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Polymerization of Actin in RBL-2H3 Cells Can Be Triggered Through Either the IgE Receptor or the Adenosine Receptor but Different Signaling Pathways Are Used

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Crosslinking of the IgE receptor on rat basophilic leukemia (RBL) cells using the multivalent antigen DNP-BSA leads to a rapid and sustained increase in the filamentous actin content of the cells. Stimulation of RBL cells through the adenosine receptor also induces a very rapid polymerization of actin, which peaks in 45–60 s and is equivalent in magnitude to the F-actin response elicited through stimulation of the IgE receptor. However, in contrast to the IgE mediated response, which remains elevated for over 30 min, the F-actin increase induced by the adenosine analogue 5′-(N-ethylcarboxamido)-adenosine (NECA) is relatively transient and returns to baseline values within 5–10 min. While previous work has shown that the polymerization of actin in RBL cells stimulated through the IgE receptor is mediated by protein kinase C (PKC), protein kinase inhibitors have no effect on the F-actin response activated through the adenosine receptor. In contrast, pretreatment of the cells with pertussis toxin completely inhibits the F-actin response to NECA but has relatively little effect on the response induced through the IgE receptor. Stimulation of RBL cells through either receptor causes increased production of phosphatidylinositol mono-phosphate (PIP) and phosphatidylinositol bis-phosphate (PIP_2), which correlates with the F-actin response. Production of PIP and PIP_2 may be important downstream signals since these polyphosphoinositides are able to regulate the interaction of gelsolin and profilin with actin. Thus the polymerization of actin can be triggered through either the adenosine receptor or the IgE receptor, but different upstream signaling pathways are being used. The IgE mediated response requires the activation of PKC while stimulation through the adenosine receptor is PKC independent but involves a G protein.

INTRODUCTION

Microfilaments are believed to be involved in a wide variety of cellular activities such as changes in cell shape, motility, endocytosis, exocytosis, intracellular transport, and directed movement of cell surface proteins (Weatherbee, 1981; Oliver and Berlin, 1982; Geiger, 1983; Jacobson, 1983; Cohen and Smith, 1985; Ishikawa, 1988). Many of these activities are specifically triggered when cells are activated through receptors on their surface. One consequence of activation is a reorganization of the cellular actin that exists in a dynamic equilibrium between monomeric (G) actin and filamentous (F) actin. This equilibrium is believed to be controlled by a series of actin binding proteins, which help to regulate the state of actin in the cell (Pollard, 1986; Hartwig and Kwiatkowski, 1991). Generally, activation of the cell leads to polymerization of actin, which is seen as an increase in F-actin and a corresponding decrease in G-actin, and recruitment of microfilaments to the cell surface. Although many different cell types undergo an increase in F-actin content when they are activated, and despite the importance of this response, the signaling mechanisms responsible have remained elusive.

Rat basophilic leukemia (RBL) cells, which are of mucosal mast cell origin, contain high affinity Fc receptors that are specific for IgE (Metzger *et al.*, 1986; Oliver *et al.*, 1988; Metzger, 1992; Beaven and Metzger, 1993). IgE binds to the receptors in a 1:1 ratio, and in this state the receptors are inactive. Crosslinking of the receptors,

generally through the addition of multivalent antigen that binds to the IgE, triggers the receptors and leads to the activation of several tyrosine kinases as well as phospholipases C (PLC), A2, and D. This leads to an increase in intracellular Ca^{2+}, activation of Protein Kinase C (PKC), changes in cellular pH, and membrane depolarization. Many of these signals appear to be important for cellular degranulation of inflammatory mediators such as histamine and serotonin. Morphologically, the cells change from a highly microvillous to a lammellar appearance when activated through the IgE receptor (Pfeiffer *et al.*, 1985). There is also increased cell spreading, increased fluid pinocytosis, clustering of the crosslinked receptors on the cells surface, and internalization of these receptors. All of these activities are correlated with an increase in F-actin, which previous studies have shown is dependent on the activation of PKC (Apgar, 1991).

RBL cells have also been shown to contain a P1-purinergic receptor of the A_3 subtype that can be activated by the addition of adenosine and its analogues such as 5'-(N-ethylcarboxamide)-adenosine (NECA) (Ramkumar *et al.*, 1993). The addition of NECA leads to a slight activation of PLC which in turn results in a small, transient increase in inositol phosphates and intracellular Ca^{2+} (Ali *et al.*, 1990; Gilfillan *et al.*, 1990). Phospholipase C is therefore activated by both receptors. However, activation through the IgE receptor is via a tyrosine kinase (Benhamou and Siraganian, 1992) while activation through the adenosine receptor utilizes a Pertussis toxin sensitive G protein (Ali *et al.*, 1990). Although activation of RBL cells through the adenosine receptor does lead to a small increase in intracellular Ca^{2+}, this level of signaling is not sufficient to lead to any appreciable degranulation or production of peptidoleukotrienes. However, it will boost the degranulation response induced by suboptimal antigen. In contrast to the degranulation response, stimulation of RBL cells through the adenosine receptor does lead to a transient but very rapid and substantial increase in F-actin. Therefore, the purpose of this study was to determine whether a common signal transduction pathway leading to the formation of F-actin is used in RBL cells when the response is triggered using the IgE receptor versus the adenosine receptor.

MATERIALS AND METHODS

Cells

RBL-2H3 cells were grown and maintained in flasks in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 5% fetal calf serum (FCS), 5% newborn calf serum, 2 mM glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and penicillin-streptomycin in a humidified, 5% CO_2 incubator at 37°C. The cells were removed from the flasks using PBS containing 5 mM EDTA and were passaged every 3–4 days.

