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CHANGES IN POLYPHOSPHOINOSITIDE METABOLISM DURING MEDIATOR RELEASE FROM STIMULATED RAT MAST CELLS

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Abstract—The metabolism of the polyphosphoinositides, diphosphoinositide (DPI) and triphosphoinositide (TPI), was studied during mediator release from rat mast cells. Serosal mast cells were purified by density gradient centrifugation and prelabeled with $^{32}PO_4$. Incorporation of $^{32}PO_4$ into DPI and TPI was determined by thin-layer chromatography on oxalic acid impregnated silica gel plates. $^{32}PO_4$ incorporation into DPI and TPI was increased by Concanavalin A (ConA) or compound 48/80. The concentration of ConA causing a half-maximal increase in DPI labeling was less than that required for a comparable change in histamine release. The increases in DPI labeling and histamine release in response to ConA were enhanced by phosphatidylserine. The addition of α-methylmannoside to mast cells after challenge with ConA rapidly halted DPI and TPI labeling. The results of these studies indicate that changes in the metabolism of polyphosphoinositides may be an intrinsic part of the biochemical mechanisms that control mediator release from mast cells.

In previous studies from this laboratory in rat mast cells [1], various stimulators of histamine release were shown to produce rapid increases in $^{32}PO_4$ incorporation into phosphatidic acid (PA†), phosphatidylinositol (PI) and phosphatidylcholine (PC). Cockcroft and Gomperts [2] have also reported rapid changes in PI turnover in activated rat mast cells. While these observations implicate the PI pathway in stimulus secretion coupling in this system, rapid changes in phospholipid methylation have also been reported [3]. The polyphosphoinositides (PPIs), phosphatidylinositol-4-monophosphate (DPI) and phosphatidylinositol-4,5-diphosphate (TPI), have apparently not been studied. Rapid increases in PPI levels or PPI labeling with $^{32}PO_4$ have been reported recently in several cell systems in association with activation [4–7]. Our interest in the possible role of PPIs in mast cell activation has been stimulated by recent studies in which we demonstrated a very active enzyme system for producing DPI in mast cell granules [8]. This enzyme system was discovered fortuitously during studies of protein phosphorylation in isolated rat mast cell granules. Granules incubated with [γ-^{32}P]ATP formed a phosphorylated lipid which was identified chromatographically as DPI. On further study, the granules were shown to contain a PI kinase which rapidly converted endogenous PI to DPI in the presence of ATP and Mg^{2+} or Mn^{2+}. The kinase is present on the exterior of the granule membrane where it is accessible to ATP and other possible regulatory molecules in the cytosol.

In the present report, intact rat mast cells were preincubated with $^{32}PO_4$, stimulated with various secretogogues, and studied for changes in PPI metabolism. Evidence for rapid increases in PPI labeling during stimulation will be presented.

MATERIALS AND METHODS

Materials. Reagents and their sources were as follows: all lipids, compound 48/80, Concanavalin A (ConA), calcium ionophore (A23187), α-methylmannoside, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES), bovine serum albumin (BSA), phosphatidylserine (PS) and Percoll (Sigma Chemical Co., St., Louis, MO); sodium chloride, potassium chloride, calcium chloride, glucose, sodium phosphate monobasic, sodium phosphate dibasic, chloroform, methanol, ammonium hydroxide, acetic acid glacial, acetone, oxalic acid, potassium oxalate, iodine and Scintiverse (Fisher Scientific Co., Philadelphia, PA); heparin sodium (O'Neal, Jones & Feldman, St. Louis, MO); silica gel plates (20 × 20 cm, 250 μm layer) (Brinkmann, Westbury, NY); [^{32}P]-orthophosphoric acid (carrier free) in HCl-free water (New England Nuclear, Boston, MA); [γ-^{32}P]ATP (4500 Ci/mmol, 0.0027 μmol/ml; ICN Pharmaceuticals, Irvine, CA).

