Activation of Protein Kinase C in Rat Basophilic Leukemia Cells Stimulates Increased Production of Phosphatidylinositol 4-Phosphate and Phosphatidylinositol 4,5-Bisphosphate: Correlation with Actin Polymerization

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Cross-linking of the immunoglobulin E receptor on rat basophilic leukemia (RBL)¹ cells by multivalent antigen activates phosphatidylinositol (PI) kinase and phosphatidylinositol 4-phosphate (PIP) kinase leading to the increased production of PIP and phosphatidylinositol 4,5-bisphosphate (PIP₂). Activators of protein kinase C (PKC), such as phorbol myristate acetate (PMA) and the synthetic diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (diC8), were found to have the same effect even though PMA and diC8 do not cause the activation of phospholipase C. Although the kinetics are different depending on the stimulant, activation of PKC using multivalent antigen, PMA or diC8 also causes the polymerization of actin and an increase in the F-actin content of the cells. In all cases, a good correlation was observed between F-actin levels, activation of PI and PIP kinases, and the increased production of PIP and PIP₂. However, in the case of antigen, there is no correlation between actin polymerization and the total amount of PIP and PIP₂. Staurosporine, an inhibitor of protein kinases, blocks the F-actin response and the increased synthesis of PIP and PIP₂ with similar dose dependencies. Furthermore, depletion of PKC activity through long-term exposure to PMA, inhibited both the F-actin response and the increased synthesis of PIP and PIP₂ induced by either DNP-BSA or diC8. These results suggest that activation of PKC precedes the activation of PI and PIP kinases and that under certain circumstances activation of the kinases and the increased synthesis of PIP and PIP₂ may be involved in the polymerization of actin in RBL cells, possibly through the interaction of the polyphosphoinositides with actin-binding proteins such as gelsolin and profilin.

INTRODUCTION

RBL cells are one of the major models for studying IgE mediated allergic reactions (Beaven and Metzger, 1993; Metzger, 1992; Metzger *et al.*, 1986; Oliver *et al.*,

1988). These cells contain a high affinity receptor which binds IgE in a 1:1 ratio. The critical event in triggering these cells to degranulate and release histamine is cross-linking of the IgE receptors by the addition of multivalent antigen. This leads to the activation of multiple tyrosine kinases (Benhamou and Siraganian, 1992) as well as phospholipases C, A₂, and D. Phospholipase C hydrolyzes the phosphoinositides to produce the inositol phosphates and diacylglycerol (Beaven and Cunha-Melo, 1988). The inositol phosphates are responsible for the increase in intracellular Ca²⁺ while diacylglycerol activates PKC. Activation of phospholipase D leads to the hydrolysis of phosphati-

¹Abbreviations used: BSA, bovine serum albumin; diC8, 1,2-dioctanoyl-*sn*-glycerol; DNP, dinitrophenyl; fMLP, formyl-Met-Leu-Phe; HEPES, *N*-2-hydroxy-piperazine-*N'*-2-ethane; IgE, immunoglobulin E; NBD-, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; RBL, rat basophilic leukemia.

dylcholine and the subsequent generation of phosphatidic acid which is metabolized into diacylglycerol. The phospholipase D mediated pathway is believed to be the major source of diacylglycerol produced during RBL cell activation (Lin *et al.*, 1991).

Triggering of RBL cells through the IgE receptor also induces polymerization of actin and recruitment of microfilaments to the plasma membrane (Pfeiffer *et al.*, 1985). Microfilaments, which are composed of polymerized actin, are believed to be involved in a wide array of functions such as changes in cell shape, motility, adhesion, endocytosis, exocytosis, and the movement of receptors on the cell surface (Cohen and Smith, 1985; Geiger, 1983; Ishikawa, 1988; Jacobson, 1983; Oliver and Berlin, 1982; Weatherbee, 1981). In many cell types, approximately 50% of the total cellular actin is found in a monomeric G-actin pool and it is maintained in that state by several actin-binding proteins. The cell then uses this pool of G-actin to form microfilaments when they are required for some particular function. Activation of cells often causes polymerization of actin and this is reflected in an increase in the F-actin content of the cell and a corresponding decrease in the G-actin content. However, despite the importance of actin, relatively little is known about the signalling mechanisms responsible for its polymerization in the cell.

Hydrolysis of the phosphoinositides is an important signaling mechanism in many cells. However, recent studies have suggested that the phosphoinositides may also play a key role in the polymerization of actin (Forscher, 1989; Isenberg, 1991; Stossel, 1989; Yin, 1987). The state of actin in the cell is regulated by a large number of actin-binding proteins and it has been shown that micelles containing PIP and PIP₂ are able to interact with many of these proteins. For example, PIP and PIP₂ are both able to dissociate gelsolin-actin and profilin-actin complexes. Based on these kinds of results, it has been hypothesized that when cells are activated, phospholipase C hydrolyzes the phosphoinositides which are then re-synthesized. Newly synthesized PIP and PIP₂ may then help regulate the polymerization of actin by interacting with actin-binding proteins such as gelsolin and profilin.

