Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 551-557

TYROSINE KINASE-DEPENDENT PHOSPHATIDYLINOSITOL TURNOVER AND FUNCTIONAL RESPONSES IN THE FcεR1 SIGNALLING PATHWAY

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Received July 24, 1991

In RBL-2H3 rat basophilic leukemia cells, FcεR1 crosslinking by multivalent antigen stimulates phosphatidylinositol (PI) turnover and Ca^{2+} influx and causes functional responses that include secretion, membrane ruffling and actin polymerization. Here, we show that the tyrosine kinase inhibitor, genistein, inhibits antigen-induced PI turnover, determined from assays of 1,4,5-inositol trisphosphate production, and impairs receptor-mediated secretion, ruffling and actin polymerization. Genistein has little effect on several functional responses to stimuli that bypass PI hydrolysis (ionomycin-induced secretion, phorbol ester-induced ruffling) but it inhibits phorbol ester-induced actin polymerization. These data implicate a common tyrosine kinase-dependent event, most likely the activation of phospholipase Cγ, in the FcεR1-mediated stimulation of PI turnover, secretion and ruffling. There may be additional tyrosine kinase-mediated events in the actin assembly pathway.

In RBL-2H3 rat basophilic leukemia cells, a model for mucosal mast cells, antigens that crosslink the high affinity receptor for IgE, FcεR1, lead to degranulation, actin polymerization and the transformation of the cell surface from a microvillous to a lamellar architecture (reviewed in 1,2). The biochemical basis for these functional responses is partially understood. Receptor crosslinking causes Ca^{2+} influx (3), most likely through the activation of a GTP-binding protein (4-7). It also stimulates a PI-specific phospholipase C (PLC), generating 1,4,5-inositol trisphosphate (1,4,5-IP_3) that mobilizes cytoplasmic Ca^{2+} stores and diacylglycerol that activates protein kinase C (8). The mechanism of antigen-induced PLC activation has remained unclear (4-7).

Recently, we studied the regulation of antigen-induced functional responses in a streptolysin O-permeabilized RBL-2H3 cell model (9). In this simplified system, several tyrosine kinase inhibitors, including genistein, abolished antigen-stimulated secretion and membrane responses. Conversely, sodium orthovanadate, whose activities include the inhibition of tyrosine phosphatase activity, mimicked the antigen-induced secretion and spreading responses in permeabilized RBL-2H3 cells. Other

Abbreviations:

anti-DNP-IgE, monoclonal immunoglobulin E directed against dinitrophenol; 1,4,5-IP_3, 1,4,5-inositol trisphosphate; DNP-BSA, dinitrophenol-conjugated bovine serum albumin; FcεR1, the high affinity Fc receptor for IgE; MEM, Minimal Essential Medium; NBD phallacidin, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin; PI, phosphatidylinositol; PLC, phospholipase C; RBL-2H3 cells, the secreting 2H3 subline of rat basophilic leukemia cells.

0006-291X/91 $1.50

recent studies have shown that FcεR1 crosslinking stimulates the tyrosine phosphorylation of several RBL-2H3 cell proteins (10, 11), very likely through the intermediate activation of a member of the *src* family of soluble tyrosine kinases (12). Together, these results link tyrosine kinase activation to the control of FcεR1-mediated signal transduction. However, they do not identify specific proteins whose tyrosine phosphorylation may affect transmembrane signalling.

Here we show in intact RBL-2H3 cells that genistein inhibits antigen-stimulated 1,4,5-IP_3 production as well as secretion, ruffling and actin polymerization. These data implicate a tyrosine kinase-activated PLC in the IgE receptor-mediated signalling pathway.

MATERIALS AND METHODS

Reagents and cells. Genistein was from Gibco (Grand Island, NY). Other reagents were obtained as described in (9). RBL-2H3 cells were cultured in Minimal Essential Medium (MEM; Gibco) with 15% fetal calf serum as described (7,9,13).

Secretion assays. RBL-2H3 cells were incubated overnight with monoclonal IgE directed against dinitrophenol (anti-DNP IgE; 14) and ^{3}H-serotonin (Dupont/NEN, Boston, MA). The IgE-primed, radiolabeled cells were washed in modified Hanks' buffer (15) with 0.05% bovine serum albumin (Hanks'-BSA), and secretion was determined from the release of radiolabel to the medium with DNP-conjugated bovine serum albumin (DNP-BSA) or other secretagogues as previously described (7,9,13). Secretion data are reported as percent of the total cell-associated radioactivity released in 20 min. They are corrected for a blank (no incubation) and for spontaneous release of serotonin when cells were incubated in Hanks'-BSA medium without any stimulus.