Reagents

The mouse monoclonal IgE, which is specific for dinitrophenyl (DNP), (Liu *et al.*, 1980) was a generous gift from Dr. Fu-Tong Liu (Scripps Research Institute, La Jolla, CA). DNP-bovine serum albumin (BSA) was prepared according to published methods (Mishell and Shiigi, 1980) and contained ~50 mol of DNP per mol of BSA. Phorbol 12-myristate 13-acetate (PMA) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin (NBD-phallacidin) were purchased from Molecular Probes (Eugene, OR). Staurosporine and K252a were obtained from Calbiochem (San Diego, CA). All other reagents were from Sigma (St. Louis, MO).

Concentrated stock solutions of PMA, staurosporine, K252a, NECA, adenosine, R-phenylisopropyladenosine, and 2-chloroadenosine were prepared in DMSO. Sphingosine, theophylline, 8-phenyltheophylline, and 3-isobutyl-1-methyl xanthine were dissolved in ethanol. Control samples were exposed to the same concentrations of solvent which in no case exceeded 0.05%.

F-Actin Assay

Filamentous actin associated with the Triton insoluble shells from RBL cells was measured with a modification (Apgar, 1991) of the method of Howard and Oresajo (Howard and Oresajo, 1985). RBL cells were removed from tissue culture flasks by incubating them for 10 min in PBS containing 5 mM EDTA. The cells were washed twice with Hank's balanced salt solution (HBSS) and resuspended in complete RPMI at a concentration of 3×10^6 cells/ml. RBL cells were incubated in the presence of 1 μg/ml of IgE on a rotator for 2 h at 37°C. In the experiments involving pertussis toxin, the cells were incubated in the presence of the toxin (0.1 μg/ml) and IgE for 4 h at 37°C. After this incubation the cells were washed three times in HBSS, resuspended in HBSS containing 0.1% BSA at a concentration of 5×10^6 cells/ml, and 200 μl aliquots (1×10^6 cells/tube) were placed in 12 × 75 mm plastic tubes in a 37°C water bath. The cells were stimulated for various lengths of time and the reaction was stopped by the addition of 3 ml of ice cold solubilizing buffer (50 mM NaCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, and 10 mM HEPES, pH 7.2). After a 15 min incubation at 4°C, the Triton shells were pelleted by centrifugation for 10 min at 750 × *g*. The supernatant was removed and 100 μl of NBD-phallacidin (3.3×10^{-7} M) was added to the pellet. The Triton shells were incubated with the NBD-phallacidin for 1 h at 4°C with occasional shaking. The shells were then washed with 3 ml of PBS, spun at 750 × *g* for 10 min, and the supernatant discarded. The bound NBD-phallacidin was extracted with 2 mls of methanol for 1 h at room temperature in the dark. Following centrifugation at 750 × *g* for 10 min, the relative fluorescence of the methanol extracts was measured using an SLM spectrofluorometer with an excitation wavelength of 465 nm and an emission wavelength of 535 nm. Control samples, which went through the entire process but contained no cells, were used to determine background fluorescence. This background fluorescence is due to methanol and residual NBD-phallacidin. The background value was then subtracted from each reading and the results are expressed as the ratio of the F-actin content in stimulated cells to unstimulated cells. Generally, unstimulated controls were sensitized cells that were not exposed to antigen. These controls yielded the same F-actin values as unsensitized cells that were exposed to DNP-BSA. In most cases, experiments were performed at least three times with representative results from one experiment being shown.

Measurement of Newly Synthesized ^{32}P-PIP and ^{32}P-PIP$_2$

A modification of the method of Wilson *et al.* (1985) was used. ^{32}P-orthophosphate was extracted with chloroform/methanol/H_2O/concentrated HCl (15:10:4:1), the aqueous layer was removed, dried down under a stream of N_2, and the material was resuspended in phosphate free media (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA, 20 mM HEPES, pH 7.2). Aliquots of IgE sensitized RBL cells (1×10^6 cells/tube) were placed in borosilicate glass tubes in phosphate free media. Cells were preincubated with extracted ^{32}P-orthophosphate for 15 min before the addition of stimulant. Inhibitors were added at the same time as the

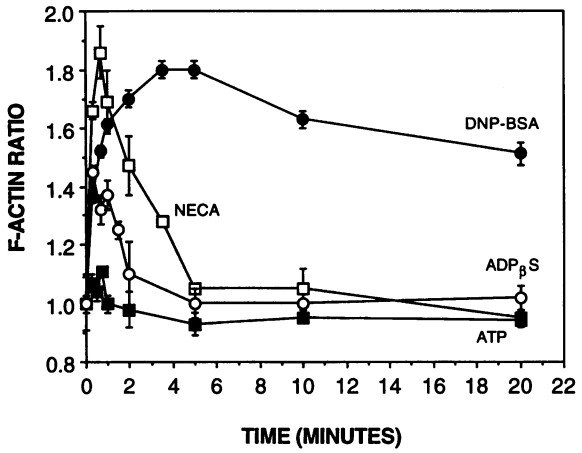

Figure 1. Kinetic analysis of the F-actin response. RBL cells were sensitized with IgE and triggered with either 25 ng/ml DNP-BSA (●), 10 μM NECA (□), 10 μM ATP (■), or 50 μM ADPβS (○) for various lengths of time at 37°C. The reactions were stopped by the addition of ice-cold solubilizing buffer containing 0.5% TX-100. The error bars represent the SE.

