Regulation of the Antigen-induced F-Actin Response in Rat Basophilic Leukemia Cells by Protein Kinase C

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Abstract. Multivalent antigen that is capable of binding to and crosslinking the IgE receptors on rat basophilic leukemia (RBL) cells, induces a rapid and sustained rise in the content of filamentous actin. This reorganization of the actin may be responsible for changes in cellular morphology during the degranulation process. The antigen-stimulated polymerization of actin can be blocked in a dose-dependent manner by protein kinase inhibitors which also block degranulation. Conversely, reagents such as PMA, 1,2-dioctanoyl-sn-glycerol (diC8), and 1-oleoyl-2-acetyl-glycerol (OAG) which stimulate protein kinase C (PKC) also activate the rise in F-actin, although they have no effect on degranulation by themselves. The actin response which can be stimulated by the PKC activators can also be blocked by protein kinase inhibitors indicating that the PMA- and OAG-induced response is probably through activation of a protein kinase. Depletion of PKC activity through long term (20 h) exposure of RBL cells to PMA, also inhibited the F-actin response when the cells were stimulated with either multivalent antigen or OAG. External Ca^{++}, which is an absolute requirement for degranulation, is not necessary for the rise in F-actin, but may modulate the response. Furthermore, ionomycin, which induces a large Ca^{++} influx, does not stimulate the F-actin increase even at doses that cause degranulation. These results suggest that activation of a protein kinase, such as PKC, may be responsible for signaling the polymerization of actin in RBL cells and that a rise in intracellular Ca^{++} is neither necessary nor sufficient for this response.

ALLERGIC reactions such as immediate hypersensitivity are triggered through the IgE receptor of mast cells and basophils. It has been shown using rat basophilic leukemia (RBL)[1] cells, a model system for studying mucosal mast cells, that crosslinking of the IgE receptor on the surface of these cells is the critical event leading to the activation of the cells and the subsequent degranulation and release of histamine, serotonin, and other mediators (30). Crosslinking of the IgE receptor complexes leads to immobility of the complexes in the plane of the membrane (29) and transmembrane signaling possibly through a G protein (32, 48). In either case, there is increased metabolism of phosphatidylinositol (2) and arachidonic acid (8) through the activation of phospholipase C and phospholipase A2. In addition, there is activation of protein kinase C (PKC) (47), a rise in cytoplasmic Ca^{++} (3), an influx of Ca^{++} through an ion channel (13) or possibly an Na^+/Ca^{++} antiporter (44), and depolarization of the cell (24, 37). Morphologically there are changes in cell shape and topography, increased fluid pinocytosis, capping and internalization of the aggregated receptor complexes, and ultimately degranulation (30).

1. *Abbreviations used in this paper:* diC8, 1,2-dioctanoyl-sn-glycerol; ³H-5HT, 5-(1,2-³H)-hydroxytryptamine; NBD-phallacidin, N-(7-nitrabenz-2-oxa-1,3-diazol-4-yl)-phallacidin; OAG, 1-oleoyl-2-acetyl-glycerol; PKC, protein kinase C; RBL, rat basophilic leukemia.

Many of the events that take place during the degranulation process are believed to be controlled in part by microfilaments. It has been shown in RBL cells (34) as well as lymphocytes (35), platelets (7, 23), and neutrophils (36) that activation of the cells is accompanied by a reorganization of the cellular actin. This is generally seen as an increase in the amount of filamentous (F) actin associated with the cell. Actin is a cytoplasmic protein that exists in a dynamic equilibrium between the monomeric (G) state and a polymerized state. Activation of the cells results in polymerization of the actin and a shift from the G-actin pool to the F-actin pool.

Although many of the signaling mechanisms utilized during the activation of RBL cells are known, the mechanism responsible for the polymerization of actin is not. Pfeiffer et al. (34) have shown that many of the morphological changes that occur can be mimicked by the addition of phorbol esters which are believed to exert their effects through the activation of PKC. The purpose of this study was to determine the role of PKC and the Ca^{++} influx on the antigen triggered polymerization of actin in RBL cells. It was found that protein kinase inhibitors could block the increase in F-actin while PKC activators had the opposite effect. Furthermore, it was shown that while an influx of extracellular Ca^{++} may modulate the actin response, it is not sufficient to trigger this response.

Materials and Methods

Cells

RBL-2H3 cells were grown in T75 flasks containing RPMI 1640 (M.A. Bio-products, Walkersville, MD) supplemented with 5% FCS, 5% newborn calf serum, 2 mM glutamine, 10 mM Hepes, 1 mM sodium pyruvate, and penicillin-streptomycin in a humidified, 5% CO_2 incubator at 37°C. Cells were passaged every 3–4 d. They were removed from the flasks by incubating the cells in PBS containing 5 mM EDTA for 10 min at 37°C.

Reagents

The mouse monoclonal IgE antibody that is specific for DNP was a generous gift from Dr. Fu-Tong Liu from the Medical Biology Institute (La Jolla, CA) (28). Ionomycin, 1 oleoyl-2-acetyl-glycerol (OAG), R29022, staurosporine, and K252a were purchased from Calbiochem-Behring Corp. (San Diego, CA), while sphingosine and PMA were from Sigma Chemical Co. (St. Louis, MO). 5-(1,2-^{3}H)-hydroxytryptamine binoxalate (^{3}H-5HT) was obtained from DuPont Co. (Wilmington, DE), while N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin (NBD-phallacidin) and 1,2-dioctanoyl-sn-glyc-erol (diC8) were from Molecular Probes Inc. (Junction City, OR). Ionomycin, PMA, OAG, diC8, sphingosine, staurosporine, and K252a were all solubilized in DMSO as concentrated stock solutions. R29022 was prepared as a stock solution in ethanol. In all experiments, control samples were exposed to the same concentration of solvent as experimental samples, and in no case did the concentration of solvent ever exceed 0.05%.