Rat mast cell preparations. Mast cells were obtained from the pleural and peritoneal cavities

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† Abbreviations: PA, phosphatidic acid; PPIs, polyphosphoinositides; PI, phosphatidylinositol; DPI, phosphatidylinositol - 4 - monophosphate; TPI, phosphatidylinositol - 4,5 - diphosphate; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; SM, sphingomyelin; CL, cardiolipin; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MCM, mast cell medium; BSSA, balanced salt solution with BSA; and BSS, balanced salt solution without BSA.

of male Sprague–Dawley rats (250–300 g; Camm, Wayne, NJ.) by lavage with mast cell medium containing 150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 5 mM sodium phosphate, 5.55 mM glucose, 1 mM PIPES, 1 mg/ml BSA and 1 unit/ml heparin, pH 6.8 (MCM-PO_4). In all subsequent steps, buffer prepared without sodium phosphate (MCM-P) was used. Cells were purified by using a BSA gradient prepared in MCM-P as described [9]. Rat mast cells were 62–76% pure by toluidine blue staining and other dye staining reactions and at least 98% viable by trypan blue dye exclusion. As a rule, twenty to twenty-five rats were used in an experiment.

Phosphate depletion and $^{32}PO_4$ incorporation in mast cells. After purification on BSA gradients, cells were washed twice with MCM-P and incubated for an additional 60 min at 37° to deplete intracellular PO_4 before labeling. The cells were then sedimented, resuspended, and used immediately for $^{32}PO_4$ labeling and histamine release experiments. Phosphate-depleted mast cells (2.5 to 8.8 × 10^5) were added to conical polypropylene tubes containing 40 μCi $^{32}PO_4$, incubated for 5 min at 37° as described previously [1], and stimulated by various agents in a final volume of 0.25 ml. Unless otherwise indicated, cells were exposed to stimulatory agents for 15 min at 37°. The most frequently used stimulatory agent was ConA which is ordinarily used at 10 μg/ml in the presence of 30 μg/ml PS. PS has been shown previously to be needed for ConA-stimulated mediator release from these cells [10]. Reactions were halted by adding 1 ml of ice-cold MCM-PO_4. The cells were rapidly centrifuged at 4°, the supernatant fractions were removed by aspiration, and the cell pellet was extracted immediately.

Isolation of mast cell PPIs. A modified Bligh and Dyer [11] lipid extraction of the cell pellet was performed: 0.6 ml of methanol, 0.3 ml of chloroform and 0.24 ml of 0.1 N HCl were added in sequence. Tubes were capped, shaken vigorously, and left for 30 min at room temperature. Insoluble material was removed by centrifugation at 1000 *g* for 10 min, and the supernatant fraction was decanted into tubes containing 0.3 ml of chloroform, 0.3 ml of 0.1 N HCl and 10 μg of unlabeled marker PPIs. Tubes were capped, shaken, and centrifuged (1000 *g* for 10 min), and the upper aqueous phase was removed carefully by aspiration. Aliquots of the organic phase were dried under N_2 at room temperature. Dried samples were dissolved in 60 μl chloroform and stored at −80° overnight.

PPIs in the above lipid extracts were separated by one-dimensional thin-layer chromatography on silica gel plates developed with chloroform/methanol/4 N NH_4OH (9:7:2, by vol.). Before use, the plates were impregnated with 1% (w/v) oxalic acid and activated as described [8]. Areas containing lipids were visualized with iodine vapor, and ^{32}P-labeled spots were detected by autoradiography on Kodak X-Omat film. Areas containing PPIs were scraped into vials and measured for radioactivity in a liquid scintillation counter after the addition of 10 ml of Scintiverse.

In three experiments, mast cell supernatant fractions as well as pellets were extracted for labeled lipid. Phosphate-depleted mast cells (3.5 × 10^6) were added to tubes containing 40 μCi $^{32}PO_4$, incubated for 5 min at 37° and stimulated with 10 μg/ml Con A and 30 μg/ml PS or PS alone for 30 min at 37°. Reactions were halted by adding 1 ml of ice-cold MCM-PO_4. The cells were rapidly sedimented at 4°, the supernatant fractions and pellets were separated, and their lipids were immediately isolated. The cell pellets were extracted as described above. The supernatant fractions were extracted by the sequential addition of 4 ml of methanol, 2 ml of chloroform and 0.35 ml of 0.457 N HCl, centrifugation, and the subsequent addition of 2 ml of chloroform and 2 ml of 0.1 N HCl.