^{32}P-orthophosphate. The reaction was terminated by the addition of 3 ml of chloroform/methanol (1:1) and 500 μl of 0.1 N HCl. The phases were separated by centrifugation, the aqueous layer removed, and the organic layer was washed with methanol/1 N HCl (1:1). The organic phase was dried under N_2 and resuspended in 100 μl chloroform/methanol/10 mM HCl (20:10:1). The phosphoinositides were separated by thin layer chromatography using polyester backed silica gel plates that had been treated with 1% potassium oxalate in H_2O/methanol (3:2). The plates were developed in chloroform/methanol/H_2O/ammonium hydroxide (136:106:23:8), and the position of standards was visualized using iodine vapor. Autoradiography was used to determine the positions of the ^{32}P-labeled polyphosphoinositides. The bands were cut out, 2 mls of Bio-Safe NA (RPI, Mount Prospect, IL) added, and ^{32}P incorporation into the polyphosphoinositides determined using a liquid scintillation counter.

RESULTS

Characteristics of the F-Actin Response Induced Through the Adenosine Receptor

The addition of multivalent antigen (DNP-BSA) leads to the stimulation of sensitized RBL cells through the IgE receptor. This leads to the polymerization of G-actin and a rapid and sustained increase in the amount of F-actin (Figure 1). The response peaks in 3–5 min and remains at nearly peak levels for over 30 min, as long as antigen is present. Polymerization of actin also occurs if the cells are stimulated with NECA through the adenosine receptor (Figure 1). However, this response is faster with a peak response being achieved in 45–60 s followed by a relatively rapid decline. Although this response reaches approximately the same levels as those achieved through the IgE receptor, it is over within 5–10 min. Recent work has shown that RBL cells also contain a purinoreceptor of the P_{2Y} type (Osipchuk and Cahalan, 1992; Qian and McCloskey, 1993). ATP was found to have no effect on the F-actin content of RBL cells although $ADP_\beta S$, which is a much more potent stimulant of P_{2Y} purinergic receptors, causes a small to moderate response which peaks in <30 s and returns to baseline values within 2–4 min (Figure 1).

The F-actin response to NECA is rapid and relatively short lived. Figure 2 (top panel) shows that the cells become unresponsive to a second dose of NECA, which was administered 15 min after the first. This indicates that the cells have become desensitized to further stimulation with NECA. However, the cells will still respond to DNP-BSA to a normal extent after previous stimulation through the adenosine receptor (Figure 2, bottom panel). Thus the desensitization is receptor specific. Desensitization of the adenosine receptor has no effect on the F-actin response triggered through the IgE receptor. Similar results have been found for the Ca^{2+} response stimulated by adenosine (Ali *et al.*, 1990).

NECA does not trigger degranulation in the absence of antigen, but it is able to stimulate an F-actin response on its own. To determine whether the F-actin responses to NECA and antigen were additive, RBL cells were

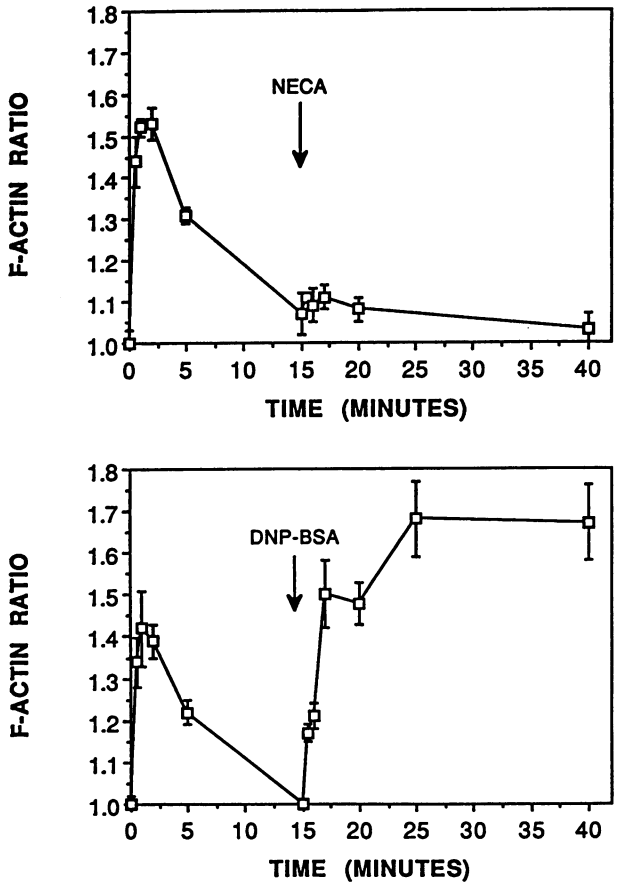

Figure 2. Desensitization of the NECA induced F-actin response. IgE primed RBL cells were stimulated with 10 μM NECA at time 0. After 15 min, the cells were restimulated with either 10 μM NECA (top panel) or 25 ng/ml DNP-BSA (bottom panel). The error bars represent the SE.

stimulated with either DNP-BSA for 5 min, NECA for 1 min, or DNP-BSA for 4 min with NECA being added for the final minute (Figure 3). As can be seen, the combination of DNP-BSA and NECA does not increase the response over that seen with NECA alone. Thus the two responses are not additive.

P1 purinergic receptors are generally classified depending on the magnitude of the response to various adenosine analogues (Bruns, 1990). Several adenosine analogues have been tested for their ability to trigger an F-actin response and the most effective stimulants were NECA and R-phenylisopropyladenosine followed by 2-chloroadenosine and adenosine. Also consistent with previous work on degranulation (Ali *et al.*, 1990; Gilfillan *et al.*, 1990), it has been seen that purinergic antagonists, such as theophylline, 8-phenyltheophylline, and isobutylmethylxanthine, do not block the F-actin response induced by NECA even at quite high concentrations. This is consistent with recent studies that indicate that RBL cells contain the newly described A3 subtype of adenosine receptors (Ramkumar *et al.*, 1993).