^{3}H-5HT Assay

RBL cells were removed from their plates and resuspended in complete media at a concentration of 3 × 10^6 cells/ml. The cells were incubated with 1 μg of IgE and 3 μCi of ^{3}H-5HT per ml for 2 h at 37°C on a rotator. After the 2-h incubation, the cells were washed three times and resuspended in HBSS containing 0.1% BSA at a concentration of 3.3 × 10^6 cells/ml. 300 μl of cells (1 × 10^6 cells total) were added to 16-mm wells and allowed to adhere for 1 h. The supernatant was then removed and 700 μl of HBSS containing 0.1% BSA and the appropriate stimulant was added. After 45 min at 37°C, the supernatants were removed from the plates and centrifuged to pellet any cells that may have become detached during the assay. 500-μl aliquots were added to 2 ml of Scintiverse II (Fisher Scientific Co., Pittsburgh, PA) and ^{3}H-5HT content was determined using a Micromedic liquid scintillation counter. The results are expressed as percent ^{3}H-5HT released ±SEM.

F-Actin Assay

A modification of the assay developed by Howard and Oresajo (19) was used to measure the amount of filamentous or F-actin associated with the detergent-insoluble shells of RBL cells. Cells were sensitized with IgE as stated above, washed three times, and 200-μl aliquots containing 1 × 10^6 cells were placed into 12 × 75-mm tubes. 200 μl of the appropriate stimulant was then added and the cells were incubated at 37°C for the indicated time. The reaction was stopped by the addition of 2.5 ml of ice-cold solubilizing buffer (50 mM NaCl, 2.5 mM MgCl$_2$, 300 mM sucrose, 1 mM PMSF, 0.5% Triton X-100, 10 mM Hepes, pH 7.2). After 15 min at 4°C, the Triton shells were pelleted by centrifuging at 2,000 rpm for 10 min. The supernatant was removed and 100 μl of NDB-phallacidin (3.3 × 10^{-7} M) was added for 1 h at 4°C. The Triton shells were then washed with 3 ml of ice cold Dulbecco's-PBS. The bound NBD-phallacidin was extracted with 2 ml of methanol for 1 h at room temperature in the dark. The relative fluorescence was measured in a spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT) using an excitation wavelength of 465 nm and an emission wavelength of 535 nm. Tubes that went through the entire process but contained no cells were used to determine the background fluorescence. The no cell background was then subtracted from each reading. The results are expressed as the ratio of F-actin content of stimulated cells to unstimulated cells.

Results

Antigen-induced crosslinking of the IgE receptor activates phospholipase C which leads to the formation of inositol phosphates and diacylglycerol. These metabolites lead to an
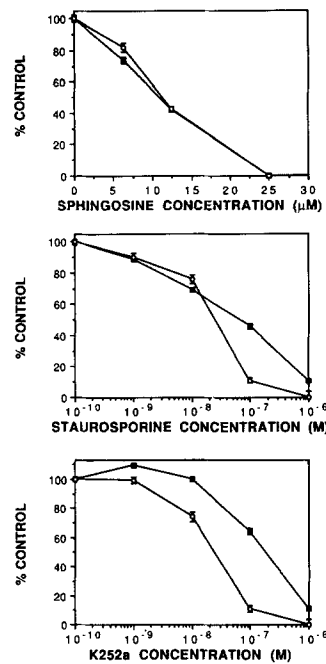

Figure 1. Inhibition of degranulation and the F-actin response by protein kinase inhibitors. Sensitized RBL cells were preincubated with the appropriate concentration of sphingosine, staurosporine, or K252a for 10 min before the addition of 25 ng/ml DNP-BSA. RBL cells were tested for their F-actin response (■) or degranulation (O) and the results are expressed as percent of control ±SEM. The control F-actin ratios (stimulated/unstimulated in the absence of inhibitors) for the sphingosine, staurosporine, and K252a experiments were 2.01, 1.71, and 1.81, respectively. The control degranulation responses were 20.8, 26.7, and 21.7%, respectively. Each experiment was performed at least three times.

influx of Ca^{++} and activation of PKC. To determine whether these events are involved in signaling for the polymerization of actin, RBL cells were stimulated with DNP-BSA in the presence of several protein kinase inhibitors. Fig. 1 shows that sphingosine, staurosporine, and K252a all inhibit the increase in F-actin with IC$_{50}$ values of 11 μM, 60 nM, and 200 nM, respectively. No effect on background levels of F-actin was detected. These protein kinase inhibitors also inhibit degranulation with very similar concentration dependencies.

The fact that the protein kinase inhibitors blocked the increase in F-actin formation indicated that protein kinases might be important in the signaling for F-actin. However, the use of inhibitors includes the potential problem of their specificity of action. Therefore, activators of PKC were tested to see if they could stimulate an increase in F-actin
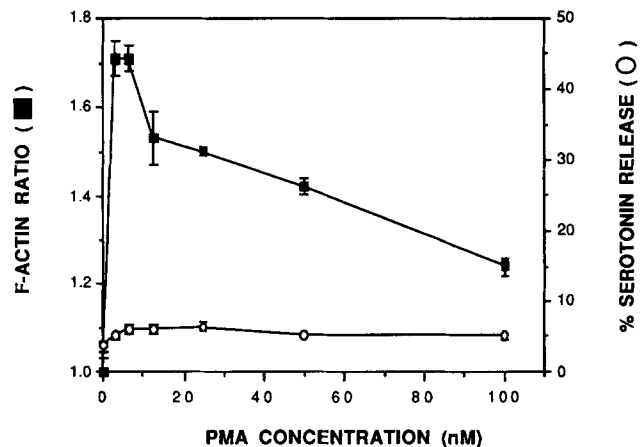