Actin polymerization. The polymerization of actin in IgE-primed cells was measured from the binding of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD phallacidin; Molecular Probes, Junction City, OR) to filamentous actin using a modification of the method described in (13). Briefly, cells were stimulated in Hanks'-BSA medium for various times, fixed and permeabilized by 60 min incubation in 2% paraformaldehyde/0.01% digitonin in phosphate-buffered saline (PBS), and incubated overnight in PBS buffer containing 0.07 μM NBD phallacidin. The cells were then washed in PBS and the fluorescence associated with a mimimum of 10,000 cells was measured in a Becton-Dickinson FACScan flow cytometer in linear mode using FACScan software for data acquisition and analysis.

1,4,5-IP_3 measurements. I,4,5-IP_3 levels were determined using the radioreceptor assay described in (16) except that bovine cerebellar membranes were used as the source of 1,4,5-IP_3 receptor. IgE-primed RBL-2H3 cells ($20x10^6$/ml, 0.75 ml/assay) were stimulated for various times in Hanks'-BSA medium and the reaction stopped by the addition of an equal volume of 16% TCA. After 15 minutes on ice, the tubes were centrifuged, the pellets were dissolved in 2ml of 0.15 NaOH for protein measurements and the supernatant fractions, containing phosphoinositides, were extracted with H_2O-saturated ether to remove TCA. The pH of the extracts was adjusted to 6.5 with $NaHCO_3$ and EDTA was added to a final concentration of 5 mM. The amount of 1,4,5-IP_3 present in the extract was determined from its ability to compete with [^3H]-1,4,5-IP_3 (3,000 cpm) for specific binding sites on cerebellar membranes prepared as described in (17,18). A standard curve was used to convert % inhibition of [^3H]-1,4,5-IP_3 binding to pmols (between 0 - 15) of 1,4,5-IP_3 in the extract.

Scanning electron microscopy. IgE-primed RBL-2H3 cell monolayers were incubated with antigen or other reagents at 37° C in Hanks'-BSA medium, then fixed with 2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4 and processed for scanning electron microscopy as described in Pfeiffer et al. (13).

RESULTS

Genestein inhibits antigen-induced secretion in RBL-2H3 cells. The effect of 100 μM genistein on secretion stimulated by antigen and also by the Ca^{2+} ionophore, ionomycin, that causes degranulation by a receptor- and PLC-independent pathway (1-3), is shown in Table I. The addition of 100 μM genistein at the same time as DNP-BSA or ionomycin has little effect on either antigen- or ionomycin-induced secretion. In cells incubated for 30 min with 100 μM genistein before the addition of DNP-BSA, antigen-induced secretion is reduced by approximately 35%. Under the same conditions,

TABLE I

Genistein Inhibits Antigen-Induced Secretion in RBL-2H3 Cells

	^{3}H-Serotonin Released in 20 min (% of Control)	
Treatment	DNP-BSA	Ionomycin
None	100	100
Genistein, no preincubation	90.5 ± 1.7	98.9 ± 0.7
Genistein, 15 min preincubation	53.8 ± 1.4	103.0 ± 2.1
Genistein, 30 min preincubation	60.9 ± 2.6	99.2 ± 0.6
Genistein, 60 min preincubation	64.5 ± 2.2	93.5 ± 2.4
Genistein, 12 hours preincubation	47.7 ± 1.9	64.9 ± 1.3

IgE-primed cells were incubated with and without 100 μM genistein in MEM-FCS, then transferred to Hanks'-BSA medium with and without inhibitor and stimulated for 20 min with DNP-BSA (0.1 μg/ml) or ionomycin (0.75 μM). Results are the average of 5 (DNP-BSA) or 3 (ionomycin) separate experiments, each performed in duplicate. Data are reported as % of secretion in untreated cells. In the absence of genistein, DNP-BSA-treated cells released 58.19 ± 0.78% of their total ^{3}H-serotonin and ionomycin-treated cells released 56.1 ± 0.78% of their total ^{3}H-serotonin in 20 min. Data are corrected for spontaneous release (approximately 4-5% of total ^{3}H-serotonin in 20 min in both untreated and genistein-treated cells).

ionomycin-induced secretion is reduced by less than 1%. Longer preincubation times result in only a slightly greater inhibition of antigen-induced secretion, but are accompanied by a progressively greater inhibition of ionomycin-induced secretion (Table I). Concentrations of genistein below 100 μM showed little inhibition of antigen-induced secretion whether cells were treated acutely or preincubated for periods up to 12 hours (data not shown). Based on these results, the experiments that follow were all done using cells preincubated for 30 min with 100 μM genistein.