As a control for possible artifactual formation of DPI in supernatant fractions (due to the extrusion of granule membranes and labeled ATP), in two experiments supernatant fractions from labeled stimulated or unstimulated cells were incubated with intact or broken membrane granules from unlabeled cells. Prelabeled mast cells (3.3 × 10^6 cells) were incubated under standard conditions with 10 μg/ml ConA and 30 μg/ml PS or PS alone in buffer for 30 min at 37°. After halting the reaction by the addition of 1 ml of ice-cold MCM-PO_4, the supernatant fractions were obtained by the rapid sedimentation of the cells. Unlabeled intact or broken membrane granules (see below) obtained from 2.4 × 10^6 mast cells were incubated with the supernatant fractions for 15 min at 37° and sedimented at 4°. The granule pellet was immediately extracted under standard conditions.

Isolation of granules and granule PPIs from labeled mast cells. After the usual phosphate depletion, mast cells (2 × 10^6) were incubated with 40 μCi $^{32}PO_4$ for 60 min at 37°. Rections were halted by adding 1 ml of ice-cold MCM-PO_4. The cells were rapidly sedimented at 4°, and the supernatant fractions were removed by aspiration. Half of the cell pellets were extracted immediately as described above to determine their total PPI content. The rest of the cells were disrupted by sonication, and the granules were isolated by centrifugation in a Percoll gradient as described [12]. Granules with intact perigranular membranes were obtained from the lower region of the Percoll gradient and granules with broken membranes from the upper region of the gradient. Granules were washed with balanced salt solution (BSS) (4.0 mM Na_2HPO_4, 2.7 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, pH 7.2) with 0.046% (w/v) BSA (BSSA), sedimented, and immediately extracted as described above.

As a control for possible losses of labeled PPI from granules during the granule purification (due to DPI breakdown or loss of granules), granules were purified from unlabeled cells and labeled with [γ-^{32}P]ATP in the presence of Mg^{2+}. They were then washed and, after removal of an aliquot for determination of the amount of label present initially in DPI, the remainder were added to sonicates of unlabeled cells (1.7 × 10^6) and the labeled granules were reisolated together with the unlabeled granules on Percoll gradients. Alternatively, the labeled granules were added to intact unlabeled cells (1.7 × 10^6), sonicated together with them, and then reisolated as just described. The recovery of radioactivity in DPI in granules during the second granule isolation was then determined by extraction, chromatography, and

liquid scintillation counting. To label the granules, intact membrane granules (from 1.7×10^6 cells) in BSSA were incubated with 5×10^6 cpm [γ-^{32}P]ATP for 15 min in the presence of 20 mM $MgCl_2$ and 20 mM Tris/HCl, pH 7.4, in a total volume of 100 μl. Reactions were halted by adding 1 ml of ice-cold BSSA, rapidly sedimenting the granules at 4°, and removing the supernatant fractions by aspiration.

As a control for possible artifactual formation of DPI from labeled ATP inside cells during cell disruption, in three experiments, 5×10^6 cpm [γ-^{32}P]ATP was added to unlabeled purified mast cell suspensions (2.7×10^6 cells) just before sonication, and mast cell granules were isolated in the usual way.

Histamine release. Because of limitations in cell number, histamine release measurements were performed under similar but not identical reaction conditions to those used for polyphosphoinositide labeling [at the same cell, ConA and PS concentrations but in a smaller volume (1.0 to 3.2×10^5 cells in a final volume of 100 μl)]; the reaction was stopped by the addition of 0.5 ml of ice-cold MCM-PO_4. The supernatant fractions were obtained by the rapid sedimentation of cells and assayed for histamine by a radioenzymatic method [13]. Total histamine was assayed in samples after two cycles of freezing and thawing. The percentage of stimulated histamine release was determined by:

$$\frac{\text{observed release—spontaneous release}}{\text{total histamine—spontaneous release}}$$

RESULTS

Thin-layer chromatography. Figure 1 shows the iodine staining pattern of control lipids separated on an oxalic acid impregnated silica gel plate. Good separation of PPIs from the other lipids was obtained.