Figure 2. Stimulation of F-actin increase by PMA. RBL cells were activated with different concentrations of PMA for 30 min for the F-actin assay and 45 min for the degranulation assay.
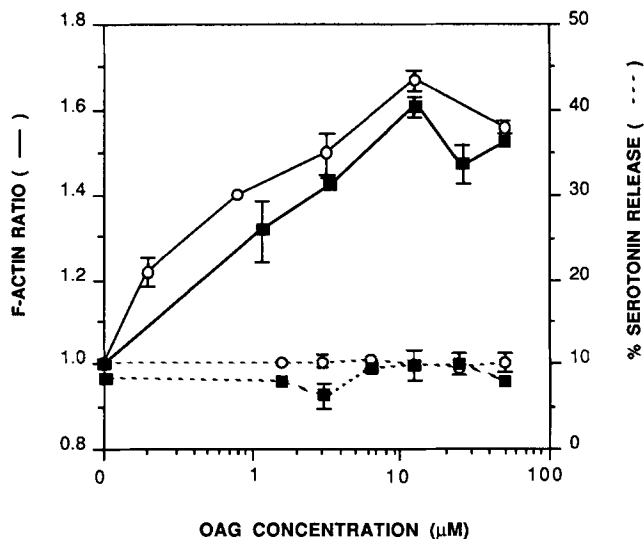
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Figure 3. Stimulation of F-actin increase by OAG and diC8. Various concentrations of OAG (o) and diC8 (■) were added to RBL cells. The reactions were allowed to proceed for 5 min for the F-actin assays (solid lines) and for 45 min for the 3H-5HT assays (dashed lines).
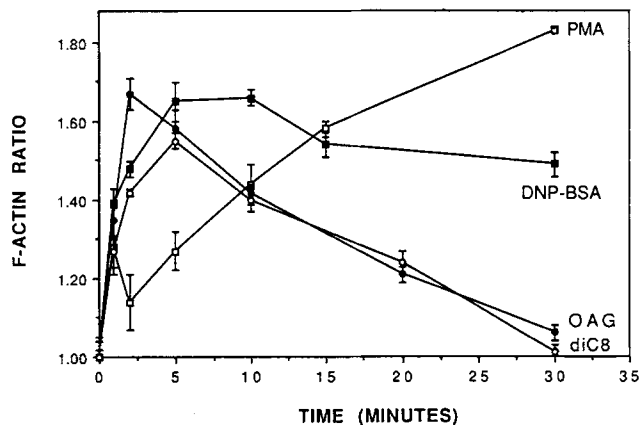

Figure 4. Kinetic analysis of the F-actin increase. Sensitized RBL cells were stimulated with 25 ng/ml DNP-BSA, 5 nM PMA, 12.5 μM OAG, or 10 μM diC8 for various lengths of time. The reactions were terminated at the appropriate times by the addition of ice-cold solubilizing buffer containing 0.5% TX-100.

content. In Fig. 2, cells were activated with varying concentrations of PMA and the F-actin response and degranulation were monitored. PMA on its own does not cause the secretion of ³H-5HT at any of the concentrations tested. However, PMA does cause an increase in the F-actin content associated with the Triton shells. Low concentrations of PMA (3–5 nM) give the best stimulation and cause an F-actin increase similar to that which is seen with antigen. Higher doses of PMA also cause an increase in F-actin, but it is not nearly as large.

However, PMA is a very potent tumor promotor and it may have other effects on the cell and it may be activating systems other than PKC. For that reason, the diacylglycerol analogues, OAG and diC8, were also tested. These synthetic diacylglycerols are known to rapidly cross the plasma membrane and stimulate PKC in the same fashion as endogenously generated diacylglycerol. Fig. 3 shows that neither OAG nor diC8 had any effect on degranulation but did cause a substantial increase in the F-actin content. The maximum response was seen at a concentration of ~10–15 μM for both reagents. The peak F-actin response was also of similar magnitude to that induced by antigen. These results further support the idea that PKC may be regulating the antigen-induced polymerization of actin in RBL cells.

The kinetic responses to antigen, PMA, OAG, and diC8 were also measured (Fig. 4). DNP-BSA causes a rapid rise in the F-actin content with a half maximal response in 30–60 s. Peak values are reached within 5 min and remain elevated with very little decrease over the duration of the experiment. In fact, F-actin levels do not decrease significantly even after 1 h (data not shown). The response to PMA is also very rapid in that an increase is seen at the earliest time point. This is followed by a reproducible dip and then a slow steady increase in the F-actin content. The reason for the dip is unknown although it also occasionally occurs when using antigen. This dip has also been reported by Pfeiffer et al. (34) using DNP-BSA. Both OAG and diC8 cause a very rapid re-

sponse. The OAG peak is reached in 2 min while the diC8 peak response is in 5 min. In both cases, the peak is followed by a sharp decline, and by 30 min, the response is back down to baseline values. These profiles are consistent with the fact that both OAG and diC8 are rapidly metabolized by the cell and the signal is presumably turned off.

To determine whether the decline in the OAG- and diC8-stimulated responses was due to metabolism of the activating reagent, RBL cells were stimulated with OAG, the response allowed to peak and decline, and then the cells were restimulated with OAG. As can be seen in Fig. 5 A, readdition of OAG causes an immediate increase in the level of F-actin back to peak levels within a few minutes. The F-actin response then decreases back towards baseline values. The cells can then be restimulated with the addition of more OAG. In addition, more frequent additions of OAG lead to a more uniformly sustained peak response (data not shown).

The primary route of metabolism for diacylglycerol is to be phosphorylated by diacylglycerol kinase, eventually leading to the resynthesis of phosphatidylinositol. Therefore, a second method for determining whether OAG and diC8 are being metabolized is to incubate the cells with R29022, a diacylglycerol kinase inhibitor (10). Fig. 5 B shows that the addition of R29022 leads to a more sustained F-actin response, presumably by inhibiting the enzyme responsible for diacylglycerol metabolism. The results of these two experiments are consistent with the hypothesis that OAG and diC8 are being rapidly metabolized which in turn leads to a decline in the response.