Genistein inhibits antigen-induced membrane ruffling in RBL-2H3 cells. The results in Figure 1A,B show that genistein alone does not alter the typical microvillous surface morphology of unstimulated cells. The transformation of the cell surface from a microvillous to a lamellar topography that occurs in response to antigen and and also to phorbol myristate acetate (PMA), a reagent that bypasses receptor and PLC activation and directly activates protein kinase C (1,2,13), is also illustrated (Figure 1C,E). Preincubation with genistein inhibits antigen-induced ruffling (Figure 1D). In contrast, PMA-induced membrane ruffling and spreading are still seen in the presence of genistein (Figure 1F).

Genistein inhibits antigen-induced 1,4,5-IP_3 *production in RBL-2H3 cells.* The results in Table II show that antigen binding causes a progressive increase in 1,4,5-IP_3 levels to more than 4 times the basal level in 2 minutes. Genistein treatment has no effect on basal levels of 1,4,5-IP_3 levels but it causes an average reduction of 36.4% in the 1,4,5-IP_3 levels measured in antigen-stimulated cells.

Genistein inhibits antigen- and PMA-induced actin assembly in RBL-2H3 cells. The effects of genistein on antigen and PMA-induced actin polymerization are reported in Figure 2. As previously described (13), antigen binding causes an increase in cytoplasmic actin polymerization to approximately 30% above control values in 10 min. PMA increases actin polymerization by approximately 20%. Geni-