It has been demonstrated that IgE-dependent polymerization of actin in RBL cells is dependent on the activation of PKC (Apgar, 1991). However, actin is not normally a phosphoprotein thus suggesting that another step is necessary. The purpose of this study was to determine whether triggering RBL cells through the IgE receptor activates PI and PIP kinases and whether the synthesis of the polyphosphoinositides could play a role in the F-actin response. It was found that multivalent antigen as well as the PKC activators, PMA and diC8, activated PI and PIP kinase leading to an increase in newly synthesized PIP and PIP₂ which correlated with an increase in F-actin. Furthermore,

PKC inhibitors were able to block both the F-actin response and the increase in PIP and PIP₂. These results indicate that activation of PKC precedes activation of PI and PIP kinase and that the synthesis of PIP and PIP₂ may be part of the signalling mechanism leading to actin polymerization.

MATERIALS AND METHODS

Cells

RBL-2H3 cells were grown as a monolayer in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 15% fetal calf serum, 5% newborn calf serum, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and penicillin-streptomycin. Cells were harvested by incubating them with 5 mM EDTA in PBS for 10 min at 37°C. The cells were sensitized by resuspending them in complete RPMI 1640 at a concentration of 3×10^6 cells/ml, adding IgE to a concentration of 1 µg/ml, and rotating the cells at 37°C for 2 h.

Reagents

The mouse monoclonal anti-DNP IgE was a generous gift from Dr. Fu-Tong Liu (Research Institute of Scripps Clinic, La Jolla, CA) (Liu *et al.*, 1980). DNP-BSA, which contained greater than 40 DNP groups per BSA molecule, was prepared according to published procedures (Mishell and Shiigi, 1980). Sphingosine and quercetin were purchased from Sigma (St. Louis, MO), whereas PMA, diC8, and *N*-(7-nitrabenz-2-oxa-1,3-diazol-4-yl) (NBD)-phalloidin were from Molecular Probes (Junction City, OR). [³²P]Orthophosphate and [³²P]ATP were obtained from DuPont (Wilmington, DE). [³H]Myo-inositol was from Amersham (Arlington Heights, IL). Staurosporine and K252a were purchased from Calbiochem-Behring (San Diego, CA). PMA, diC8, sphingosine, staurosporine, K252a, and quercetin were all solubilized as concentrated stock solutions in dimethyl sulfoxide. In all experiments, the concentration of dimethyl sulfoxide did not exceed 0.05% and control samples were exposed to the same concentrations of solvent.

F-Actin Assay

The F-actin content of RBL cells was measured using a modification (Apgar, 1991) of the method of Howard and Oresajo (1985). IgE sensitized RBL cells were washed 3 times in Hanks' buffered saline solution containing 0.1% BSA, resuspended in the same buffer at a concentration of 5×10^6 cells/ml and 200 µl aliquots (1×10^6 cells total) were added to 12 × 75 mm tubes. 200 µl of buffer or the appropriate stimulant was then added, and the cells were incubated for the indicated lengths of time at 37°C. The stimulants used were DNP-BSA (25 ng/ml final), PMA (5 nM final), or diC8 (10 µM final). Cell stimulation was terminated by the addition of 3 ml of ice-cold solubilizing buffer (50 mM NaCl, 2.5 mM MgCl₂, 200 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10 mM HEPES, pH 7.2) and incubated on ice for 15 min. The Triton shells, which are composed of the detergent-insoluble membrane skeleton and cytoskeleton as well as a nuclear remnant, were then pelleted by centrifugation at 2,000 rpm for 10 min. The supernatant was removed and 100 µl of NBD-phalloidin (3.3×10^{-7} M), which had been dried down and resuspended in PBS, was added. After a 1-h incubation at 4°C, the Triton shells were washed with 3 ml of PBS and centrifuged as before. The supernatant was removed and 2 ml of methanol was added in order to extract the bound NBD-phalloidin. After 1 h at room temperature in the dark, the tubes were centrifuged and the relative fluorescence of the methanol extract was measured in a spectrofluorometer (SLM 8000) using an excitation wavelength of 465 nm and an emission wavelength of 535 nm. Control tubes which went through the entire process but which contained no cells were used to determine background fluorescence.

This value was then subtracted from each reading and the results are expressed as the ratio of the F-actin content in stimulated cells divided by the F-actin content in unstimulated cells.

Assay for Inositol Phosphate Production

The polyphosphoinositides were labeled with ³H by growing RBL cells in Eagle's basal medium containing 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% (v/v) each of Eagle's essential and nonessential amino acids, 20 mM HEPES, penicillin-streptomycin, and 3–5 μ Ci/ 10^6 cells of [³H]myoinositol for 24 h. The cells were washed and sensitized with IgE for 2 h at 37°C. After sensitization, the cells were washed three times to remove unbound IgE and resuspended in Hanks' buffered saline solution containing 0.1% BSA and 10 mM LiCl at a concentration of 5×10^6 cells/ml, and 200- μ l aliquots (1×10^6 cells total) were placed in 12 $\times$ 75-mm glass tubes. The cells were activated with the appropriate stimulant in 200 μ l for 20 min at 37°C. The reaction was stopped by the addition of 2 ml of chloroform/methanol (1:2, v/v) and 500 μ l of 0.044 M HCl. The phases were separated by centrifugation at 1000 rpm for 5 min. The aqueous phase was removed, the organic phase was re-extracted with 1 ml of water, and the phases were separated by centrifugation. The two aqueous phases were combined and the pH returned to neutrality by the addition of NaOH. Dowex 1-X8 in the formate form was used to isolate the total [³H]inositol phosphates using a modification of the method of Berridge *et al.* (1982).