Stimulated $^{32}PO_4$ incorporation into PPI. PPIs were examined for basal and stimulated $^{32}PO_4$ labeling after exposure to agents known to cause mediator release from mast cells. ConA and PS in combination or compound 48/80 each produced increases in labeling both in DPI and TPI (stimulation ratios of approximately 1.5), whereas A23187 was ineffective under the same experimental conditions. Stimulation ratios are shown in Table 1. Since the combination of ConA and PS stimulated PPI synthesis in this system has the advantage in that stimulation can be rapidly blocked by α-methylmannoside [14], it was used in subsequent experiments.

Comparisons with other phospholipids. In some experiments, the effect of 10 μg/ml ConA and 30 μg/ml PS on $^{32}PO_4$ incorporation into PA, PI, PC and

Table 1. Stimulation ratios for mast cell DPI and TPI labeling by agents that cause mediator release

Agent	Stimulation ratios for lipid labeling	
	DPI	TPI
ConA + PS	1.51 ± 0.10	1.55 ± 0.05
A23187	1.10 ± 0.03	1.08 ± 0.02
48/80	1.34 ± 0.04	1.44 ± 0.07

Phosphate-depleted mast cells (6.3×10^5) were prelabeled at 37° with $^{32}PO_4$ (40 μCi/tube) for 5 min before stimulation with the secretory agonist. Fifteen minutes later, incorporation was halted by the addition of ice-cold MCM-PO_4, the cell pellet was obtained by centrifugation, and the phospholipids were extracted and chromatographed, as described in Materials and Methods. The data represent results from three experiments, each one in duplicate, and are given as the mean ± SEM. The concentrations of the agents used were: ConA, 10 μg/ml; PS, 30 μg/ml; A23187, 0.3 μg/ml; and compound 48/80, 1.0 μg/ml. The stimulation ratio = ^{32}P cpm (stimulated)/^{32}P cpm (unstimulated). Histamine release was determined in one of the experiments by stimulation of phosphate-depleted cells under identical conditions but with 2.5×10^5 mast cells, as described in Materials and Methods. The mean net histamine release was 5.7% for ConA + PS, 7.7% for A23187, and 15.7% for 48/80.

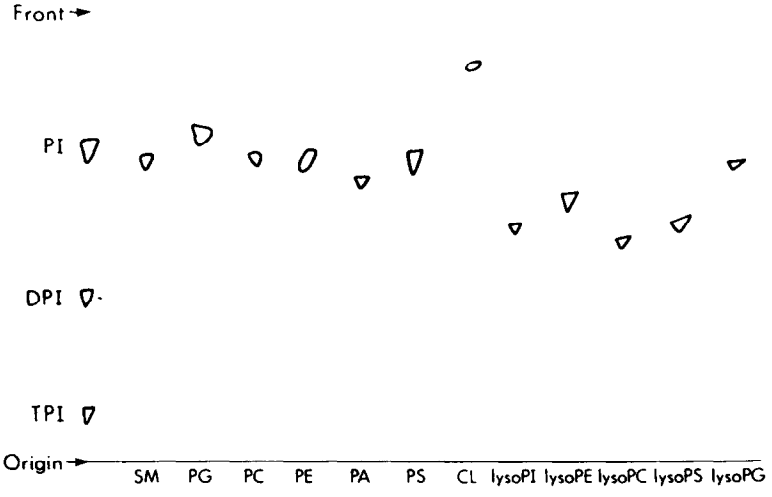

Fig. 1. Position of various lipids as determined by staining with iodine vapor after chromatography on an oxalic acid impregnated silica gel plate.