To confirm that PMA and OAG were causing a rise in F-actin due to activation of a protein kinase, RBL cells were stimulated with these two activators in the presence of the protein kinase inhibitors. The PMA- and OAG-induced increases in filamentous actin content were inhibited by sphingosine, staurosporine, and K252a (Fig. 6). The same results were seen when cells were stimulated with diC8 (data not shown). In all cases, the IC_{50} values are very similar for inhibiting the response due to antigen and PMA. In general, the OAG-induced response was more susceptible to the inhibitors than DNP-BSA or PMA.

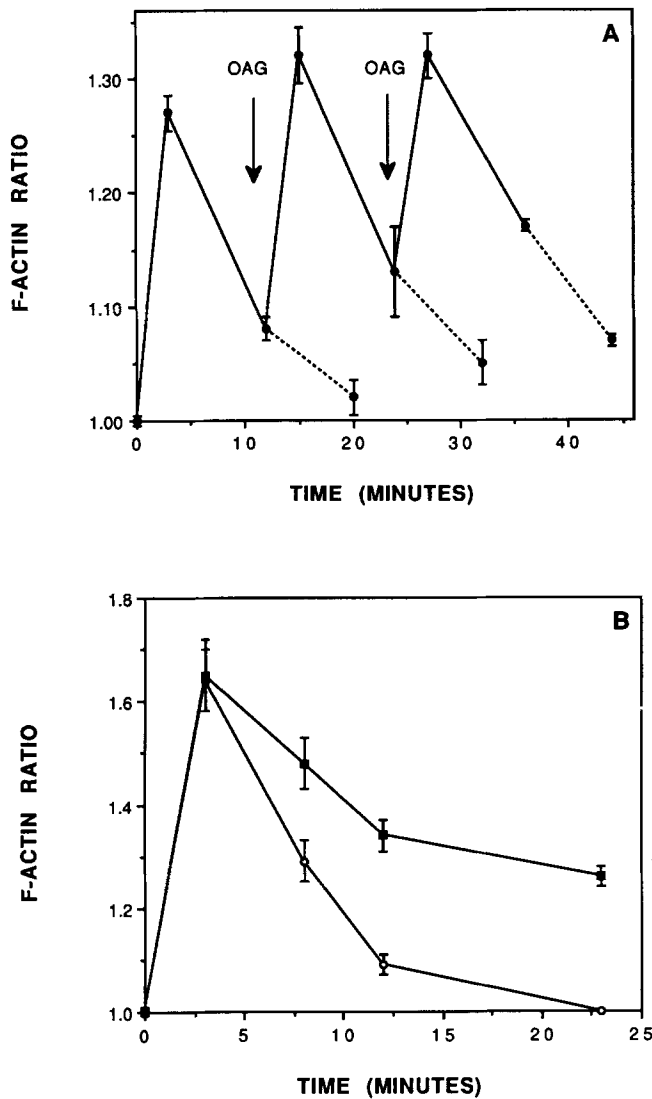Results from the previous experiments, utilizing both acti-

A

F-ACTIN RATIO

OAG   OAG

1.30

1.20

1.10

1.00

TIME (MINUTES)

B

F-ACTIN RATIO

1.8

1.6

1.4

1.2

1.0

TIME (MINUTES)

Figure 5. Extended stimulation of the F-actin response either by repeated application of OAG or by the diacylglycerol kinase inhibitor, R29022. (*A*) RBL cells were stimulated with 12.5 μM of OAG at time 0 and were restimulated with OAG at 12 and 24 min. The F-actin response in cells that were not restimulated is indicated by dashed lines. (*B*) Cells were preincubated with either solvent (o) or 10 μM R29022 (■) for 5 min before the addition of 12.5 μM OAG. The reaction was allowed to proceed for various lengths of time and was stopped by the addition of ice-cold solubilizing buffer.

% CONTROL

SPHINGOSINE CONCENTRATION (μM)

% CONTROL

STAUROSPORINE CONCENTRATION (M)

% CONTROL

K252a CONCENTRATION (M)

Figure 6. Inhibition of the PMA- and OAG-induced F-actin response. RBL cells were preincubated with the appropriate inhibitor for 10 min before the addition of the stimulant. Cells were activated with either 5 nM PMA (■) or 12.5 μM OAG (o). The F-actin content for each point was determined and the results are expressed as percent of the no inhibitor control. The actual F-actin ratios for the 100% PMA control values were 1.70, 1.49, and 1.50, respectively. The control ratios for the OAG-induced responses were 1.89, 1.33, and 1.31, respectively.

sumably because of the loss of PKC activity. These cells cannot be activated to increase their F-actin levels either by antigen or by OAG. Thus, loss of PKC activity, even in the presence of other signaling mechanisms, leads to the inhibition of the F-actin response.

In addition to activation of PKC, antigen-induced stimulation of RBL cells leads to an increase in intracellular Ca^{++}. Although the role of intracellularly released stores of Ca^{++} is somewhat controversial in the degranulation process, it is well known that there is an absolute requirement for the opening of an ion channel and the influx of extracellular Ca^{++} into the cell (30). The effect of extracellular Ca^{++} on the F-actin increase was tested using ionomycin which is a Ca^{++} ionophore. The influx of extracellular Ca^{++} due to ionomycin is concentration dependent and although all concentrations of ionomycin cause a rise in intracellular Ca^{++} (data not shown), release of ^3H-5HT is not seen until the ionomycin concentration is above 200 nM (Fig. 7). Progressively higher concentrations of ionomycin cause higher levels of degranulation. However, at all of the concentrations tested, there was never a detectable rise in the level of F-actin associated with the Triton shells. These results indicate that