Measurement of Polyphosphoinositide Levels

Total or steady state levels of the polyphosphoinositides were measured by growing RBL cells in the presence of [³H]myoinositol for 24 h as described above. Aliquots of IgE-sensitized RBL cells (1×10^6 cells in 200 μ l) were placed in 12 $\times$ 75-mm glass tubes and were stimulated for various lengths of time by the addition of 200 μ l of either DNP-BSA, PMA, or diC8 at 37°C. The reactions were terminated by the addition of 3 ml of chloroform/methanol (1:1, v/v) and 500 μ l of 2.4 N HCl. The phases were separated by centrifugation and the aqueous phase was removed. The organic phase was washed two times with chloroform/1 N HCl (1:1, v/v) and finally dried under N₂.

Levels of newly synthesized PIP and PIP₂ were assayed by measuring the incorporation of ³²P using a modification of the method of Wilson *et al.* (1985). IgE sensitized RBL cells (1×10^6 cells/tube) were resuspended in phosphate-free medium (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgSO₄, 1 mM CaCl₂, 0.1% BSA, 20 mM HEPES, pH 7.2) and were aliquoted into glass tubes. [³²P]Orthophosphate, which had been previously extracted with chloroform/methanol/H₂O/concentrated HCl (15:10:4:1) to remove lipid-soluble ³²P contaminants, was added 5 min before the addition of a stimulant. After the appropriate length of time, the reactions were terminated and the polyphosphoinositides were extracted as described above.

The dried samples were resuspended in 50 μ l of chloroform/methanol/10 mM HCl (20:10:1) and spotted onto polyester backed silica gel plates which had been treated with 1% potassium oxalate in methanol/water (2:3, v/v). The polyphosphoinositides were then separated by developing the plates in either chloroform/acetone/acetic acid/methanol/water (40:15:15:13:8), v/v or chloroform/methanol/ammonium hydroxide/water (136:106:8:23, v/v). Lanes containing authentic PIP and PIP₂ were run as standards and were visualized by iodine staining. The experimental lanes were cut up into 0.5-cm slices, placed in tubes containing 2 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) and radioactivity determined by liquid scintillation counting.

PI and PIP Kinase Assay

Membrane preparations were produced by resuspending RBL cells in Dulbecco's phosphate-buffered saline containing 0.4 mM phenyl-

methylsulfonyl fluoride at a concentration of 10^7 cells/ml and subjecting the cells to one round of freezing and thawing. The cells were then further disrupted using a Dounce homogenizer. This material was centrifuged at 1,500 rpm for 10 min to pellet nuclei and unlysed cells. The supernatant was then removed and the pellet resuspended in kinase assay buffer (20 mM MgCl₂, 20 mM Tris, pH 7.3). The material was then re-centrifuged at 1,500 rpm for 10 min. The supernatant was removed and the pellet was discarded. The two supernatants were combined and centrifuged at $100,000 \times g$ for 45 min in order to pellet the membranes. The membrane pellets were resuspended in kinase assay buffer at a concentration of 2×10^7 cellular equivalents/ml, and 50- μ l aliquots (1×10^6 cellular equivalents/tube) were placed in 12 $\times$ 75-mm glass tubes. The reaction was initiated by adding 50- μ l aliquots of 0.1 mM ATP (γ -³²P]ATP specific activity, 500 cpm/pmol) in kinase assay buffer for the indicated lengths of time. The reactions were terminated by the addition of 2 ml of ice-cold chloroform/methanol (1:1 v/v) followed by the addition of 500 μ l of 2.4 N HCl. The organic phase was washed twice with 1 ml of methanol/1 N HCl (1:1, v/v), dried down under N₂, and resuspended in 50 μ l of chloroform/methanol/10 mM HCl (20:10:1, v/v). The polyphosphoinositides were separated by thin layer chromatography using oxalate-coated silica gel plates as stated above. The bands were visualized by autoradiography, and the appropriate areas were cut out and counted by liquid scintillation. All assays were run in quadruplicate and the results are expressed as ³²P cpm incorporated $\pm$ SEM.

RESULTS

Multivalent antigen (DNP-BSA), PMA, and diC8 are all capable of stimulating the polymerization of actin in RBL cells (Apgar, 1991). In IgE-sensitized cells, DNP-BSA induced cross-linking of the IgE receptor leads to the activation of phospholipase C which hydrolyzes the polyphosphoinositides to produce the inositol phosphates (Table 1). Diacylglycerol, which is also produced upon hydrolysis of the polyphosphoinositides, then activates PKC which is believed to be a key signal for the F-actin response triggered through the IgE receptor. Neither PMA nor diC8 activates phospholipase C and thus no increase in inositol phosphate production can be detected (Table 1). These two reagents are able to cause polymerization of actin by directly activating PKC thus bypassing PLC mediated hydrolysis of the polyphosphoinositides.