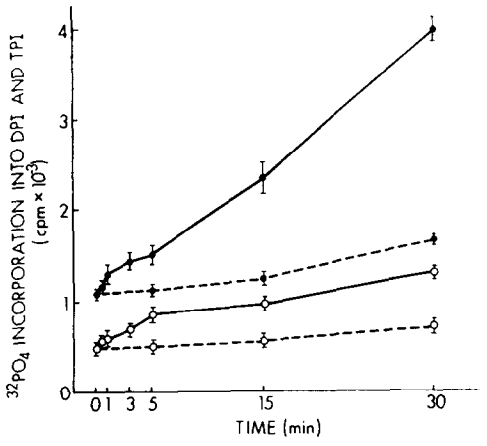

Fig. 2. Kinetic analysis of ConA-mediated changes in PPI metabolism. Mast cells (4×10^5) were prelabeled for 5 min with $^{32}PO_4$ and stimulated with 10 μg/ml ConA and 10 μg/ml PS (——) or PS alone (---) at 37°. After the time indicated, the incorporation of label was stopped by the addition of ice-cold MCM-PO_4. The cell pellets were extracted, and the lipids were separated by thin-layer chromatography and counted as described in Materials and Methods. The data are the mean ± SEM of triplicate values. Key: (●) DPI, (○) TPI.

PS was also examined using potassium oxalate impregnated silica gel plates for separation of the lipids [8]. As reported previously [1], ConA increased $^{32}PO_4$ incorporated into PA, PI and PC (stimulation ratios of 2.00, 3.42 and 2.22 respectively), but not into PS (stimulation ratio, 1.09).

Kinetic analysis of alterations in PPI metabolism. The rate at which ConA stimulates changes in PPI labeling by $^{32}PO_4$ was investigated. After a brief lag period, DPI and TPI labeling increased at a linear rate in ConA-stimulated cells (Fig. 2). A slight increase of $^{32}PO_4$ incorporation into DPI and TPI was observed in unstimulated cells at 30 min.

Effect of various concentrations of ConA on histamine release and PPI metabolism. The dose–response curve for histamine release roughly paralleled that of $^{32}PO_4$ incorporation into DPI and TPI (Fig. 3). However, somewhat higher concentrations of ConA were required for half-maximal histamine release than for half-maximal DPI labeling.

Phosphatidylserine effect on PPI metabolism. Since PS has marked potentiating effects on mediator release in the ConA system [13], its effect on PPI metabolism was studied (Fig. 4). Although some stimulation of PPI metabolism was observed with ConA alone, the addition of PS (3–30 μg/ml) clearly enhanced DPI labeling. The dose–response curve for histamine, release paralleled that of $^{32}PO_4$ incorporation into DPI.

Blocking of the ConA response. In previous studies, the removal of ConA from mast cells by the addition of α-methylmannoside resulted in a rapid cessation of continued mediator release [14]. To evaluate whether the continued presence of cell-bound ConA is necessary for late increases in PPI labeling, 50 mM α-methylmannoside was used to elute bound ConA from mast cells. A reduced ConA concentration (1 μg/ml) was used in these experiments in order to facilitate the displacement of ConA from cells. Figure 5 shows that α-methylmannoside largely inhibited the further labeling of polyphosphoinositides.

$^{32}PO_4$ incorporation into PPI in cell pellets and the supernatant fractions. If some of the increase in PPI labeling in stimulated cells is occurring in granules, at least some labeled DPI and TPI might be expected to be present in the supernatant fractions, in associ-

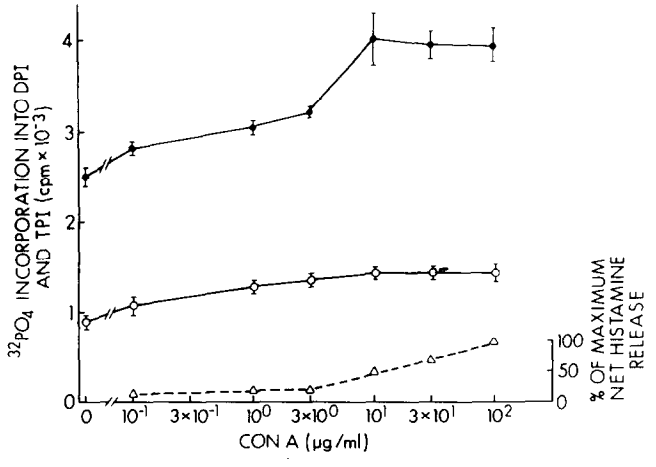

Fig. 3. Effect of various ConA concentrations on histamine release and labeling of PPI. After a 5-min prelabeling period with $^{32}PO_4$, 8.8×10^5 mast cells were challenged with various concentrations of ConA in the presence of 10 μg/ml PS. After 15 min, the incorporation was halted by addition of ice-cold MCM-PO_4, and the phospholipids were isolated from the cell pellet by extraction and thin-layer chromatography as described in Materials and Methods. Histamine release was determined by stimulation of phosphate-depleted cells with ConA except that 3.2×10^5 mast cells were used. The data on PPI labeling are the mean ± SEM of triplicate values and for histamine release the mean of triplicate values. Key: (●) DPI, (○) TPI, and (△) histamine.