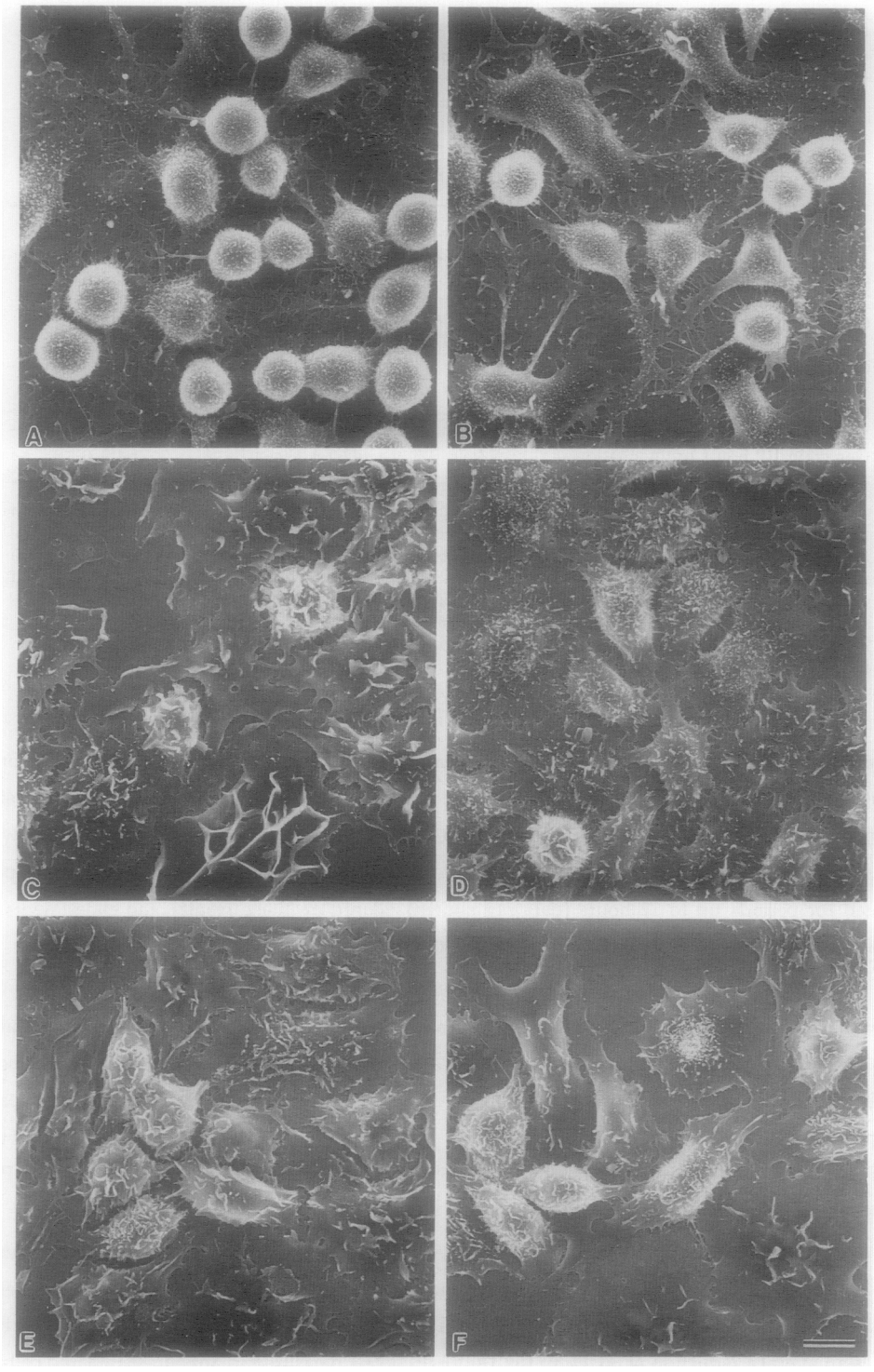

Figure 1. The effect of genistein on RBL-2H3 cell morphology. IgE-primed RBL-2H3 cell monolayers were incubated with or without 100 μM genistein for 30 min in MEM-FCS, followed by 10 min incubation in Hanks'-BSA medium with no addition, 0.1 μg/ml DNP-BSA or 50 nM PMA. In the absence of stimulation, control (A) and genistein-treated (B) cells share the same microvillous surface morphology. Control cells show a ruffling response to antigen (C), whereas antigen-induced ruffling is inhibited in genistein-treated cells (D). There is a ruffling response to PMA in both control (E) and genistein-treated (F) cells. Bar = 10 μm.

TABLE II

Genistein Inhibits Antigen-Induced 1,4,5-IP_3 Synthesis in RBL-2H3 Cells

	1,4,5-IP_3 Concentration (pmol/mg protein)	
Treatment	Control Cells	Genistein-Treated Cells
None	7.35 ± 0.52	7.00 ± 0.39
DNP-BSA, 30 sec	13.94 ± 1.81	10.10 ± 0.70
DNP-BSA, 60 sec	22.60 ± 1,46	11.69 ± 1.00
DNP-BSA, 120 sec	30.85 ± 1.90	20.56 ± 3.10

IgE-primed cells were incubated for 30 min in MEM-FCS with or without 100 μM genistein, then transferred to Hanks'-BSA medium and stimulated for various times with 0.1 μg/ml DNP-BSA. 1,4,5-IP_3 levels in TCA extracts of these cells were measured as described in Methods. Results are the average ± SEM of 3 separate experiments, each performed in duplicate.

stein reduces the basal levels of polymerized actin in RBL-2H3 cells by approximately 10% and it limits the actin assembly response to antigen to approximately 10%. In genistein-treated cells, PMA-stimulated actin polymerization is inhibited at least as much as the antigen-stimulated response.

DISCUSSION

Recently, we reported that tyrosine kinase inhibitors block, and tyrosine phosphatase inhibitors mimic, antigen-induced cell activation in permeabilized RBL-2H3 cells (9). Other investigators (10, 11) have demonstrated that antigen binding to IgE-receptor complexes stimulates the tyrosine phos-

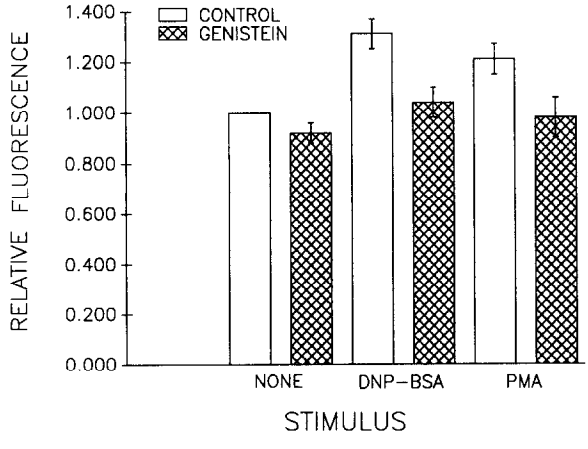

Figure 2. The effect of genistein on actin polymerization. IgE-primed RBL-2H3 cells were incubated with (hatched columns) or without (open columns) 100 μM genistein for 30 min in MEM-FCS, then harvested and incubated for 10 min with no addition, 0.1 μg/ml DNP-BSA or 50 nM PMA. In the absence of stimuli, genistein reduces the amount of polymerized actin by approximately 10%. Antigen and PMA both stimulate a strong actin polymerization response in control cells but cause very little actin assembly in genistein-treated cells. Results, expressed as % of the mean channel number for NBD-phallacidin fluorescence in unstimulated control cells, are the average +/- SEM of four separate experiments.

phorylation of several proteins in RBL-2H3 cells. Together, these results indicate that antigen-induced tyrosine kinase activation, most likely mediated by interactions of receptor subunits with *src*-family tyrosine kinases (12), may play an essential role in IgE receptor-mediated signal transduction.