Table 1. Inositol phosphate production by DNP-BSA, PMA, and diC8

Stimulant	cpm
Control	3,708 $\pm$ 241
DNP-BSA (25 ng/ml)	17,558 $\pm$ 422
PMA (2.5 nM)	3,617 $\pm$ 260
diC8 (10 μ M)	3,735 $\pm$ 79

RBL cells were labeled overnight with [³H]myoinositol. The IgE-sensitized cells were incubated with the appropriate concentration of stimulant in the presence of LiCl for 20 min at 37°C. Inositol phosphates were isolated by Dowex ion exchange chromatography as described in MATERIALS AND METHODS.

Polyphosphoinositide metabolism was further investigated by measuring the kinases responsible for their production. In order to directly assay kinase activity, RBL cells were sensitized with IgE and stimulated with DNP-BSA for various lengths of time. The reaction was stopped and membranes were isolated according to methods outlined in MATERIALS AND METHODS. The membranes were resuspended in buffer, [γ - 32 P]ATP was added for 3 min, and incorporation of 32 P into endogenous PIP and PIP₂ was determined by thin layer chromatography. As can be seen in Figure 1A, triggering of the cells through the IgE receptor induces an increase in both PI and PIP kinase activity. The activity peaks in approximately 5 minutes and remains elevated for at least 20 min.

Measurement of antigen-induced production of PIP and PIP₂ was achieved by labeling the cells with either [3 H]myoinositol or 32 P. In the case of [3 H]myoinositol, the cells were cultured overnight with the radiolabel, thus allowing equilibrium labeling of the phosphoinositides. Under these conditions, approximately 85% of the 3 H label was found in PI, 9% in PIP, and 6% in PIP₂. Equilibrium labeling allows shifts in total or steady state levels of the phosphoinositides to be monitored. In the second labeling procedure, cells were incubated with [32 P]orthophosphate for only 5 min before the addition of the stimulant. Thus, 32 P incorporation is seen only in the polyphosphoinositides that are synthesized during the time span of the assay. This assay therefore measures the rate of newly synthesized PIP and PIP₂. Figure 1B shows that antigen causes increased synthesis of PIP and PIP₂ as detected by an increase in 32 P incorporation in both of the polyphosphoinositides. This is due to the activation of the PI and PIP kinases (Figure 1A). The levels of 3 H-PIP and PIP₂ decrease when the cells are activated through the IgE receptor (Figure 1C). This is consistent with the data in Table 1 which shows that antigen causes PLC mediated hydrolysis of the phosphoinositides. These results show that the phosphoinositide cycle is activated when IgE receptors are crosslinked. Activation of RBL cells through the IgE receptor leads to both hydrolysis of the phosphoinositides due to increased activity of PLC as well as increased synthesis of PIP and PIP₂ due to stimulation of PI and PIP kinases. Thus, both hydrolysis and re-synthesis of the polyphosphoinositides is occurring.

PMA, a potent tumor promoter and PKC activator, does not activate PLC as is shown in Table 1. However, it does lead to the very rapid activation of both PI and PIP kinases (Figure 2A). This leads to the production of newly synthesized PIP and PIP₂ (Figure 2B) as measured by 32 P incorporation. There is also a rapid increase in the steady state levels of PIP and PIP₂ as measured by [3 H]inositol labelling of the polyphosphoinositides (Figure 2C). The steady state levels peak in approximately 1 min and then plateau.

Figure 1. Kinetic analysis of activation of PI and PIP kinases, steady state levels of the phosphoinositides, and synthesis of PIP and PIP₂ induced by DNP-BSA. IgE sensitized RBL cells were activated with 25 ng/ml DNP-BSA for various lengths of time at 37°C. (A) PI and PIP kinase activity was measured by isolating plasma membranes from IgE sensitized cells which had been stimulated with DNP-BSA (25 ng/ml) for various lengths of time. [γ - 32 P]ATP was added to the membranes for 3 min at 24°C and the activity of PI and PIP kinases was assayed by measuring the incorporation of 32 P into endogenous phosphoinositides. No difference in the kinetics of the reactions were seen if exogenous PI and PIP were added separately. The error in all cases was less than 10%. (B) RBL cells were preincubated with [32 P]orthophosphate for 5 min before the addition of either buffer or DNP-BSA. The phosphoinositides were separated by thin layer chromatography and incorporation of 32 P into PIP (□) and PIP₂ (■) measured. The results are presented as (cpm in stimulated cells) - (cpm in unstimulated cells) or net incorporation of 32 P $\pm$ SEM. Incorporation of 32 P into unstimulated cells is linear over the time course of the experiment. (C) Changes in steady state levels of PIP (□) and PIP₂ (■) were measured in RBL cells labeled overnight with [3 H]myoinositol. Cells were stimulated with DNP-BSA and the reaction was terminated by the addition of chloroform/methanol (1:1). The phosphoinositides were extracted and then separated by thin layer chromatography as outlined in MATERIALS AND METHODS. The error in all cases was less than 6%.