PKC Activation Is Crucial for the Antigen Induced Response but Not for the NECA Induced Response

PLC, which has been shown to be transiently activated by NECA, hydrolyzes the polyphosphoinositides to produce the inositol phosphates and diacylglycerol (Ali *et al.*, 1990). The inositol phosphates lead to an increase in intracellular Ca^{2+} while diacylglycerol is important in the activation of PKC. PKC has been shown to be an important signal leading to the polymerization of actin when RBL cells are stimulated through the IgE receptor (Apgar, 1991). Staurosporine, K252a, and sphingosine are protein kinase inhibitors with broad specificity which have been shown to inhibit PKC as well as various tyrosine kinases (Tamaoki, 1991; Yamada *et al.*, 1992). As can be seen in Figure 4, these protein kinase inhibitors all block the antigen-induced F-actin response in a dose dependent manner with IC_{50} values of 120 nM, 60 nM, and 14 μM, respectively. However, the polymerization of actin induced by NECA is relatively unaffected by these kinase inhibitors (Figure 4). This indicates that the F-actin response is dependent on a kinase, probably PKC, when initiated by the IgE receptor and independent of protein kinases when initiated by the adenosine receptor.

The fact that PKC is not involved in signaling for the F-actin response through the adenosine receptor was further confirmed by incubating the cells overnight in PMA. Long-term exposure of RBL cells to PMA leads to the total loss of certain isoforms of PKC from the cell but leaves other signaling mechanisms intact (Cunha-Melo *et al.*, 1989; Ozawa *et al.*, 1993). Table 1 shows that overnight exposure of RBL cells to PMA results in almost total inhibition of the increase in F-actin due to DNP-BSA but has relatively little effect on the response triggered by NECA.

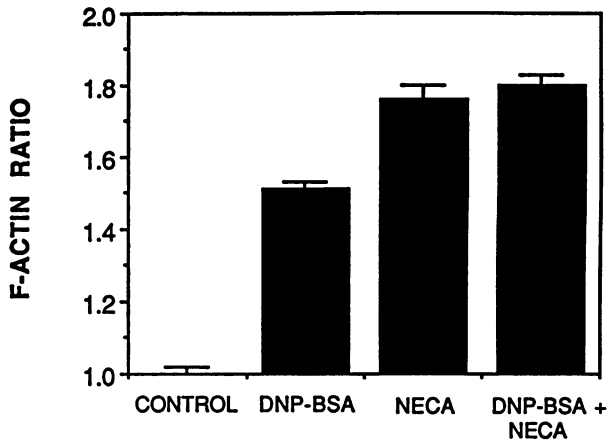

Figure 3. F-actin increase in response to DNP-BSA, NECA, or a combination of both reagents. RBL cells were sensitized with IgE and stimulated with either 25 ng/ml DNP-BSA for 5 min, 10 μM NECA for 1 min, or 25 ng/ml DNP-BSA for 4 min with 10 μM NECA being added for the final minute. The results are presented as the F-actin ratio ± SE.

The F-Actin Response is Relatively Insensitive to Ca^{2+} Levels

The other major signal that is produced due to phosphoinositide hydrolysis is an increase in intracellular Ca^{2+}. Table 2 shows the F-actin ratios that are induced by DNP-BSA and NECA. In the presence of Ca^{2+}, DNP-BSA caused a 1.78-fold increase in the level of polymerized actin while NECA caused a 1.61-fold increase. La^{3+} ions have been shown to block the increase in intracellular Ca^{2+} (Beaven *et al.*, 1984), presumably by blocking the Ca^{2+} channels since it does not enter the cells. This was confirmed in this study by measuring the influx of $^{45}Ca^{2+}$. In the presence of La^{3+} there is no change in the F-actin ratio elicited by either antigen or NECA. In addition, no change is seen in the F-actin response when using Ca^{2+}-free buffer or Ca^{2+}-free buffer in the presence of 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Table 2).

The Role of G Proteins

The above experiments indicated that IgE receptor- and adenosine receptor-mediated F-actin responses involve different signaling pathways. In a further attempt to separate the two systems, RBL cells were pre-incubated with pertussis toxin for 4 hours. This toxin can inhibit some G protein mediated responses by catalyzing the ADP-ribosylation of the α subunit of certain G proteins (Birnbaumer *et al.*, 1990; Spiegel, 1992). As can be seen in Table 3, the F-actin response induced by DNP-BSA is relatively unaffected by the toxin. This is consistent with other studies which have shown that the signaling pathways associated with the IgE receptor are pertussis toxin insensitive (Ali *et al.*, 1990; Gilfillan *et al.*, 1990).

