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Isoprenoid pathway activity is required for IgE receptor-mediated, tyrosine kinase-coupled transmembrane signaling in permeabilized RBL-2H3 rat basophilic leukemia cells

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Previously, we reported that the isoprenoid pathway inhibitor, lovastatin, blocks the activation by IgE receptor cross-linking of $^{45}Ca^{2+}$ influx, 1,4,5-inositol trisphosphate production, secretion, and membrane changes (ruffling, spreading) in intact RBL-2H3 rat basophilic leukemia cells. These results indicated that an isoprenoid pathway intermediate, very likely an isoprenylated protein, is importantly involved in the control of IgE receptor-mediated signal transduction. Here, we show that 20 h of pretreatment with lovastatin also inhibits antigen-induced secretion and membrane responses in streptolysin O-(SLO)-permeabilized cells. However, lovastatin does not inhibit secretion stimulated by the nonhydrolyzable GTP analog, GTPγS. Furthermore, the membrane responses to GTPγS persist, although in an attenuated form, in lovastatin-treated permeabilized cells. The relative insensitivity of GTPγS-induced responses to lovastatin was one of several indications that antigen and GTPγS may activate separate pathways leading to transmembrane responses in permeabilized cells. Further experiments showed that the β-thio derivative of GDP, GDPBAS, inhibits the secretory and membrane responses to GTPγS, as expected for a GTP-binding protein-dependent signaling pathway, while having little effect on antigen-induced responses. Conversely, genistein blocks the secretory and membrane responses to antigen, as expected for a tyrosine kinase-dependent pathway, without altering the GTPγS-induced responses. From these results, and from additional data from cells treated with tyrphostins and sodium orthovanadate, we propose that IgE receptor-mediated secretion from permeabilized RBL-2H3 cells occurs by a tyrosine kinase-dependent pathway that requires isoprenoid pathway activity for function. We propose further that RBL-2H3 cells contain a separate GTP-binding protein-mediated signaling pathway whose direct activation by GTPγS is either independent of isoprenoid pathway activity or depends on the activity of an isoprenylated protein that is not significantly depleted after 20 h of lovastatin treatment.

Introduction

In RBL-2H3 rat basophilic leukemia cells, a model for mucosal mast cells, antigens that cross-link IgE-receptor complexes activate an inositol phospholipid-specific phospholipase C (PLC),[1] generating 1,4,5-inositol trisphosphate (1,4,5-IP_3) that is responsible for the mobilization of cytoplasmic Ca^{2+} stores and diacylglycerol that activates protein kinase C (Beaven *et al.,* 1984a). Receptor cross-linking also causes the increased uptake of Ca^{2+} from the medium (Beaven *et al.,* 1984b; Narasimhan *et al.,* 1988; Wilson *et al.,* 1989), and it stimulates protein tyrosine phosphorylation in RBL-2H3 cells (Benhamou *et al.,* 1990). These and other biochemical events lead to the release of serotonin and other substances from granules, the assembly of F-actin, and the transformation of the cell surface from a microvillous to a lamellar architecture (reviewed in Metzger *et al.,* 1986; Oliver *et al.,* 1988). There is evidence that a GTP-binding protein regulates the antigen-stimulated influx of Ca^{2+} that is required for secretion (McCloskey, 1988; Narasimhan *et al.,* 1988;

[1] Abbreviations used: DMSO, dimethyl sulfoxide; DNP-BSA, dinitrophenol-conjugated bovine serum albumin; EGF, epidermal growth factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTPγS, the γ-thio-derivative of GTP; GDPβS, the β-thio derivative of GDP; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; 1,4,5-IP_3, 1,4,5-inositol triphosphate; MEM, Eagle's minimal essential medium; MVA, mevalonic acid; PG, Pipes-glutamate; PLC, phospholipase C; RBL-2H3, the 2H3 secreting subline of RBL rat basophilic leukemia cells; SLO, streptolysin O.

Wilson *et al.,* 1989, 1991). The coupling events linking IgE receptor cross-linking to PLC activation and other biochemical and functional responses have not been clearly defined.