Figure 2. Kinetic analysis of activation of PI and PIP kinases, steady state levels of the phosphoinositides, and synthesis of PIP and PIP₂ induced by PMA. RBL cells were activated with 2.5 nM PMA for various lengths of time at 37°C. (A) Kinetic analysis of the activation of PI and PIP kinases. (B) Production of newly synthesized PIP (□) and PIP₂ (■) as measured by incorporation of ³²P. (C) Changes in steady state levels of PIP (□) and PIP₂ (■) in RBL cells labeled overnight with [³H]myoinositol. The error in all cases was less than 7%. These experiments were performed in the same manner as those in Figure 1.

diC8 is a synthetic, short chain diacylglycerol that activates PKC presumably by mimicking endogenously produced diacylglycerol. diC8 also did not cause phosphoinositide hydrolysis (Table 1), although it did stimulate the PI and PIP kinases (Figure 3A). Unlike stimulation with antigen or PMA, kinase activity reached a peak in 5 min and then returned to baseline values within 20 min. This led to a transient increase in newly synthesized PIP and PIP₂ (Figure 3B). There was also a transient increase in the overall level of the polyphosphoinositides which peaked and then returned to baseline values over the time course of the experiment (Figure 3C).

Figure 3. Kinetic analysis of activation of PI and PIP kinases, steady state levels of the phosphoinositides, and synthesis of PIP and PIP₂ induced by diC8. RBL cells were activated with 10 μM diC8 for various lengths of time at 37°C. (A) Changes in PI and PIP kinase activity. (B) Production of newly synthesized PIP (□) and PIP₂ (■) as measured by incorporation of ³²P. (C) Changes in steady state levels of PIP (□) and PIP₂ (■) in RBL cells labeled overnight with [³H]myoinositol. In all cases the error was less than 5%. These experiments were performed in the same manner as those in Figure 1.

Multivalent antigen, PMA, and diC8 all cause polymerization of cellular actin which is reflected in an increase in the level of F-actin. Figure 4 shows that activation through the IgE receptor by multivalent antigen causes a rapid increase which plateaus in 3–5 min. The level of F-actin remains high as long as antigen is present. PMA also causes a very rapid increase in F-actin which reaches a plateau level in approximately 1 min. The F-actin level remains high as the cell is unable to metabolize PMA. It should be noted that the kinetics of the F-actin response induced by PMA are different than those reported in an earlier study (Apgar, 1991). The exact reason for this is not

Figure 4. Kinetic analysis of the changes in F-actin content in cells stimulated with DNP-BSA, PMA, or diC8. IgE sensitized RBL cells were activated with either DNP-BSA (25 ng/ml), PMA (2.5 nM), or diC8 (10 μ M) for various lengths of time at 37°C. The reaction was stopped by the addition of ice-cold solubilizing buffer. F-actin content was measured using NBD-phalloidin in stimulated versus unstimulated cells and the results are expressed as the F-actin ratio $\pm$ SEM.

known except that the early studies used a different batch of PMA. All subsequent batches of PMA have given results identical to those shown in Figure 4. Polymerization of actin induced by diC8 peaks in 5 min and then returns to baseline values within 20 min because it is metabolized by diacylglycerol kinase (Apgar, 1991). In all cases, the peak levels of F-actin are comparable in magnitude. Furthermore, the kinetics of the F-actin response by all three stimulants is similar to the kinetics of activation of the PI and PIP kinases.

If PKC is involved in the regulation of PI and PIP kinases, then inhibitors of PKC should block activation of these kinases as well. Figure 5 shows that the increased production of PIP and PIP₂ induced by DNP-BSA and measured by ³²P incorporation are inhibited in a dose dependent manner. However, at these concentrations, staurosporine and K252a will also completely inhibit degranulation and the hydrolysis of the phosphoinositides. This is due to the fact that these reagents also inhibit tyrosine kinases which are important in activating PLC γ 1 thus leading to polyphosphoinositide hydrolysis (Yamada *et al.*, 1992). Therefore, the effect of these inhibitors was tested on diC8 which is also able to induce increased synthesis of PIP and PIP₂ by stimulating PKC and bypassing phosphoinositide hydrolysis. Figure 6 shows that staurosporine and K252a also inhibit the increased production of PIP and PIP₂ induced by diC8. Similar results were obtained when the cells were stimulated with PMA. These results indicate that

Figure 5. Inhibition of DNP-BSA induced increases in F-actin and the phosphoinositides by protein kinase inhibitors. IgE-sensitized RBL cells were preincubated with various concentrations of staurosporine or K252a for 15 min before the addition of 25 ng/ml DNP-BSA. The cells were stimulated with DNP-BSA for 5 minutes in each case before the reaction was terminated. The F-actin response (●) and the production of ³²P labelled PIP (□) and PIP₂ (■) were measured. The results are expressed as percent of control $\pm$ SEM.

diC8, as well as PMA, is causing PIP and PIP₂ production by activating PKC and that this increased synthesis of the polyphosphoinositides can be blocked by inhibiting PKC activation. Previous work has shown that the IgE receptor mediated F-actin response is also dependent on the activation of PKC and that the kinase inhibitors staurosporine and K252a are able to inhibit this response (Apgar, 1991). Figure 5 shows that the F-actin response to DNP-BSA is also inhibited by the protein kinase inhibitors with a dose dependency that is very similar to that for the inhibition of PIP and PIP₂ production. Figure 6 shows the same type of result using diC8 as the stimulant.