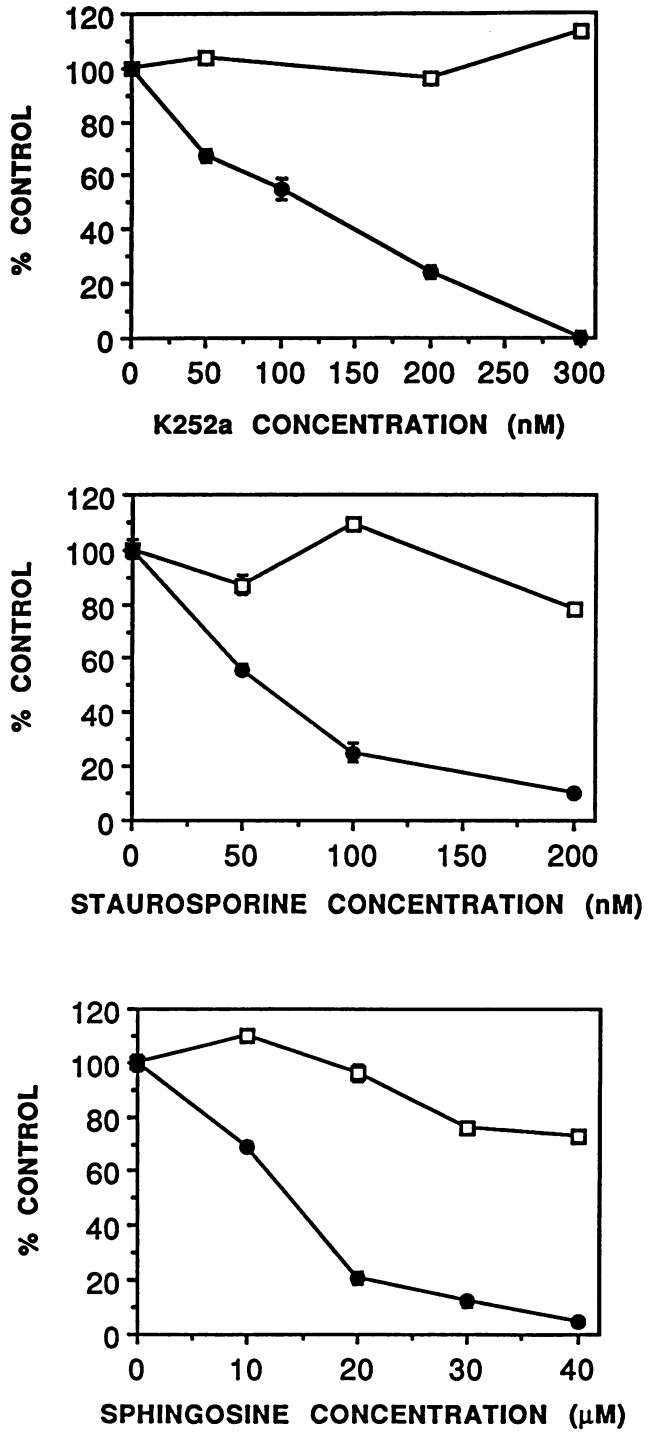

Figure 4. Effect of protein kinase inhibitors on the F-actin response. IgE sensitized cells were preincubated for 15 min with different concentrations of K252a, staurosporine, or sphingosine. The cells were triggered with either 25 ng/ml DNP-BSA (●) for 5 min or 10 μM NECA (□) for 1 min. The results are expressed as percent of control ± SE. The F-actin ratio for DNP-BSA was 1.66 and 1.54 for NECA.

Table 1. Effect of long-term exposure of RBL cells to PMA on the F-actin response

	Stimulant	
	DNP-BSA	NECA
Control	100.0 ± 0.4	100.0 ± 2.8
PMA Treatment[a]	3.5 ± 5.2	89.1 ± 4.3

The results are expressed as percent of control ±SE. The F-actin ratio for DNP-BSA was 1.54 while the NECA induced response was 1.59.

[a] RBL cells were cultured for 24 h in the presence of 40 nM PMA in complete RPMI media. Long-term treatment of the cells with PMA did not affect the unstimulated level of F-actin.

However, preincubation of the cells with the toxin almost completely inhibits the increase in F-actin triggered through the adenosine receptor. This experiment provides further confirmation that the two receptors use different signaling pathways leading to the polymerization of actin.

cAMP Levels Do Not Influence the F-Actin Response Mediated Through the Adenosine Receptor

The results indicate that neither protein kinases nor Ca^{2+} are important in the signaling for the F-actin response through the adenosine receptor, and that some other signals must be responsible, probably through a G protein. Since many purinergic receptors are associated with adenylate cyclase through a G protein, these receptors tend to modulate responses induced through other receptors by altering cyclic 3'5'-adenosine monophosphate (cAMP) levels inside the cell (Bruns, 1990). To determine whether the F-actin response was sensitive to cAMP levels, RBL cells were incubated with either forskolin or dibutyryl cAMP. These two reagents had no effect on the F-actin content on their own (Figure 5, bottom panel). In addition, dibutyryl cAMP had no effect on the F-actin level induced with either suboptimal (Figure 5, bottom panel) or optimal (Figure 5, top panel) concentrations of NECA. Thus polymerization of actin ac-

Table 2. Effect of Ca^{2+} on F-actin response

	DNP-BSA	NECA
HBSS	100 ± 7.3	100 ± 3.9
HBSS + 1 mM La^{3+}	115 ± 5.6	107 ± 2.4
Ca^{2+} free HBSS	101 ± 2.5	88 ± 2.1
Ca^{2+} free HBSS + 1 mM EGTA	118 ± 9.9	97 ± 3.0

The results are expressed as the percent of control ± SE. The F-actin ratio using HBSS was 1.78 for DNP-BSA and 1.61 for NECA. IgE sensitized RBL cells were preincubated in media for 15 min before the addition of 25 ng/ml DNP-BSA for 5 min or 10 μM NECA for 1 min. The various treatments did not affect the unstimulated level of F-actin as compared with the controls.

Table 3. Effect of pertussis toxin treatment on the F-actin response

	DNP-BSA	NECA
Control	100 ± 5.3	100 ± 2.1
Pertussis toxin[a]	75 ± 2.3	10 ± 1.7

The results are expressed as percent of control ± SE. Cells were stimulated with either DNP-BSA (25 ng/ml) for 5 min at 37°C or NECA (10 μM) for 1 min at 37°C. The F-actin ratio for the control samples was 1.75 for DNP-BSA and 1.60 for NECA.

RBL cells were preincubated with pertussis toxin (0.1 μm/ml) for 4 h at 37°C. F-actin values for unstimulated cells treated with pertussis toxin were the same as the F-actin values for unstimulated control cells.

tivated through the adenosine receptor does not appear to be controlled by cAMP levels. It has also been reported that cAMP plays no role in adenosine mediated inositol phosphate production in RBL cells (Ali *et al.*, 1990).