vators and inhibitors, indicate that stimulation of PKC is involved in the F-actin response. An alternate approach to testing this hypothesis is to culture RBL cells for relatively long periods of time in the presence of PMA. This leads to the loss of PKC activity while having no effect on other signaling routes such as phosphatidylinositol metabolism or Ca^{++} influx (9). Growing RBL cells in 40 nM PMA for 20 h leads to an 85% decrease in the F-actin response generated by antigen, and a complete inhibition of the response triggered by OAG (Table I). In addition, the level of F-actin in unstimulated cells is the same for both control and PMA-treated cells. PMA itself will cause a rise in F-actin in untreated cells over a relatively short period of time. However, prolonged exposure leads to a return to baseline levels of F-actin, pre-
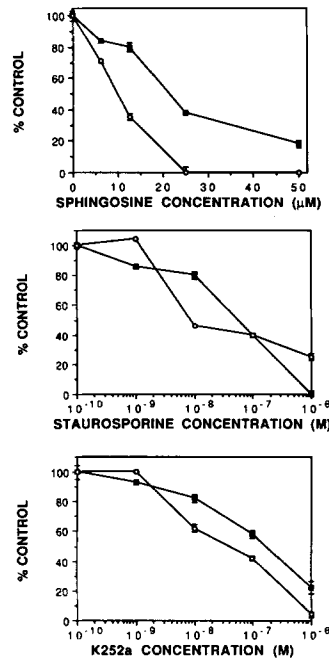
Table I. Effect of Long Term PMA Treatment on the F-Actin Response*

	Stimulant	
	DNP-BSA	OAG
Control	100	100
PMA treatment‡	15	0

* The results are expressed as percent of control. The unstimulated F-actin level in control cells and PMA-treated cells was the same, indicating that the PMA was no longer stimulating an increase on its own. The actual increase in F-actin was 1.58 for DNP-BSA and 1.51 for OAG stimulated cells. In all cases, the standard error was <5%.

‡ RBL cells were grown overnight in complete RPMI media containing 40 nM PMA. In all subsequent steps of the experiment, 40 nM PMA was present.
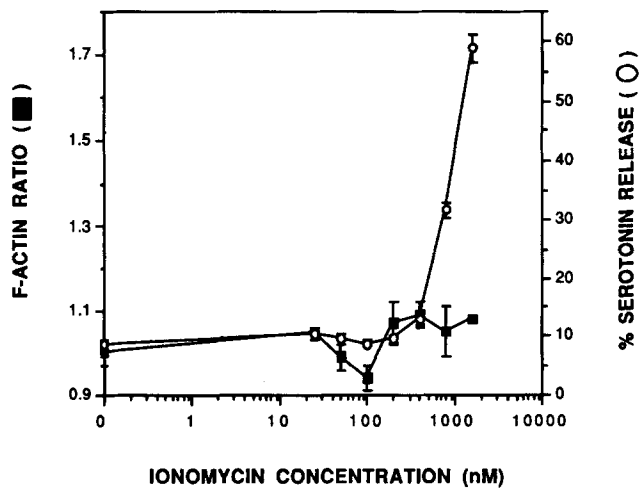
IONOMYCIN CONCENTRATION (nM)

Figure 7. Effect of ionomycin on degranulation and the F-actin response. RBL cells were incubated with various concentrations of ionomycin for 20 min for the F-actin response and 45 min for the degranulation assay.

the antigen-triggered influx of extracellular Ca^{++} is not sufficient to induce the rise in F-actin.

Although an influx of extracellular Ca^{++} does not appear to trigger the increase in F-actin, it might be necessary for the response. IgE-sensitized RBL cells were therefore activated with DNP-BSA in Ca^{++}-free media. As can be seen in Table II, the absence of extracellular Ca^{++} causes a 97% inhibition in the level of ^{3}H-5HT release. However, the presence or absence of extracellular Ca^{++} has relatively little effect on the rise in F-actin induced by DNP-BSA, PMA, or OAG. The slight decrease which is seen using DNP-BSA is very reproducible indicating that extracellular Ca^{++} is not required for actin polymerization but may have a modulating effect. PMA and OAG, which activate PKC directly, are essentially not affected at all within the limits of experimental error.

Discussion

One of the responses induced by crosslinking of the IgE receptor on the surface of RBL cells is a rapid increase in the amount of F-actin in the cell. The fact that the cells are solubilized in Triton before the addition of NBD-phallacidin may further restrict the analysis to F-actin associated with the plasma membrane. Generally, there is a 1.5–1.8-fold increase in the level of F-actin. This DNP-BSA–induced increase can be blocked in a dose-dependent fashion by protein kinase inhibitors such as sphingosine, K252a, and staurosporine. Sphingosine is reported to be specific for PKC (16), while K252a and staurosporine are capable of inhibiting several different kinases (18, 25). In addition to inhibiting the F-actin response, it was also found that the protein kinase inhibitors had a profound effect on the extent of degranulation. For each inhibitor, the inhibition curves for F-actin and ^{3}H-5HT were similar. This indicates that either activation of PKC is necessary for degranulation or that the inhibitors are blocking indirectly by affecting other signaling pathways.

Although the inhibitors that were used are believed to be quite specific for certain protein kinases, the use of inhibi-

*Table II. Effect of Ca^{++}-free Buffer on Degranulation and the F-Actin Response**

	F-actin‡			^{3}H-5HT§
	DNP-BSA	PMA	OAG	DNP-BSA
HBSS	100	100	100	100
Ca^{++}-free HBSS	67	105	95	3

* The results are expressed as percent of control with the value obtained using HBSS as the 100% value. The actual increase in F-actin was 1.48 using DNP-BSA, 1.38 using PMA, and 1.39 using OAG as the stimulant. Specific degranulation using HBSS as the buffer system was 33.5%.
‡ The F-actin ratio was measured after activating RBL cells with 25 ng/ml DNP-BSA for 30 min, 5 nM PMA for 30 min, or 5 µg/ml OAG for 3 min.
§ The cells were activated with 25 ng/ml DNP-BSA and ^{3}H-5HT release was measured as described in Materials and Methods.