An alternate method for blocking PKC activation is by culturing RBL cells overnight in 100 nM PMA. It has been shown that long term culture of RBL cells in PMA leads to the total loss of certain isoforms of PKC while having no effect on antigen-induced increases in intracellular Ca²⁺ levels or PLC activity as measured

Figure 6. Inhibition of diC8 induced increases in F-actin and the phosphoinositides by protein kinase inhibitors. RBL cells were pre-incubated with various concentrations of staurosporine or K252a for 15 min before the addition of 10 μ M diC8. The reactions were terminated 5 min after the addition of diC8. The F-actin response (●) and the production of ³²P labeled PIP (□) and PIP₂ (■) were measured. The results are expressed as percent of control $\pm$ SEM.

by the production of inositol phosphate (Cunha-Melo *et al.*, 1989; Ozawa *et al.*, 1993a, b). Thus PKC can be selectively inhibited without interfering with PLC activity. The results in Table 2 show that depletion of PKC by PMA pretreatment resulted in a total inhibition of the antigen- and diC8-induced rise in ³²P incorporation into PIP₂ and almost total inhibition of PIP production. This result correlates with the inhibition of the F-actin response induced by these two stimulants and supports the kinase inhibitor data.

The results shown above indicated that DNP-BSA, PMA, and diC8 all cause an increase in PIP and PIP₂ synthesis due to increased activity of the PI and PIP kinases. In addition, kinase inhibitors block the increased synthesis of these phosphoinositides in a dose dependent manner. All of these results suggest that activation of PKC is an important step leading to the increased production of PIP and PIP₂. However, one possibility is that these PKC stimulants and inhibitors are acting directly on the PI and PIP kinases. This

Table 2. Effect of long-term PMA exposure on the F-actin response and production of PIP and PIP₂ induced by DNP-BSA and diC8

	DNP-BSA		diC8			
	F-actin	PIP	PIP ₂	F-actin	PIP	PIP ₂
Control	100	100	100	100	100	100
PMA treatment ^a	12	28	0	2	13	0

Results are expressed as percent of control. In all cases, the standard error was less than 7%. Cells were briefly incubated with [³²P]orthophosphate, except when measuring F-actin, and then stimulated with 25 ng/ml DNP-BSA or 10 μ M diC8 for 5 min at 37°C. The F-actin ratio was 1.87 for DNP-BSA and 1.63 for diC8 after 5 min at 37°C. DNP-BSA caused an increase of 884 cpm in the PIP fraction and 688 cpm for PIP₂ as compared to unstimulated control values. There was an increase of 446 cpm for PIP and 326 cpm for PIP₂ when the cells were stimulated with diC8.

^aRBL cells were incubated with 100 nM PMA for 20 h before use.

possibility was tested by incubating membranes isolated from RBL cells with [γ -³²P]ATP for increasing lengths of time in the presence of PMA, diC8, staurosporine, K252a, and sphingosine. As Figure 7 shows, these activators and inhibitors of PKC had no effect on the enzyme kinetics. Although it is possible that PMA and diC8 did not activate PI and PIP kinases because some co-factor may be missing, the fact that the inhibitors had no effect indicates that the modes of action of the inhibitors and activators in whole cells are being mediated by PKC.

DISCUSSION

Stimulation of IgE-sensitized RBL cells with multivalent antigen leads to the activation of several tyrosine kinases and the phosphorylation of PLC γ 1 (Benhamou and Siraganian, 1992). This in turn leads to the hydrolysis of the phosphoinositides to produce the inositol phosphates and diacylglycerol. Diacylglycerol is then important in the activation of PKC. The results reported here show that activation of RBL cells through the IgE receptor also leads to the activation of PI and PIP kinases and the synthesis of PIP and PIP₂ (Figure 1). Thus, stimulation of these cells with antigen activates the phosphoinositide cycle.

It was also found that activation of PKC using either PMA or diC8 also caused activation of the PI and PIP kinases as measured by direct enzyme activity and product formation (Figures 2 and 3). This occurred despite the fact that these reagents do not lead to the hydrolysis of the polyphosphoinositides (Table 1). Thus, an increase in the total levels of PIP and PIP₂ can be seen when [³H]inositol is used to monitor the response, and is almost certainly due to the fact that the ³H label is not lost in the form of [³H]inositol phos-

Figure 7. Effect of PKC activators and inhibitors on PI and PIP kinase activity. Plasma membranes were isolated from unstimulated RBL cells. PI and PIP kinase activity was measured by following the incorporation of ^{32}P into endogenous phosphoinositides by adding $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to the isolated membranes for various lengths of time. PMA (5 nM) was added to the membranes at the same time as the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The isolated membranes were preincubated with staurosporine (500 nM), sphingosine (40 μM), or K252a (500 nM) for 15 min before the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

phate (Figures 2 and 3). Although phosphate exchange cannot be ruled out as a possibility, the fact that increases in steady state levels of the polyphosphoinositides is seen when cells are activated with PMA or diC8 indicates that at least some of the increased incorporation of ^{32}P is due to increased synthesis. Another possible source of increased PIP and PIP_2 that cannot be excluded is the inhibition of a phosphatase. Since PMA and diC8 do not directly activate the PI and PIP kinases (Figure 7), this result indicates that activation of PKC precedes the production of PIP and PIP_2 in the signalling pathway and that PKC may be involved in the activation of PI kinase and PIP kinase. In general, the regulation of these kinases is poorly

understood (Carpenter and Cantley, 1990; Pike, 1992). As in the case of RBL cells, there are several reports in other systems implicating PKC while some studies have shown that cAMP and tyrosine kinases may play a role.

