minimal Essential Medium and incubated further in the absence of LiCl with or without vasopressin for the times indicated. Values represent means ± 1 S.D. of quadruplicate determinations. Similar results were observed in two other experiments.

		Radioactivity (^{3}H c.p.m. per well)		
Time (min)	VP	Phospho-inositides	Inositol	Inositol phosphates
0	−	23940 ± 3324	10916 ± 1632	4144 ± 612
0	+	8860 ± 720	7384 ± 992	30924 ± 2148
15	−	10924 ± 1710	3652 ± 696	24672 ± 5312
15	+	11250 ± 804	4224 ± 460	31252 ± 1600
30	−	13704 ± 1814	5116 ± 476	14304 ± 1680
30	+	13720 ± 1382	5356 ± 704	18840 ± 3232
45	−	18592 ± 2378	10812 ± 1204	7068 ± 1564
45	+	17628 ± 892	12668 ± 2276	11688 ± 1872
60	−	20610 ± 2136	8424 ± 388	1098 ± 84
60	+	18646 ± 940	11068 ± 744	3404 ± 256

DISCUSSION

The data presented here suggest a coupling between the hydrolytic and resynthetic phases of the phosphoinositide cycle. If regeneration of free inositol from the inositol phosphates is inhibited by Li^+, resynthesis of phosphatidylinositol is slowed down. The LiCl-induced inhibition of hormone-induced [^{32}P]P_i incorporation into phosphatidylinositol can be overcome by the addition of high concentrations of exogenous inositol. Thus the inositol generated as a result of lipid hydrolysis is required for full functioning of the resynthetic phase of the cycle. Similar observations have been made by other investigations [8–11]. The fact that [^{32}P]P_i incorporation rates do not fall below basal levels in the presence of hormone and Li^+ suggests that the inositol depletion may be localized such that only resynthesis and not synthesis is affected. This is further supported by the data previously reported, which demonstrate that incorporation of radiolabelled inositol into phosphoinositides is not inhibited in the presence of Li^+ and vasopressin [3]. There is, rather, a shift in radioactivity from phosphoinositides to inositol phosphates. To appear in the inositol phosphates, inositol must first have been incorporated into phosphoinositide. However, one cannot rule out the possibility that incorporation of inositol is by exchange or reversal of the synthetase activity, rather than by synthesis *de novo*, as has been suggested by others [13,14], and that this exchange requires lower inositol concentrations than does synthesis *de novo*.

That regeneration of inositol is required for resynthesis of phosphatidylinositol does not mean that the presence of hormone is not also required. For example, the enzymes responsible for resynthesis of phosphatidylinositol may be activated by hormonal stimulation. The results of additional experiments described here, however, indicate that the resynthetic phase of the cycle does not require the presence of hormone. [^{32}P]P_i incorporation is stimulated in the absence of hormone if cells are first stimulated in the presence of Li^+. Furthermore, the rate of reincorporation of endogenous labelled inositol into phosphoinositides after removal of Li^+ is not increased by the presence of hormone;

however, since regeneration of free inositol from inositol phosphates is occurring at the same time, it is difficult to determine whether this regeneration step has become rate-limiting, such that any effect of vasopressin would be obfuscated. Nevertheless, it is clear that resynthesis can take place quite well in the absence of hormone. In the experiment shown (Fig. 2), the level of $[^{32}P]P_i$ incorporation was greater in the continued presence of hormone. Likewise, in Fig. 3, resynthesis of phosphoinositides from endogenous labelled inositol reaches only 40% of the starting level. Taken together, these data suggest that, in the presence of a 90 min treatment with hormone and Li^+, approximately half of the resynthetic potential is lost. For example, perhaps some of an accumulated substrate is shunted into another pathway.

To understand what is triggering the hormone-independent resynthesis, it is necessary to examine the differences observed when Li^+ is added. The three obvious possibilities are (1) a decrease in the concentration of phosphatidylinositol, (2) an increase in the level of diacylglycerol, and (3) an increase in the accumulation of inositol phosphates. As mentioned above, the experiment shown in Fig. 2 by-passes any increase in the phosphorylated precursors, phosphatidic acid and CDP-diacylglycerol. With respect to accumulation of inositol phosphates, the data shown in Table 4 indicate that this alone is not sufficient to activate synthesis, although it may be required. Likewise, increasing the endogenous inositol concentration in the absence of phosphoinositide depletion does not activate synthesis (Fig. 1). It is difficult to analyse the contribution of increased diacylglycerol levels (Table 3), since this phenomenon is not specific for phosphoinositide cycling, and may reflect phosphatidylcholine turnover as well. Imai & Gershengorn have presented data that suggest that it is the decrease in phosphatidylinositol levels which triggers resynthesis [5]. Our own data regarding ionophore-stimulated phospholipase C activity would tend to confirm this. Concomitant with increased hydrolysis of phosphoinositides, A23187 also stimulates $[^{32}P]P_i$ incorporation into phosphatidylinositol [15]. This is interesting in light of the fact that Ca^{2+} inhibits phosphatidylinositol synthetase *in vitro*.

Thus we conclude that (1) resynthesis requires regeneration of free inositol, (2) increasing the inositol concentration in the absence of a decrease in phosphatidylinositol will not stimulate synthesis, and (3) resynthesis does not require the presence of hormone and, under the conditions of the experiments described here, is not stimulated by hormone. Whether the triggering event for resynthesis is the generation of substrate or the decrease in product, or some as yet unidentified event, cannot be determined from these experiments.

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