Increased Synthesis of PIP and PIP_2 Induced by DNP-BSA and NECA. Because of the ability of PIP and PIP_2 to dissociate actin from gelsolin and profilin, it has been hypothesized that these polyphosphoinositides may be involved in actin polymerization (Yin, 1987; Forscher, 1989; Stossel, 1989; Isenberg, 1991). When RBL cells are triggered through the IgE receptor, the changes in the F-actin content, activation of PI and PIP kinases, and the production of newly synthesized PIP and PIP_2 parallel each other. This work has demonstrated that PKC is an upstream signal and may be involved in the regulation of PI and PIP kinases thus regulating the synthesis of PIP and PIP_2 (Apgar, unpublished observations). Stimulation of the F-actin response through the adenosine receptor appears to be independent of PKC. To determine whether the two signalling pathways were completely different or whether they possibly merged at a downstream signal, RBL cells were incubated with ^{32}P-orthophosphate for only 15 min before the addition of NECA. Using this type of a labeling strategy, only PIP and PIP_2, which are being produced at that moment, will be labeled with ^{32}P. This assay therefore measures flux through this pathway. Figure 6 is a kinetic analysis of the production of newly synthesized PIP and PIP_2 when the cells are stimulated through either the IgE receptor (Figure 6A) or the adenosine receptor (Figure 6B). NECA causes a rapid increase in ^{32}P-labeled PIP and PIP_2, which peaks in ~1 min and then begins to decline. Because this assay measures only what is being produced and not total amounts of the polyphosphoinositides, the fact that the curve peaks and then declines indicates that relatively little PIP and PIP_2 are being produced past 1 min. In contrast, the response to DNP-BSA rises more slowly but continues to increase over the course of the assay. The kinetics of these responses parallels the kinetics of the F-actin response thus suggesting that production of PIP and PIP_2 may be involved in the polymerization of actin induced through both of these receptors.

If synthesis of PIP and PIP_2 is important in actin polymerization, then the various inhibitors and reagents which affect the F-actin response should also affect polyphosphoinositide production. Table 4 shows that staurosporine inhibits the responses induced by the IgE receptor but not those of the adenosine receptor. Pertussis toxin, which inhibits the NECA induced F-actin response, also completely inhibits the production of ^{32}P-labeled PIP and PIP_2 but has no effect on either response activated by DNP-BSA. Finally, performing the assays in Ca^{2+}-free buffer has no effect on any of the responses regardless of which receptor is being used. These results confirm the previous results and indicate that the F-actin response induced through the adenosine receptor is independent of PKC but may be regulated by production of PIP and PIP_2. Furthermore, the results sug-

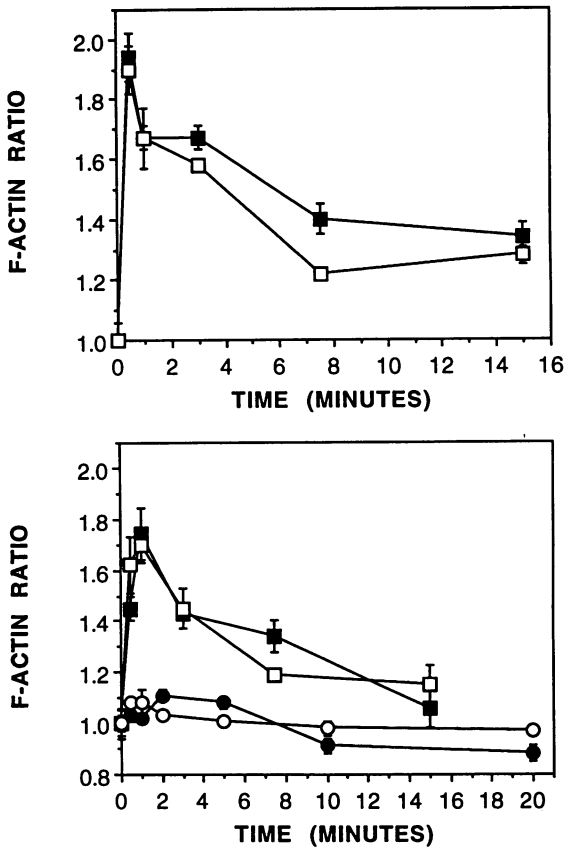

Figure 5. Effect of forskolin and dibutyryl cAMP on the F-actin response. In the top panel, RBL cells were stimulated for various lengths of time with an optimal (10 μM) concentration of NECA for 1 min at 37°C in the presence (□) or absence (■) of 1 mM dibutyryl cAMP. Dibutyryl cAMP was added 5 min before the addition of NECA. In the bottom panel, cells were stimulated with suboptimal (0.5 μM) NECA for 1 min in the presence (□) or absence (■) of dibutyryl cAMP. RBL cells were also incubated with just 1 mM dibutyryl cAMP (○) or 30 μM forskolin (●) for the indicated lengths of time. Error bars represent the SE.