The goal of the present study was to begin the identification of specific proteins whose tyrosine phosphorylation is critical for cell activation. First, we established that genistein inhibits antigen-induced secretion and membrane ruffling in intact RBL-2H3 cells. This inhibition requires a 15-30 min preincubation of cells with drug before antigen stimulation, most likely because intact RBL-2H3 cells are relatively impermeable to genistein. We were unable to discover conditions where intact cells reproduce the responses of permeabilized cells to either tyrphostins or sodium orthovanadate, indicating that the permeability barrier may be greater for other tyrosine kinase and tyrosine phosphatase inhibitors (G.G. Deanin and J.M. Oliver, unpublished results).

Antigen-induced secretion in intact cells was reduced by around 40% by 100 μM genistein and the antigen-induced membrane ruffling and spreading response was also not completely inhibited. In contrast, we showed before that antigen-induced secretion and ruffling are virtually abolished by 20 μM genistein in permeabilized cells (9). This difference in the extent of inhibition between intact and permeabilized cells may be another reflection of the limited permeability of intact cells to genistein. An alternative explanation is that antigen binding may activate both tyrosine kinase-dependent and other pathways leading to functional responses in intact RBL-2H3 cells. We speculate that permeabilized cells may lack substrates for the redundant pathways, GTP for example, thus increasing their dependence on the tyrosine kinase-mediated pathway.

Although it inhibits receptor-mediated responses, genistein has very little effect on secretion and ruffling in response to reagents that bypass receptor crosslinking and PLC activation. Thus, genistein-treated cells secrete normally in response to ionomycin, presumed to mimic both the $1,4,5-IP_3$-mediated release of Ca^{2+} stores and the GTP-binding protein-mediated influx of Ca^{2+}. The membrane ruffling response to PMA, a phorbol ester that mimics the activation of protein kinase C by diacylglycerol, also persists in the presence of genistein. From these results, we infer that tyrosine phosphorylation is essential for an early step in the receptor-mediated signalling cascade.

Studies with the receptor tyrosine kinases have identified a long list of potential substrates for stimulated tyrosine phosphorylation, including PLCγ, the 85 kDa subunit of phosphatidylinositol 3-kinase, GAP, the protein that controls the activity of $p21^{ras}$, the non- *ras* GTP-binding protein, G25K, the *src* family of soluble protein kinases, the family of extracellular signal-regulated serine/threonine kinases (ERKs), the $p^{72}raf$ serine/threonine kinase and *vav*, a protein that may have transcription enhancing activity (19-22). We determined the effect of genistein on antigen-stimulated PI turnover because FcεR1-mediated signal transduction requires PLC activation and because of recent evidence that the T cell receptor, which shares homologies with the FcεR1 (23), is coupled through a *src* family tyrosine kinase to PLCγ (24). It was found that the antigen-stimulated synthesis of $1,4,5-IP_3$, the product of phosphatidylinositol bisphosphate hydrolysis by PLC, is reduced by approximately 40% in genistein-treated RBL-2H3 cells. From these data, we propose that the activation by tyrosine phosphorylation of a PLC, most likely PLCγ, is a principal event in the antigen-stimulated signalling cascade in RBL-2H3 cells. We propose further that the inhibition of this enzyme activity by genistein is responsible, at least in part, for the reduced antigen-stimulated secretion and membrane ruffling in intact RBL-2H3 cells.

In contrast with the partial inhibition by genistein of antigen-induced 1,4,5-IP_3 production, secretion and ruffling, genistein almost completely inhibits antigen-induced F-actin assembly. PMA-induced actin assembly is also strongly inhibited by genistein. Since actin assembly appears to be controlled in part by the association of phosphoinositides with actin-binding proteins (25), it is possible that this inhibition is linked to the inhibition of PI turnover in genistein-treated cells. However, the greater inhibition by genistein of antigen-stimulated actin polymerization than of 1,4,5-IP_3 production makes it likely that there are additional roles for protein tyrosine phosphorylation in the pathway leading to actin assembly. The inhibition in genistein-treated cells of PMA-induced actin polymerization but not membrane ruffling provides the first evidence that these responses can be independently regulated.

Acknowledgments. This work was supported in part by NIH Grant GM37202, NSF Grant DIR8912441 and by funds from the University of New Mexico School of Medicine and Cancer Center. We thank Mr. L. Seamer for assistance with flow cytometry.

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