tors always entails the problem that unknown or unwanted reactions may take place. For that reason, the rise in F-actin was also stimulated by PMA, OAG, and diC8 which activate PKC. PMA is a potent tumor promotor with wide ranging effects on cellular morphology and the cytoskeleton (33). It is believed that its mode of action is by mimicking diacylglycerol which is the endogenous activator of PKC. OAG and diC8 are synthetic diacylglycerols that can activate PKC both in vivo and in vitro (11, 14, 15). These two activators presumably work in the same fashion as endogenously generated diacylglycerol. Although all of these PKC activators cause substantial increases in F-actin, there are differences in the kinetics of the response. Crosslinking of the IgE receptor by multivalent antigen induces a rapid and sustained increase in the level of F-actin. The half-maximal response is 30–60 s and a plateau is reached by 5 min. This level is sustained for at least 30 min and only a slight decrease can be seen after 60 min. There is also a progressive release of ^{3}H-5HT during this time period. Addition of the monovalent antigen DNP-lysine immediately inhibits further degranulation (34) and returns the amount of F-actin to background levels (1). This indicates that there is positive signaling occurring throughout this period of time and this may account for the sustained peak level of F-actin. PMA causes a slow but steady increase in F-actin levels so that by 30 min, the increase in F-actin is as great as that seen for antigen. The reason for this slow increase is not known although experiments by Phatak et al. (35) using lymphocytes suggest that higher concentrations of PMA may lead to a more rapid response. Stimulation by OAG and diC8 is similar to that of antigen in peak levels attained and in that it is very rapid with a maximum effect being reached within a few minutes. However, the F-actin increases which are stimulated by OAG and diC8 return to baseline levels within 15–20 min, reflecting the fact that these stimulants are rapidly metabolized by the cell. This was further demonstrated by the fact that peak levels of F-actin could be sustained by repeated stimulation with OAG. Furthermore, inhibition of diacylglycerol kinase, the major route of diacylglycerol metabolism, leads to a more sustained increase in F-actin.

It has also been shown that the F-actin response induced by PMA and OAG can be blocked by protein kinase inhibitors. This indicates that PMA and OAG are probably exerting their effects directly through PKC and are not working indirectly by activating other pathways. This is further supported by the fact that PMA and OAG do not activate degran-

ulation, Ca^{++} influx, or phosphatidylinositol metabolism in RBL cells (4). The effects of PMA and OAG appear to be rather specific in these cells.

It has been shown that incubation of RBL cells for 20 h leads to the total loss of PKC activity (47) as well as the loss of the enzyme from the cell (9). Although this causes partial inhibition of degranulation, it has relatively little effect on phosphatidylinositol metabolism and Ca^{++} influx. Thus, long term exposure to PMA leads to a loss of PKC activity while other signaling mechanisms remain intact. In this paper, it has been shown that prolonged exposure of RBL cells to PMA leads to inhibition of the F-actin response using either antigen or OAG as the stimulating agent. This result further strengthens the conclusion that activation of PKC appears to be a step leading to the increase in F-actin.

The rise in F-actin triggered by DNP-BSA, PMA, and OAG was also relatively insensitive to external Ca^{++} levels. Whereas degranulation is totally dependent on the presence of extracellular Ca^{++}, only a small reduction in the F-actin increase was seen when cells were activated with antigen. The stimulation due to PMA, OAG, and diC8, which activate PKC directly, were not affected at all by a lack of extracellular Ca^{++}. Furthermore, although ionomycin caused release of ^3H-5HT, it did not trigger polymerization of actin. These results indicate that the F-actin response is neither activated by a Ca^{++} influx nor dependent on its occurrence. However, these experiments were performed using Ca^{++}-free buffers in the absence of EGTA, so it is possible that there are changes in the distribution of intracellular stores. The fact that there was a slight but consistent decrease in the F-actin increase using antigen suggests that Ca^{++} from either intracellular or extracellular sites may modulate the response.

Activation of neutrophils by chemotactic peptides is one of the most actively studied systems for analyzing F-actin regulation. The addition of peptides such as formylmethionyl-leucyl-phenylalanine causes very rapid changes in F-actin levels, cell motility, cell shape changes, phagocytosis, and degranulation. Although very rapid, transient changes in the F-actin levels are well documented, the question of which mechanisms are signaling for these changes is obviously complex. It has clearly been shown with intact cells and pertussis toxin (39) and with permeabilized cells and GTP analogues (46) that a G protein is involved in the F-actin response. However, the role of second messengers such as Ca^{++}, PKC, phosphoinositides, and intracellular pH is much more tenuous. A number of studies have shown that Ca^{++} changes are neither sufficient nor necessary to trigger the F-actin rise although they may modulate the response (38, 40). PMA in large doses is capable of stimulating a small rise in F-actin but it is generally believed that activation of PKC is not the prime signaling mechanism (5, 20). Furthermore, although some reports have suggested that changes in intracellular pH and phosphoinositides may be responsible for actin polymerization, recent studies have been able to dissociate the responses (6, 12, 31).

There are clear differences in the activation of actin in RBL cells and neutrophils. Ca^{++} does not appear to be crucial in either system while a protein kinase does appear to be important in RBL cells but not in neutrophils. These differences may reflect the fact that there may be several different ways of activating polymerization of actin. Alternatively, it could be that the F-actin has different functions in

the two cell types and therefore the activation mechanisms are different.