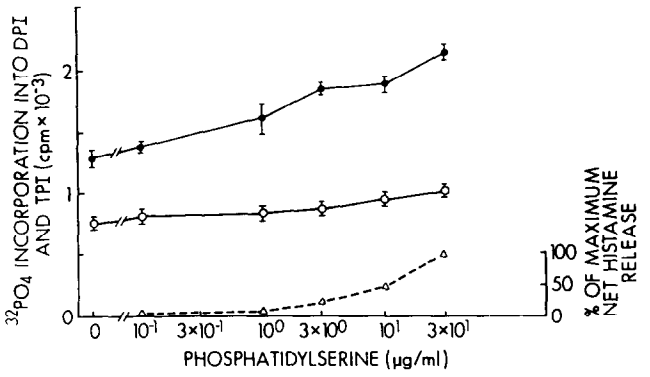

Fig. 4. Effect of exogenous PS on the metabolism of PPI. Mast cells (2.5×10^5) were preincubated for 5 min with $^{32}PO_4$ before stimulation with 10 μg/ml ConA in the presence of various concentrations of PS (0–30 μg/ml). The PS had been dissolved in chloroform, dried, and sonicated in MCM-P to obtain a uniform suspension. Histamine release was determined by stimulation of phosphate-depleted cells (see Materials and Methods). The data on PPI labeling are the mean ± SEM of triplicate values and that for histamine release the mean of triplicate values. Key: (●) DPI, (○) TPI, and (△) histamine.

ation with discharged granules. Therefore, the supernatant fractions were examined for labeled PPIs after exposure to ConA. Obvious DPI labeling was demonstrated in the supernatant fractions of mast cells exposed to ConA (Table 2), although the absolute level of labeling was low. Since the extent of granule release is not known precisely (some of the histamine in the medium may be coming from granules that remain in the cell but communicate with the medium), the quantitative interpretation of this experiment is uncertain.

$^{32}PO_4$ incorporation into PPI in granules. The question of how much of the labeled DPI and TPI in mast cells is present in the granule fraction was considered. Unfortunately, attempts to isolate granules from stimulated cells were unsuccessful, presumably because of changes in the granules associated with the release response. However, we were able to determine the amount of labeled polyphosphoinositide in granules in unstimulated cells. Table 3 shows that substantial amounts of labeled DPI and TPI are present in both broken and intact granules, particularly the former. Control experiments indicated that some loss of label from the granules was occurring during granule isolation. When labeled intact granules were added to unlabeled mast cells, sonicated, and reisolated in the Percoll gradient, the recovery of labeled DPI was 22.4% in intact and 18.7% in the broken membrane granule fraction. When labeled granules were added to already sonicated unlabeled mast cells and reisolated in the gradient, 37.4% of the label was recovered in intact, and 24.1% in broken, granules. Less than 30% of the labeled TPI was lost during

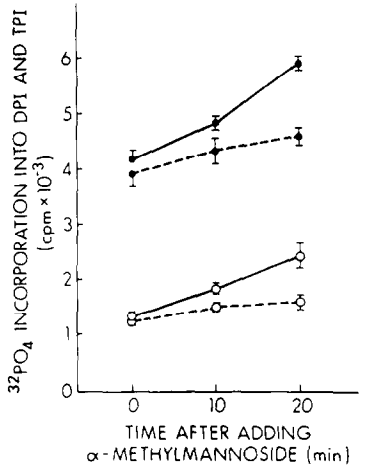

Fig. 5. Effect of α-methylmannoside on ConA-induced changes in the metabolism of PPI. After incubation of 6.8×10^5 mast cells with $^{32}PO_4$ for 5 min, the cells were stimulated by suboptimal levels of ConA (1 μg/ml) and PS (10 μg/ml). Ten minutes after stimulation, α-methylmannoside (50 mM) was added and the cells were allowed to incubate for the indicated times before cessation of the reaction. The data are the mean ± SEM of triplicate values. Key: (●) DPI, and (○) TPI; (——) without α-methylmannoside and (---) with α-methylmannoside.