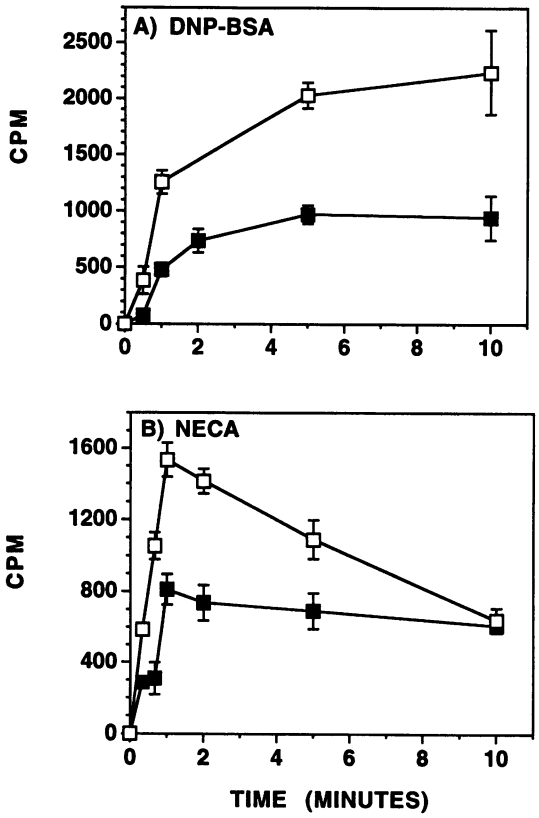

Figure 6. Production of newly synthesized PIP and PIP_2 in antigen (Panel A) and NECA (Panel B) stimulated cells. RBL cells (1×10^6 cells/tube) were pre-incubated with ^{32}P-orthophosphate for 15 min before the addition of either buffer, 25 ng/ml DNP-BSA, or 10 μM NECA for various lengths of time at 37°C. The reaction was stopped by the addition of chloroform/methanol (1:1). The phosphoinositides were extracted, separated by thin layer chromatography, and ^{32}P incorporation into PIP (□) and PIP_2 (■) was determined. CPM from unstimulated samples was subtracted from CPM in DNP-BSA and NECA stimulated samples to yield net ^{32}P incorporation ± SE.

gest that PI and PIP kinases may be activated through a G protein in the case of the adenosine receptor and through PKC when the IgE receptor is used.

DISCUSSION

The results presented here show that an F-actin response can be induced in RBL cells through either the IgE receptor or the adenosine receptor. Triggering of the cells through either receptor leads to a very rapid 1.6- to 1.9-fold increase in the F-actin content. Furthermore, because the cells are solubilized in Triton prior to the addition of NBD-phallacidin, the F-actin increase that is measured is probably restricted to actin, which is associated with the plasma membrane. The response induced through the IgE receptor reaches a peak level in 3–5 min and remains at an elevated level for over 30 min as long as antigen is present. The F-actin response elicited through the adenosine receptor peaks in <1 min and returns to baseline values in 5–10 min. Although the F-actin response to NECA is transient, it is as large in magnitude as the response to antigen. The two stimulants together do not cause an additive increase in F-actin levels indicating that either one response predominates over the other or they are competing for a limited pool of actin and responses of greater than twofold are not possible. In addition, stimulation with NECA desensitizes the cells to further activation with NECA, although a normal response to multivalent antigen will still occur. The response to antigen is prolonged and there is evidence that continuous positive signaling throughout is needed to maintain the response (Apgar, 1991). The fact that the adenosine receptor becomes desensitized so rapidly may explain why the F-actin response to NECA is relatively short lived.

Stimulation of RBL cells through the IgE receptor leads to the activation of several tyrosine kinases such as $pp60^{c\text{-}src}$ and $p56^{lyn}$ (Eiseman and Bolen, 1992). Tyrosine phosphorylation of phospholipase Cγ1 (Park *et al.*, 1991; Rhee, 1991; Li *et al.*, 1992) leads to the hydrolysis of the phosphoinositides and the generation of inositol phosphates which lead to an increase in intracellular Ca^{2+} and diacylglycerol, which activates PKC (Beaven and Cunha-Melo, 1988). Phospholipase A2 is activated through a G protein (Narasimhan *et al.*, 1990) with the subsequent production of arachidonic acid and its metabolites, the leukotrienes and prostaglandins (Crews *et al.*, 1981). Phospholipase D hydrolyzes phosphatidylcholine to produce phosphatidic acid, which can be further metabolized into diacylglycerol. Research involving both RBL cells (Lin *et al.*, 1991) and mast cells (Gruchalla *et al.*, 1990; Kennerly, 1990) indicates that this route is probably the major source of diacylglycerol and the continued activation of PKC. Several of these signals are required for degranulation while the polymerization of actin is dependent on PKC activation but relatively insensitive to changes in Ca^{2+}.

In contrast to the F-actin response, NECA does not cause any appreciable degranulation on its own nor does it lead to the production of leukotrienes (Ali *et al.*, 1990; Gilfillan *et al.*, 1990). Stimulation through the adenosine receptor causes a transient activation of PLC which leads to a small increase in inositol phosphate production and a small rise in intracellular Ca^{2+}. It is believed that the influx of Ca^{2+} acts to amplify the other signals thus leading to degranulation but because NECA causes such a small, transient rise in Ca^{2+}, it is unable to induce degranulation on its own (Ali *et al.*, 1990). However, NECA is able to boost degranulation in response to suboptimal concentrations of DNP-BSA. The synergistic signal that is supplied by the adenosine receptor is coming from PLC mediated hydrolysis of the polyphosphoinositides although a different isoform of PLC may be involved (Park *et al.*, 1991).

Although NECA does not cause any degranulation, it is able to elicit a strong, although transient, F-actin response. The signaling pathway responsible for the

Table 4. Effect of inhibitors on the F-actin response and ^{32}P-phosphoinositide production

	DNP-BSA			NECA		
	F-Actin	PIP	PIP_2	F-Actin	PIP	PIP_2
Control	100 ± 2.1	100 ± 8.2	100 ± 4.8	100 ± 3.3	100 ± 6.9	100 ± 9.4
Staurosporine[a]	15 ± 1.7	11 ± 8.1	27 ± 4.4	87 ± 5.3	69 ± 12.3	121 ± 6.9
Pertussis toxin[b]	88 ± 2.5	92 ± 1.6	107 ± 5.1	8 ± 2.1	23 ± 4.4	0 ± 2.3
Ca^{2+} free buffer[c]	99 ± 1.1	121 ± 3.9	96 ± 4.8	106 ± 4.9	81 ± 5.9	113 ± 10.8

Results are expressed as percent of control ± SE. IgE sensitized RBL cells were stimulated with DNP-BSA (25 ng/ml) for 5 min or with NECA (10 μM) for 1 min at 37°C. F-actin experiments were performed using HBSS + 0.1% BSA while ^{32}P-phosphoinositide experiments used phosphate free media + 0.1% BSA.