Although the data in this paper indicate that a protein kinase is important in the signaling for the rise in F-actin, the substrate and the mechanism of action are not known. However, it has been shown by other investigators that gelsolin and profilin bind to actin and prevent polymerization, thus helping to regulate the state of actin in the cell (42, 43, 49). In vitro studies have also shown that the phosphoinositides, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, can cause dissociation of gelsolin and profilin from actin (22, 26, 27). Recent investigations using platelets and neutrophils have been able to demonstrate a temporal correlation between an increase in cellular F-actin and a decrease in gelsolin-actin (21) and profilin-actin complexes (17, 41). The results from these studies suggest that stimulation of neutrophils leads to the activation of several enzymes, including phospholipase C, which hydrolyzes the phosphoinositides, and the kinases which are responsible for phosphorylating phosphatidylinositol and phosphatidylinositol 4-phosphate. This increased turnover of the phosphoinositides causes the dissociation of gelsolin-actin complexes thus leading to the polymerization of actin. It is not known whether the same mechanisms are involved in the F-actin response in RBL cells, and unfortunately, relatively little is known about the phosphoinositide kinases and their regulation. However, it has been shown that PMA stimulation of platelets causes increased phosphorylation of the phosphoinositides leading to an increase in the levels of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (45). Thus, one possibility is that PKC may be an earlier step in the signaling pathway, and that it may be regulating the activity of the phosphoinositide kinases. If this is the case, then the results presented in this paper are consistent with the idea that stimulation of RBL cells leads to phospholipase C hydrolysis of the phosphoinositides with the production of diacylglycerol and the activation of PKC. PKC in turn may activate the phosphoinositide kinases leading to the production of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate which in turn cause dissociation of gelsolin and profilin from actin thus allowing filament formation and elongation to occur.

References

1. Apgar, J. R. 1990. Antigen induced crosslinking of the IgE receptor leads to an association with the detergent-insoluble membrane skeleton of rat basophilic leukemia (RBL-2H3) cells. *J. Immunol.* 145:3814–3822.
2. Beaven, M. A., J. P. Moore, G. A. Smith, T. R. Hesketh, and J. C. Metcalfe. 1984. The calcium signal and phosphatidylinositol breakdown in 2H3 cells. *J. Biol. Chem.* 259:7137–7142.
3. Beaven, M. A., J. Rogers, J. P. Moore, T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. 1984. The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J. Biol. Chem.* 259:7129–7136.
4. Beaven, M. A., D. F. Guthrie, J. P. Moore, G. A. Smith, T. R. Hesketh, and J. C. Metcalfe. 1987. Synergistic signals in the mechanism of antigen-induced exocytosis in 2H3 cells: evidence for an unidentified sig-

nal required for histamine release. *J. Cell Biol.* 105:1129–1136.

5. Bengtsson, T., O. Stendahl, and T. Andersson. 1986. The role of the cytosolic free Ca^{++} transient for fmet-leu-phe induced actin polymerization in human neutrophils. *Eur. J. Cell Biol.* 42:338–343.

6. Bengtsson, T., I. Rundquist, O. Stendahl, M. P. Wymann, and T. Andersson. 1988. Increased breakdown of phosphatidylinositol 4,5-bisphosphate is not an initiating factor for actin assembly in human neutrophils. *J. Biol. Chem.* 263:17385–17389.

7. Carlsson, L., F. Markey, I. Blikstad, T. Persson, and U. Lindberg. 1979. Reorganization of actin in platelets stimulated by thrombin as measured by the DNase I inhibition assay. *Proc. Natl. Acad. Sci. USA.* 76:6376–6380.

8. Crews, F. T., Y. Morita, A. McGivey, F. Hirata, R. P. Siraganian, and J. Axelrod. 1981. IgE-mediated histamine release in rat basophilic leukemia cells: receptor activation, phospholipid methylation, Ca^{++} influx, and release of arachidonic acid. *Arch. Biochem. Biophys.* 212:561–571.

9. Cunha-Melo, J. R., H. M. S. Gonzaga, H. Ali, F. L. Huang, K. P. Huang, and M. A. Beaven. 1989. Studies of protein kinase C in the rat basophilic leukemia (RBL-2H3) cell that reveal that antigen-induced signals are not mimicked by the actions of phorbol myristate acetate and Ca^{++} ionophore. *J. Immunol.* 143:2617–2625.

10. de Chaffoy de Courcelles, D., P. Roevens, and H. VanBelle. 1985. R 59 022, a diacylglycerol kinase inhibitor. *J. Biol. Chem.* 260:15762–15770.

11. Ebeling, J. G., G. R. Vandenbark, L. J. Kuhn, B. R. Ganong, R. M. Bell, and J. E. Niedel. 1985. Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation. *Proc. Natl. Acad. Sci. USA.* 82:815–819.

12. Faucher, N., and P. H. Naccache. 1987. Relationship between pH, sodium, and shape changes in chemotactic-factor-stimulated human neutrophils. *J. Cell. Physiol.* 132:483–491.

13. Fewtrell, C., and E. Sherman. 1987. IgE receptor-activated calcium permeability pathway in rat basophilic leukemia cells: measurement of the unidirectional influx of calcium using quin2-buffered cells. *Biochemistry.* 26:6995–7003.

14. Ganong, B. R., C. R. Loomis, Y. A. Hannun, and R. M. Bell. 1986. Specificity and mechanism of protein kinase C activation by sn-1,2-diacylglycerols. *Proc. Natl. Acad. Sci. USA.* 83:1184–1188.

15. Gilmore, T., and G. S. Martin. 1983. Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. *Nature (Lond.).* 306:487–490.

16. Hannun, Y. A., C. R. Loomis, A. H. Merrill, and R. M. Bell. 1986. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J. Biol. Chem.* 261:12604–12609.

17. Hartwig, J. H., K. A. Chambers, K. L. Hopcia, and D. J. Kwiatkowski. 1989. Association of profilin with filament-free regions of human leukocyte and platelet membranes and reversible membrane binding during platelet activation. *J. Cell Biol.* 109:1571–1579.

18. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. 1977. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry.* 23:5036–5041.

19. Howard, T. H., and C. O. Oresajo. 1985. A method for quantifying F-actin in chemotactic peptide activated neutrophils: study of the effect of tBOC peptide. *Cell Motil.* 5:545–557.

20. Howard, T. H., and D. Wang. 1987. Calcium ionophore, phorbol ester, and chemotactic peptide induced cytoskeleton reorganization in human neutrophils. *J. Clin. Invest.* 79:1359–1364.

21. Howard, T., C. Chaponnier, H. Yin, and T. Stossel. 1990. Gelsolin-actin interaction and actin polymerization in human neutrophils. *J. Cell Biol.* 110:1983–1991.