Recently, we established that the isoprenoid synthetic pathway, whose products include cholesterol and dolichol, is essential for IgE receptor-mediated transmembrane signaling (Deanin *et al.,* 1991). RBL-2H3 cells were incubated for 20 h with 10 μM lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (Endo *et al.,* 1976; Kaneko *et al.,* 1978; Glomset *et al.,* 1990; Goldstein and Brown, 1990). This treatment reduced cholesterol synthesis by 98% without inhibiting RBL-2H3 cell division or reducing IgE receptor density. It did, however, strongly inhibit antigen-stimulated $^{45}Ca^{2+}$ influx, PLC activation, secretion, and membrane responses. The inhibitory effects of lovastatin were reversed by the addition of the isoprenoid pathway precursor, mevalonic acid (MVA), but not by the addition of dolichol or cholesterol (end products of isoprenoid metabolism), implicating an isoprenoid pathway intermediate in the control of signal transduction in RBL-2H3 cells. The ability of reagents that bypass the receptor to stimulate transmembrane responses (ionomycin-induced secretion, phorbol myristate acetate-induced membrane ruffling responses) in lovastatin-treated cells indicated that isoprenoid pathway activity is required for an early event in the IgE receptor-activated signal transduction pathway. It was postulated that isoprenoid pathway intermediates are required for the post-translational isoprenylation of proteins involved in IgE receptor-mediated signaling. The known substrates for this modification include several members of the *ras*- related family of small GTP-binding proteins as well as the γ subunit of the heterotrimeric GTP-binding proteins (reviewed in Glomset *et al.,* 1990; Goldstein and Brown, 1990; Maltese, 1990).

In this paper, we take advantage of the streptolysin O (SLO)-permeabilized cell model developed by Ahnert-Hilger, Gomperts, and others (reviewed in Ahnert-Hilger *et al.,* 1989) and adapted for RBL-2H3 cells by Beaven and colleagues (Maeyama *et al.,* 1988; Ali *et al.,* 1989a,b) to further explore the role of isoprenoid pathway activity in mast cell signaling. We first confirm the ability of permeabilized cells to secrete in response to antigen and the γ-thio derivative of GTP (GTPγS), and we show for the first time that antigen and GTPγS also cause membrane ruffling and spreading in IgE-primed permeabilized cells. Further studies in the permeabilized cell model indicate that antigen-induced signaling in permeabilized cells occurs by a tyrosine kinase-coupled pathway that appears to be separate from the GTP-binding protein-coupled pathway activated by GTPγS. Our data strongly suggest that isoprenoid metabolism is required for the antigen-activated, tyrosine kinase-coupled signaling pathway.

Results

Characteristic properties of secretion from intact and SLO-permeabilized RBL-2H3 cells

The results in Table 1B confirm previous evidence (Ali *et al.,* 1989a,b) that dinitrophenol-conjugated bovine serum albumin (DNP-BSA) stimulates secretion from anti-DNP IgE-primed intact cells in Hanks'-BSA medium and from anti-DNP IgE-primed permeabilized cells in Pipes-glutamate medium with SLO (PG-SLO medium). Intact cells typically release approximately twice as much ^{3}H-serotonin as permeabilized cells in response to antigen. There was no secretion from cells incubated in PG buffer with Ca^{2+} but without SLO (data not shown). As noted by Maeyama *et al.* (1988), higher concentrations of DNP-BSA are required for maximum secretion from permeabilized than intact cells.

The amount of spontaneous secretion (Table 1A) is higher in permeabilized than intact cells. No clear explanation for this difference has been obtained. It is apparently not the result of increased levels of cytoplasmic Ca^{2+} in permeabilized cells, because the high levels of spontaneous secretion are maintained when cells are permeabilized in nominally Ca^{2+}-free PG-SLO medium (that contains 1–2 μM Ca^{2+}) and in nominally Ca^{2+}-free PG-SLO medium with 25 μM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (calculated to reduce free Ca^{2+} levels from 3 μM to <50 nM in medium prepared with 7 mM Mg^{+} at pH 7.0 and 37°C). In contrast with spontaneous release, antigen-stimulated secretion (Table 1B) is Ca^{2+}-sensitive in both intact and permeabilized cells. Antigen-induced secretion from intact cells is abolished simply by omitting Ca^{2+} from the Hanks'-BSA medium. Antigen-induced secretion from permeabilized cells persists in nominally Ca^{2+}-free PG-SLO medium but is inhibited by the addition of EGTA.