[a] Cells were pre-incubated with 150 nM staurosporine for 15 min before the addition of stimulant.

[b] RBL cells were pre-treated with pertussis toxin (0.1 μg/ml) for 4 h at 37°C.

[c] Cells were washed twice in Ca^{2+} and phosphate-free media, and the experiment was performed in this same buffer.

polymerization of actin through the adenosine receptor is clearly different than the pathway being used for the IgE receptor mediated response. The results using protein kinase inhibitors indicate that neither PKC nor tyrosine kinases are involved in the adenosine receptor mediated response. It has been reported that NECA on its own does not cause any appreciable production of arachidonic acid or its metabolites (Gilfillan *et al.*, 1990), and results reported here show that the response is relatively insensitive to levels of extracellular Ca^{2+}. An F-actin response could not be elicited by either forskolin or dibutyryl cAMP nor could these reagents alter the response to suboptimal or optimal concentrations of NECA, thus indicating that cAMP levels are not involved. The results of experiments in which cells were incubated with pertussis toxin indicate that a toxin-sensitive G protein is important in the NECA-induced F-actin response, but not the antigen-induced response. This result further separates the two signaling pathways. Pertussis toxin blocks the F-actin response, inositol phosphate production, and the augmentation of degranulation elicited by NECA but has no effect on antigen-induced responses (Ali *et al.*, 1990; Collado-Escobar *et al.*, 1990a,b; Gilfillan *et al.*, 1990). Therefore, polymerization of actin induced through the adenosine receptor is mediated by a G protein but appears to be independent of arachidonic acid metabolism, PKC activation, tyrosine kinase activation, Ca^{2+} influx, and cAMP levels.

Studies reported here have also shown that stimulation of RBL cells through either receptor leads to increased production of PIP and PIP_2. Experiments have shown that both PIP and PIP_2 can cause the dissociation of actin from both gelsolin (Janmey and Stossel, 1987; Janmey *et al.*, 1987) and profilin (Lassing and Lindberg, 1985), two proteins which are thought to help regulate the state of actin (Yin, 1987; Forscher, 1989; Stossel, 1989; Isenberg, 1991). Gelsolin is known to sever microfilaments and cap the barbed end of actin. Thus it may be acting as a regulator of actin nuclei whose formation is the rate limiting step in actin polymerization. Profilin was originally thought to be a monomer sequestration protein, but more recent studies suggest that it may actually be involved in polymerization by increasing the conversion of ADP-actin to ATP-actin (Goldschmidt-Clermont *et al.*, 1992). In RBL cells which have been activated with DNP-BSA, changes in the activation of PI and PIP kinases, the production of both PIP and PIP_2, and actin polymerization parallel each other (Apgar, unpublished observations). Thus, in the case of antigen, PKC activation may be an upstream signal that leads to the activation of the PI and PIP kinases. There is also a good correlation between the kinetics of production of PIP and PIP_2 and the kinetics of the F-actin response induced through the adenosine receptor. Activation of PI and PIP kinases and the production of PIP and PIP_2 is also inhibited by pertussis toxin but unaffected by staurosporine or lack of Ca^{2+}. This indicates that the PI and PIP kinases may be regulated by PKC when the IgE receptor is engaged but may be activated by a G protein when the adenosine receptor is used. These experiments certainly do not prove that PIP and PIP_2 are involved in actin polymerization, but they are consistent with that hypothesis. Thus polymerization of actin may involve different upstream signals, but the two pathways may merge at this downstream step.

The results reported here support the work with neutrophils that has shown that activation of the cells leads to an increase in F-actin content and a concomitant decrease in actin-gelsolin complexes and an increase in barbed-end nucleating activity (Howard *et al.*, 1990). In addition, there is an increase in phosphatidylinositol-4-phosphate kinase activity in neutrophils which correlates with the long-term maintenance of actin filaments (Pike *et al.*, 1991). However, these results do not agree with other studies that have indicated that PIP_3 is the crucial polyphosphoinositide and not PIP_2 (Eberle *et al.*, 1990). Furthermore, Dadabay *et al.* (1991), using A431 cells, have been able to dissociate increases in

bulk levels of PIP and PIP_2 from actin polymerization under certain conditions. Clearly, different cells can use different mechanisms to signal an F-actin response and even different receptors on the same cells can use distinct pathways. The F-actin response in RBL cells is dependent on PKC when signaling through the IgE receptor but is independent of this kinase when using the adenosine receptor. However, both pathways may involve the production of the polyphosphoinositides. Neutrophils are the most widely studied cell for the polymerization of actin. However, the signaling mechanisms in these cells remains somewhat obscure and the role of PKC is controversial (Howard and Wang 1987; Downey *et al.*, 1992). It has been shown that actin polymerization can be induced by chemotactic peptides, adherence, and immune complexes in neutrophils (Southwick *et al.*, 1989; Brennan *et al.*, 1991). It has also been found that the response to chemotactic peptides is blocked by pertussis toxin while the response to the other two stimuli is not. Furthermore, the response is dependent on Ca^{2+} for an adherence induced F-actin increase but independent of Ca^{2+} for a response to chemotactic peptides. Thus neutrophils may also use alternate pathways to signal for actin polymerization. Further studies will be necessary to determine whether the signaling pathways used by different cells are completely different or whether there are similarities in the downstream signals.

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