Activation of RBL cells through the IgE receptor also leads to a rapid and sustained increase in the content of F-actin. Because the cells are solubilized in Triton before the addition of NBD-phalloidin, the F-actin content which is measured is probably restricted to membrane-associated F-actin. This pool of actin is probably analogous to the stable, highly crosslinked, Triton-insoluble actin described by other groups (Watts and Howard, 1993; Cassimeris *et al.*, 1990). Previous studies have shown that activation of PKC is crucial to the IgE mediated response in RBL cells (Apgar, 1991). This is based on the fact that 1) protein kinase inhibitors such as staurosporine block the F-actin response, 2) the response can be mimicked by PKC activators such as PMA and diC8, 3) the PMA and diC8 induced responses can be inhibited by staurosporine, and 4) long-term exposure to PMA which leads to the loss of PKC activity in the cell also completely blocks the F-actin response induced by antigen or diC8. These studies have also shown that Ca^{2+} influx and inositol phosphate production are not important to the response (Apgar, 1991). However, since actin is not normally phosphorylated and PKC is not necessary for actin polymerization *in vitro*, another intermediate signal is required to link PKC activation to F-actin formation.

There has been considerable speculation on the possible role of the polyphosphoinositides in the polymerization of actin (Forscher, 1989; Isenberg, 1991; Stossel, 1989; Yin, 1987). This is based on the fact that the interaction of several actin-binding proteins with actin can be altered by PIP and PIP_2 . Exactly what is happening inside the cell is unknown but there are several possible ways in which the polyphosphoinositides could influence actin polymerization. Under the appropriate conditions, gelsolin is known to sever actin filaments and bind to the barbed end thus preventing elongation (Hartwig and Kwiatkowski, 1991; Pollard, 1986; Weeds and Maciver, 1993). Incubation of gelsolin-actin complexes with micelles containing PIP or PIP_2 leads to the dissociation of these complexes which allows polymerization to take place (Janmey and Stossel, 1987; Janmey *et al.*, 1987). Thus one hypothesis is that activation of cells leads to an increase in intracellular Ca^{2+} which causes gelsolin to sever and cap actin filaments. Activation of the phosphoinositide cycle would lead to increased synthesis of PIP and PIP_2 which would dissociate the gelsolin from the actin thus creating a large number of actin nucleation sites which is the rate limiting step in actin polymerization. The binding of a variety of other actin binding proteins such as profilin (Lassing and Lindberg, 1985), Cap100 (Hofmann *et al.*, 1992), destrin (Yonezawa *et al.*,

1990), and cofilin (Yonezawa *et al.*, 1990) are also probably regulated by the polyphosphoinositides. One of the most important and complex of these proteins is profilin which binds to monomeric actin (Goldschmidt-Clermont and Janmey, 1991; Theriot and Mitchison, 1993). Although originally believed to be an actin sequestration protein, it is now thought to be actively involved in the polymerization of actin. Profilin is probably normally bound to the polyphosphoinositides while actin monomers are sequestered by thymosin β 4 (Nachmias, 1993; Safer, 1992). When cells are stimulated, PLC hydrolyzes the polyphosphoinositides thus dissociating profilin (Goldschmidt-Clermont *et al.*, 1990, 1991) and allowing it to promote filament formation. Two different mechanisms have been proposed in which profilin either enhances nucleotide exchange thus increasing F-actin formation (Goldschmidt-Clermont *et al.*, 1992) or reduces the critical concentration necessary for actin polymerization (Pantaloni and Carlier, 1993). Although the interaction of profilin with actin is influenced by PIP and PIP₂, the role of the polyphosphoinositides in profilin regulated actin polymerization is not known.

Evidence concerning the role of the polyphosphoinositides in actin polymerization in various cell types is contradictory. One study involving human neutrophils (Howard *et al.*, 1990) and another using platelets (Lind *et al.*, 1987) has shown a temporal correlation between F-actin content and the levels of gelsolin-actin complexes. Furthermore, receptor mediated increases in actin nucleating activity have been shown to correlate with increases in F-actin (Carson *et al.*, 1986; Howard *et al.*, 1990). Pike and colleagues (1991) have shown that activation of neutrophils through the formyl peptide (fMLP) receptor leads to increased PIP kinase activity and actin polymerization. Based on kinetic studies, they believe that the kinase is probably involved in the maintenance of the F-actin response rather than its initiation. However, their results are in apparent conflict with other studies in neutrophils which found a better correlation between phosphatidylinositol triphosphate levels and F-actin content when the cells were stimulated by fMLP (Eberle *et al.*, 1990) or mastoparan (Norgauer *et al.*, 1992). Furthermore, in A431 cells it was found that bradykinin stimulates an increase in F-actin while having no effect on polyphosphoinositide levels or actin-gelsolin complexes (Dadabay *et al.*, 1991). When these same cells were activated with epidermal growth factor, there was an increase in PIP and PIP₂ levels, a decrease in actin-gelsolin complexes, and an increase in membrane ruffling and F-actin content. However, if the cells were stimulated with epidermal growth factor in the presence of cholera toxin, then no change was observed in PIP levels but actin-gelsolin complexes still decreased. They concluded that changes in PIP and PIP₂ levels were probably not involved in actin polymerization in A431 cells.