Table IC confirms that GTPγS stimulates secretion from permeabilized RBL-2H3 cells. The secretory response of cells in PG-SLO buffer to GTPγS is typically as great or greater than antigen-induced secretion. Results obtained in EGTA-containing media indicate that the Ca^{2+}

Table 1. Secretory properties of intact and permeabilized RBL-2H3 cells

Incubation condition	% ^{3}H-serotonin released in 20 min	
	Intact cells in Hanks'-BSA medium	Permeabilized cells in PG-SLO medium
A. Spontaneous release		
Complete medium	4.0 ± 1.5	20.0 ± 3.7
Nominally Ca^{2+}-free medium	3.7 ± 1.2	19.8 ± 0.9
Ca^{2+}-free medium + 25 μM EGTA	3.9 ± 1.4	20.0 ± 2.1
B. DNP-BSA-stimulated release[a]		
Complete medium	50.0 ± 2.3	19.8 ± 1.3
Nominally Ca^{2+}-free medium	0	21.1 ± 1.1
Ca^{2+}-free medium + 25 μM EGTA	0	0.6 ± 0.1
C. GTPγS-stimulated release[a]		
Complete medium	—	25.6 ± 0.9
Nominally Ca^{2+}-free medium	—	23.1 ± 1.7
Ca^{2+}-free medium + 25 μM EGTA	—	15.1 ± 0.8

IgE-primed, ^{3}H-serotonin-loaded cells were incubated for 20 min at 37°C in Hanks'-BSA medium (intact cells) or PG-SLO medium (permeabilized cells) with A, no additions; B, DNP-BSA (0.1 μg/ml in intact cells and 2 μg/ml in permeabilized cells); and C, 20 μM GTPγS. $CaCl_2$ was added (1.8 mM) to complete Hanks'-BSA medium, whereas 0.6 μM $CaCl_2$ was added to complete PG-SLO medium. Nominally Ca^{2+}-free medium contained 1–2 μM Ca^{2+}. Results are the average ± SEM of 3 experiments, each performed in duplicate.

[a] Note that the data for release stimulated by antigen (B) and GTPγS (C) are corrected for spontaneous release (given in A).

requirement for GTPγS-induced secretion from permeabilized cells is less than the Ca^{2+} requirement for antigen-induced secretion. Intact cells are impermeable, and so unresponsive, to GTPγS.

Antigen and GTPγS stimulate membrane responses in intact and permeabilized cells

It has been established that IgE receptor cross-linking activates a series of membrane and cytoskeletal responses in intact RBL-2H3 cells, including membrane ruffling, spreading and adhesion, fluid pinocytosis, and F-actin assembly (Pfeiffer *et al.*, 1985). Figure 1 compares the morphological responses of intact and permeabilized cells to cross-linking antigen and to GTPγS. The morphology of resting, SLO-permeabilized cells differs from intact cells only by a small reduction in the density of surface microvilli on some cells and the presence of more filopodia-like extensions from the cell bodies (compare Figure 1, A and B). The addition of antigen stimulates ruffling and spreading responses in both IgE-primed intact and SLO-permeabilized cells (Figure 1, C and D). The cells in Figure 1C were stimulated in Hanks'-BSA medium; intact cells could also spread and ruffle when stimulated with antigen in PG buffer lacking SLO (not illustrated). The membrane responses of permeabilized cells, like those of intact cells, do not depend on the presence of extracellular Ca^{2+} and in fact are not inhibited by concentrations of EGTA that abolish antigen-induced secretion (data not shown). As expected, the membrane-impermeant GTP analog, GTPγS, does not alter the morphology of intact cells (Figure 1E). However, GTPγS induces a Ca^{2+}-independent ruffling and spreading response in permeabilized cells (Figure 1F).