Table 2. $^{32}PO_4$ incorporation into DPI and TPI in mast cell pellets and supernatant fractions with and without ConA stimulation

	$^{32}PO_4$ incorporation (cpm)	
	DPI	TPI
Mast cell pellets		
ConA + PS	11,100 ± 459	3,760 ± 169
PS	8,110 ± 200	2,040 ± 197
Supernatant fractions		
ConA ± PS	203 ± 0.08	50 ± 0.07
PS	48 ± 0.07	22 ± 0.02

Phosphate-depleted mast cells (3.5×10^6) at 37° were prelabeled with $^{32}PO_4$ (40 μCi/tube) for 5 min and then incubated with either 10 μg/ml ConA and 30 μG/ml PS or PS alone. Thirty minutes later, incorporation was halted by the addition of ice-cold MCM-PO_4, and PPI labeling was determined as described in Materials and Methods and the legend to Table 1. The data represent means ± SEM from three experiments, each done in duplicate.

Table 3. $^{32}PO_4$ incorporation into DPI and TPI in mast cell pellets and granules

	$^{32}PO_4$ incorporation (cpm)	
	DPI	TPI
Cell pellets	6,173.3 ± 411.4	1,005.0 ± 38.5
Intact membrane granules	522.7 ± 90.2	103.3 ± 16.8
Broken membrane granules	1,608.3 ± 192.6	712.0 ± 97.6

Phosphate-depleted mast cells (2×10^6) were incubated with $^{32}PO_4$ (40 μCi/tube) for 60 min at 37°. Further labeling was inhibited by adding 1 ml of ice-cold MCM-PO_4. After sedimenting the cells, half of the cell pellets was extracted immediately under standard conditions The remainder of the cells was resuspended in 1 ml BSSA, and their granules were isolated as described in Materials and Methods. The lipid was extracted from the isolated granules under standard conditions. The data are the mean ± SEM of triplicate values.

granule isolation in each condition. These observations indicated that as much as 50% of the labeled DPI may be lost from intact granules during granule purification. These losses are surprisingly high and raise the possibility that phosphorylated granules after isolation may be more difficult to isolate.

The possibility was considered that the incorporation of radioactivity into DPI and TPI in isolated granules was artifactual due to rapid uptake of radioactivity from [γ-^{32}P]ATP or $^{32}PO_4$ during granule purification. As a control, in three experiments unlabeled mast cells were sonicated in the presence of [γ-^{32}P]ATP before the separation of granules. No obvious ^{32}P incorporation into polyphosphoinositides was observed in either intact or broken membrane granules. As a further control, in two experiments intact and broken membrane granules were incubated with the supernatant fractions of either ConA-stimulated or unstimulated mast cells prelabeled with $^{32}PO_4$. Again, no obvious $^{32}PO_4$ incorporation into polyphosphoinositides was seen.

DISCUSSION

Alterations in phospholipid metabolism have been reported in association with secretion by platelets, pancreas, thyroid and parotid glands [15–18]. In these systems, the kinetic or dose–response relationships of stimulated cell functions have not always corresponded well with the changes in phospholipid metabolism, and it has sometimes been difficult to determine the precise role of enhanced phospholipid metabolism in the systems. Previous studies in mast cells [1] have indicated a close relationship between increased incorporation of $^{32}PO_4$ into PI, PC and PA and secretion. The present report extends these observations to PPI. Mast cells preincubated with $^{32}PO_4$ stimulated with ConA and PS in combination or the multifunctional amine, compound 48/80, showed a rapid increase in PPI labeling which continued for at least 30 min. While a precise relationship between PPI labeling and histamine release was not observed, a number of observations suggest that changes in PPI metabolism are part of the cellular response to secretogogues: (1) the increase in labeling was rapid being demonstrable within 30 sec which is about the time histamine release is first readily detected [19], (2) exogenous PS enhanced the PPI response to ConA and also potentiated histamine release; and (3) α-methylmannoside, which removes bound ConA from the surface of mast cells, rapidly stops histamine release [14] and inhibited further PPI labeling, indicated that the continued presence of ConA at the cell surface is required for both responses. Thus, the conditions that enhance or inhibit the changes in PPI metabolism are associated with parallel changes in mediator release. The only important exception to the overall correlation between mediator release and PPI labeling is the response to A23187, which stimulated histamine release but not PPI labeling. While a number of explanations might be considered, one possibility is that A23187 acts at a relatively late stage of the secretory process, bypassing some of the metabolic changes associated with other secretagogues.