The data shown here present a potentially complex picture of actin polymerization in RBL cells. Changes in F-actin, activation of PI and PIP kinases, and the production of PIP and PIP₂ parallel each other when the cells are stimulated with either DNP-BSA, PMA, or diC8. These results are consistent with the hypothesis that PIP and PIP₂ may be regulating actin polymerization but are only correlative and certainly do not prove that they are involved. DNP-BSA causes the activation of PLC and thus profilin could be released to help regulate actin assembly. Although DNP-BSA causes an increase in kinase activity and an increase in newly synthesized PIP and PIP₂, there is an overall decrease in the levels of the phosphoinositides due to hydrolysis by PLC. One possible explanation for this lack of correlation with F-actin content is that local rather than global concentrations of the polyphosphoinositides may be very crucial to the regulation of the actin-binding proteins. However, once again, there is no evidence that this is true. An alternate explanation is that release of profilin is driving actin polymerization under these conditions and that PIP and PIP₂ are not involved except to replenish the polyphosphoinositides that are being hydrolyzed. However, activation of PLC and hydrolysis of the phosphoinositides is not sufficient since experiments have shown that PKC activity is required. In the case of PMA, there is a rapid increase in the activity of both PI and PIP kinases which peaks and then remains elevated for at least 20 min. diC8 also induces increased activity of PI and PIP kinase which peaks in 5 min but then declines back to baseline values. In both cases, these changes are very similar to the time course for the changes in F-actin content. Both PMA and diC8 cause increases in the steady state levels of PIP and PIP₂ which also mirror the changes in F-actin content. However, the fact that they do not activate PLC suggests that either profilin is not involved in PMA and diC8 induced actin polymerization or that hydrolysis of the polyphosphoinositides is not the releasing mechanism for profilin. This finding is supported by earlier work with RBL cells which showed that in the absence of Ca²⁺ there is no inhibition of actin polymerization but complete inhibition of PLC mediated inositol phosphate production (Apgar, 1991).

It has also been found that inhibitors of PKC such as staurosporine and K252a block the F-actin response and the production of PIP and PIP₂ in a similar dose dependent manner when cells are stimulated with either DNP-BSA (Figure 5) or diC8 (Figure 6). Furthermore, it has been shown that staurosporine and the PKC activators do not have any direct effect on the PI and PIP kinases (Figure 7). A more selective inhibition of PKC can be achieved by incubating the cells overnight in the presence of PMA. PKC depletion also inhibits the F-actin response and the production of PIP and PIP₂ when the cells are stimulated with either

DNP-BSA or diC8 (Table 2) thus adding support to the hypothesis that PKC activation occurs prior to PI and PIP kinase activation.

Recently, it has been shown that an F-actin response can be induced in RBL cells through the adenosine receptor (Apgar, 1994). This response is independent of PKC but dependent on a Pertussis toxin sensitive G-protein. Activation through this receptor also leads to increased synthesis of PIP and PIP₂ which correlates with the F-actin response. Furthermore, Pertussis toxin inhibits the F-actin response as well as the increased production of PIP and PIP₂. However, this toxin has no effect on either response when the cells are triggered through the IgE receptor. Conversely, PKC inhibitors block both responses triggered through the IgE receptor but have no effect on the polymerization of actin or the production of the polyphosphoinositides if the cells are activated through the adenosine receptor. Thus in both cases, production of the polyphosphoinositides and the F-actin response are coupled, at least in terms of their sensitivity to various inhibitors. These studies offer additional correlative evidence for the involvement of PIP and PIP₂ in the polymerization of actin in RBL cells.

The data presented here, coupled with many studies showing that the polyphosphoinositides can affect the behavior of several actin-binding proteins, provides circumstantial evidence that the increased production of PIP and PIP₂ may play a role in actin polymerization in RBL cells under certain conditions. However, it is also possible that in these cells, PKC regulates both actin polymerization and increased synthesis of PIP and PIP₂, but that these are independent events. Furthermore, it is not known why the polyphosphoinositides appear to play a role in one system but clearly do not in others. However, it is certainly a fact that different cells are capable of using different signalling pathways leading to the polymerization of actin. For example, PMA at very low concentrations causes a rapid and sustained polymerization of actin in RBL cells while even relatively high concentrations cause very little effect in neutrophils (Downey *et al.*, 1992; Howard and Wang, 1987). The F-actin content of IIC9 fibroblasts is unaffected by PMA and diacylglycerol but can be stimulated by phosphatidic acid (Ha and Exton, 1993). Of potential interest is the report that phosphatidic acid is a specific activator of PIP kinase (Moritz *et al.*, 1992) and therefore the polyphosphoinositides could be involved. Another possible problem is that all of these assays are measuring the changes of signals in whole populations of cells while it is probably local concentrations within the cell that are important. Ultimately, the state of actin inside the cell is probably a delicate balance between many factors and perturbing any one factor may alter filament formation without necessarily affecting the other fac-

tors. Thus, which mechanism is used may depend not only on the cell type but also the stimulus which is used.

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