The effect of lovastatin on antigen- and GTPγS-stimulated secretion from intact and permeabilized RBL-2H3 cells

The results in the first column of Table 2 confirm previous evidence (Deanin *et al.*, 1991) that 20 h of incubation with 10 μM lovastatin essentially abolishes DNP-BSA-induced secretion from anti-DNP IgE-primed intact RBL-2H3 cells and that 4 h incubation with 0.1 mM MVA reverses the inhibition of secretion due to lovastatin. This inhibition of antigen-induced secretion persists in permeabilized cells. The results in the second column of Table 2 show that lovastatin inhibits antigen-induced secretion from permeabilized

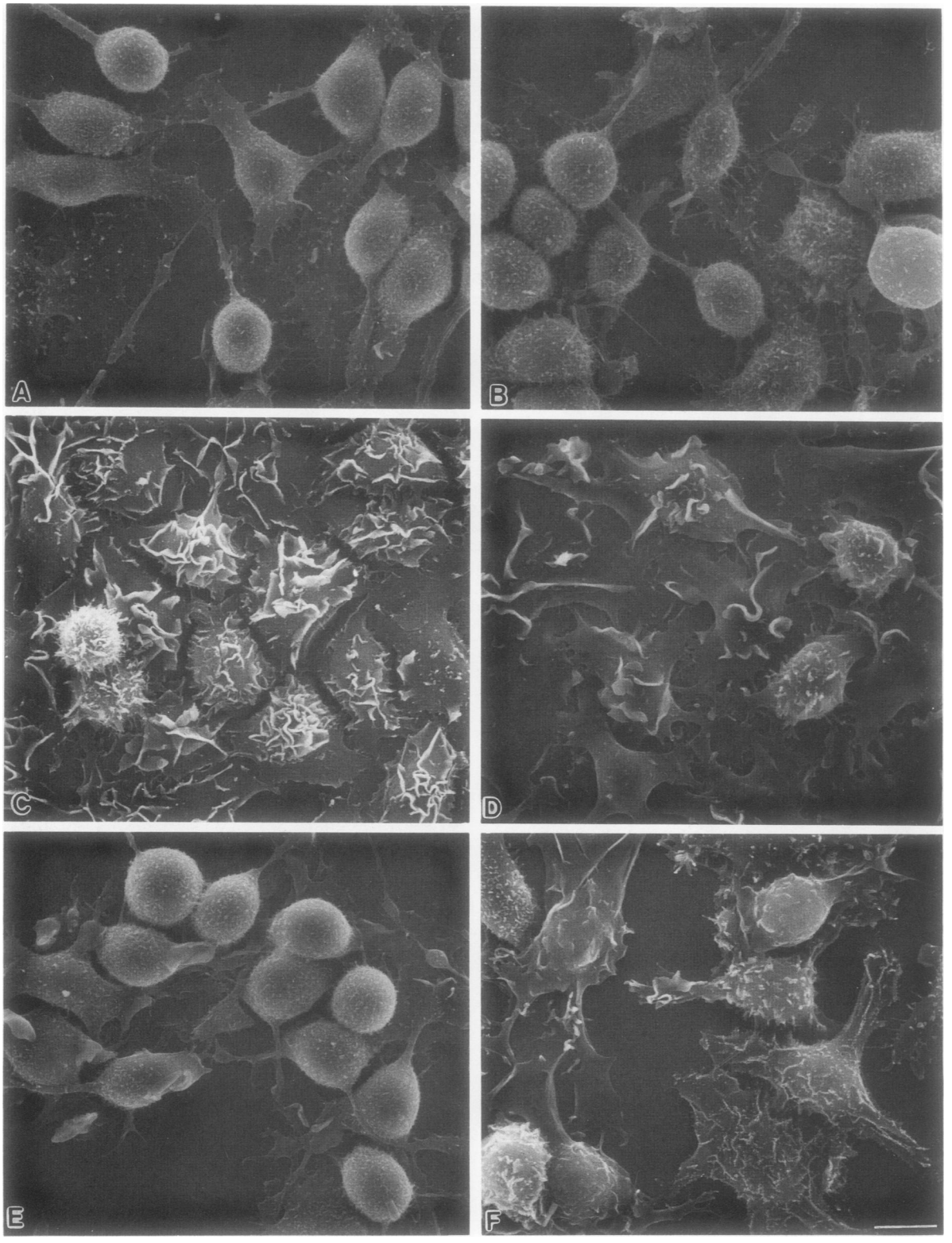


Figure 1. **Morphology of intact and permeabilized cells.** IgE-primed RBL-2H3 cells incubated for 10 min at 37°C in Hanks'-BSA medium (intact cells) without added stimuli display a microvillous surface architecture (A). They ruffle and spread in response to antigen (0.1 μg/ml DNP-BSA, 10 min; C) but show no morphological response to 10 min incubation with the impermeant nucleotide, GTPγS (20 μM; E). Cells incubated for 10 min in PG-SLO medium (permeabilized cells) are also microvillous in the absence of stimulation (B). Permeabilized cells ruffle and spread in response to 10 min incubation with antigen (2 μg/ml DNP-BSA; D) and GTPγS (20 μM; F). Bar = 10 μm.

Table 2. The effect of lovastatin on stimulated secretion from intact and permeabilized RBL-2H3 cells

Condition	% ^{3}H-serotonin release in 20 min	
	Intact cells in Hanks'-BSA medium	Permeabilized cells in PG-SLO medium
Control cells		
+DNP-BSA	43.7 ± 1.3	23.8 ± 1.4
+GTPγS	—	33.1 ± 2.7
Lovastatin-treated cells		
+DNP-BSA	1.0 ± 0.5	5.5 ± 1.0
+GTPγS	—	37.5 ± 3.7
MVA-treated cells		
+DNP-BSA	39.1 ± 2.1	24.2 ± 1.6