The localization of the PPI response in stimulated mast cells will require further study. Studies in unstimulated cells labeled with $^{32}PO_4$, including controls for artifactual incorporation of label during granule isolation, suggest that at least 50% of the total cellular radiolabel in PPI is in the granules. Since other studies in our laboratory indicate that mast cell granules contain a very active PI kinase [8], a response at this level would not be surprising.

Unfortunately, because of the difficulty in isolating granules from stimulated mast cells, $^{32}PO_4$ incorporation into PPI could not be investigated directly in the granules that remained associated with cells. The ability to identify labeled PPI in cell supernatant fractions after stimulation of labeled cells with ConA indicates that at least some labeled PPI is being released at the same time as the granules, presumably within the ganule membranes, but the amounts are small and not easily interpretative in quantitative terms.

It has been suggested that PPIs may help to control calcium concentrations through direct interactions with calcium and effects on membrane permeability [20, 21]. Calcium is essential for chemical mediator release from immunologically activated rat mast cells. Moreover, DPI and TPI are subject to degradation by phosphodiesterases and could be significant sources of diacylglycerol in activated mast cells. By acting as a fusogen, as a source of monoacylglycerol, free fatty acid and PA and through effects on calcium-dependent protein kinases [22], diacyl-

glycerol may contribute to intracellular activation in at least several different ways.

A more complex issue concerns the kinetics of the polyphosphoinositide response, particularly that involving DPI. As is apparent in Fig. 2, the labeling of DPI continued to increase between 15 and 30 min with a similar trend for TPI. The continuing increase in DPI radioactivity raises questions about the relationship between DPI formation and histamine release which is largely or entirely complete within 5 min. There are several possible interpretations: (1) It should be kept in mind that only a 5-min prelabeling time with $^{32}PO_4$ was used. The specific activity of ATP, which is the precursor of the phosphate introduced on the inositol moiety of PI, may well be changing quite significantly over the time course of these experiments, resulting in an overestimate of the magnitude of the late response. Moreover, DPI is metabolized by several different pathways including conversion to TPI, cleavage by phospholipase C to create diacylglycerol, or conversion back to PI. A late increase in activity of the phosphatase converting DPI to PI might create an increase in specific ctivity of DPI while its concentration was remaining constant or even falling. (2) It seems likely that there is DPI present at the level of the plasma membrane as well as on the granule surface so it is possible that the DPI labeling response is occurring with different kinetics in different DPI pools within the cell. (3) The heterogeneity of DPI metabolism may extend to the granules themselves which may include granules open to the extracellular fluid but still physically within mast cells as well as deeper granules which remain largely or entirely intact and still maintain their original content of histamine. Possibly granules which have already released their mediators continue to participate in the changes in DPI metabolism. In other words, if the granules in which increased DPI formation is occurring remained in the cell despite having already responded maximally in terms of mediator release, increased formation of DPI might continue even though secretory response was essentially over. (4) The DPI and TPI responses may represent a repair mechanism which continues after mediator release is completed and ultimately enables the cells to reconstitute their granules and regain their original responsiveness to acute secretory stimuli. Unfortunately because of the present inability to isolate pure granules from cells during their response to a secretory stimulus, these questions cannot be addressed directly at this time. What is clear is that the rapid initial increase in labeling of DPI and TPI in stimulated cells does correspond to the time during which mediator release is occurring.

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