22. Jamney, P. A., and T. P. Stossel. 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature (Lond.).* 325:362–364.

23. Jennings, L. K., J. E. B. Fox, H. H. Edwards, and D. R. Phillips. 1981. Changes in the cytoskeletal structure of human platelets following thrombin activation. *J. Biol. Chem.* 256:6927–6932.

24. Kanner, B. I., and H. Metzger. 1983. Crosslinking of the receptors for immunoglobulin E depolarizes the plasma membrane of rat basophilic leukemia cells. *Proc. Natl. Acad. Sci. USA.* 80:5744–5748.

25. Kase, H., K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, and M. Kaneko. 1987. K252a compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* 142:436–440.

26. Lassing, I., and U. Lindberg. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature (Lond.).* 314:472–474.

27. Lind, S. E., P. A. Jamney, C. Chaponnier, T. J. Herbert, and T. P. Stossel. 1987. Reversible binding of actin to gelsolin and profilin in human plate-

let extracts. *J. Cell Biol.* 105:833–842.

28. Liu, F. T., J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman, and D. H. Katz. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J. Immunol.* 124:2728–2737.

29. Menon, A. K., D. Holowka, W. W. Webb, and B. Baird. 1985. Clustering, mobility, and triggering activity of small oligomers of immunoglobulin E on rat basophilic leukemia cells. *J. Cell Biol.* 102:534–540.

30. Metzger, H., G. Alcaraz, R. Hohman, J. P. Kinet, V. Pribluda, and R. Quarto. 1986. The receptor with high affinity for immunoglobulin E. *Annu. Rev. Cell Biol.* 4:419–470.

31. Naccache, P. H., S. Therrien, A. Caon, N. Liao, C. Gilbert, and S. R. McColl. 1989. Chemoattractant-induced cytoplasmic pH changes and cytoskeletal reorganization in human neutrophils. *J. Immunol.* 142:2438–2444.

32. Narasimhan, V., D. Holowka, C. Fewtrell, and B. Baird. 1988. Cholera toxin increases the rate of antigen-stimulated calcium influx in rat basophilic leukemia cells. *J. Biol. Chem.* 263:19626–19632.

33. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.).* 308:693–698.

34. Pfeiffer, J. R., J. C. Seagrave, B. H. Davis, G. G. Deanin, and J. M. Oliver. 1985. Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. *J. Cell Biol.* 101:2145–2155.

35. Phatak, P. D., C. H. Packman, and M. A. Lichtman. 1988. Protein kinase C modulates actin conformation in human T lymphocytes. *J. Immunol.* 141:2929–2934.

36. Rao, K. M. K., and J. Varani. 1982. Actin polymerization induced by chemotactic peptide and Concanavalin A in rat neutrophils. *J. Immunol.* 129:1605–1607.

37. Sagi-Eisenberg, R., and I. Pecht. 1983. Membrane potential changes during IgE mediated histamine release from basophilic leukemia cells. *J. Membr. Res.* 75:97–104.

38. Sha'afi, R. I., J. Shefcyk, R. Yassin, T. F. P. Molski, M. Volpi, P. H. Naccache, J. R. White, M. B. Feinstein, and E. L. Becker. 1986. Is a rise in the intracellular concentration of free calcium necessary or sufficient for stimulated cytoskeletal-associated actin? *J. Cell Biol.* 102:1459–1463.

39. Shefcyk, J., R. Yassin, M. Volpi, T. F. P. Molski, J. H. Naccache, J. J. Munoz, E. L. Becker, M. B. Feinstein, and R. I. Sha'afi. 1985. Pertussis but not cholera toxin inhibits the stimulated increase in actin association with the cytoskeleton in rabbit neutrophils: role of the "G proteins" in stimulus-response coupling. *Biochem. Biophys. Res. Commun.* 126:1174–1181.

40. Sklar, L. A., G. M. Omann, and R. G. Painter. 1985. Relationship of actin polymerization and depolymerization to light scattering in human neutrophils: dependence on receptor occupancy and intracellular Ca^{++}. *J. Cell Biol.* 101:1161–1166.

41. Southwick, F. S., and C. L. Young. 1990. The actin released from profilin-actin complexes is insufficient to account for the increase in F-actin in chemoattractant-stimulated polymorphonuclear leukocytes. *J. Cell Biol.* 110:1965–1973.

42. Stossel, T. P. 1989. From signal to pseudopod: how cells control cytoplasmic actin assembly. *J. Biol. Chem.* 264:18261–18264.

43. Stossel, T. P., C. Chaponnier, R. Ezzel, J. H. Hartwig, P. A. Jamney, D. J. Kwiatkowski, S. E. Lind, D. B. Smith, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin-binding proteins. *Annu. Rev. Cell Biol.* 1:353–402.

44. Stump, R. F., J. M. Oliver, E. J. Cragoe, and G. G. Deanin. 1987. The control of mediator release from RBL-2H3 cells: roles for Ca^{++}, Na^+, and protein kinase C. *J. Immunol.* 139:881–886.

45. Taylor, M. V., J. C. Metcalfe, T. R. Hesketh, G. A. Smith, and J. P. Moore. 1984. Mitogens increase phosphorylation of phosphoinositides in thymocytes. *Nature (Lond.).* 312:462–465.

46. Therrien, S., and P. H. Naccache. 1989. Guanine nucleotide-induced polymerization of actin in electropermeabilized human neutrophils. *J. Cell Biol.* 109:1125–1132.

47. White, K. N., and H. Metzger. 1988. Translocation of protein kinase C in rat basophilic leukemia cells induced by phorbol ester or by aggregation of IgE receptors. *J. Immunol.* 141:942–947.

48. Wilson, B. S., G. G. Deanin, J. C. Standefer, D. Vandenjagt, and J. M. Oliver. 1989. Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. *J. Immunol.* 143:259–265.

49. Yin, H. L. 1987. Gelsolin: calcium and phosphoinositide regulated actin modulating protein. *Bioessays.* 7:176–179.