+GTPγS	—	33.8 ± 2.1
Lovastatin and MVA-treated cells		
+DNP-BSA	43.4 ± 1.0	22.6 ± 1.1
+GTPγS	—	34.4 ± 1.7

Cells were incubated for 20 h with 10 μM lovastatin. MVA (0.1 mM) was present during the last 4 h of incubation. IgE-primed ^{3}H-serotonin-loaded cells were resuspended to a concentration of ~1.5×10^6/ml in Hanks'-BSA medium and 1.8 mM $CaCl_2$ or PG medium with 0.6 μM $CaCl_2$, plus lovastatin and MVA as indicated. Cells in Hanks'-BSA medium were incubated at 37°C for 20 min with DNP-BSA (0.01 μg/ml). Cells in PG medium were incubated at 37°C for 20 min with SLO (0.2 U/ml), plus DNP-BSA (2 μg/ml) or GTPγS (20 μM). Data are corrected for spontaneous secretion (4.2 ± 0.4% in 20 min for intact cells and 22.2 ± 1.3% in 20 min for permeabilized cells) that is not altered by lovastatin or MVA treatment. Results are the average ± SEM of three separate experiments, each performed in duplicate. GTPγS has no effect in intact cells (—).

cells by >75%. It is also shown that secretion is fully restored by 4 h incubation of lovastatin-treated cells with MVA before permeabilization.

In contrast with antigen-induced secretion, pretreatment with lovastatin does not inhibit the secretory response of permeabilized cells to GTPγS (Table 2, second column).

The effect of lovastatin on antigen- and GTPγS-stimulated membrane responses in permeabilized cells

We showed before that lovastatin causes the rounding of intact RBL-2H3 cells, very likely through an effect on cytoskeletal organization that is separate from its effect on transmembrane signaling (discussed in Deanin *et al.*, 1991). We also demonstrated that lovastatin strongly inhibits antigen-induced ruffling and spreading. Figure 2 shows that lovastatin-treated permeabilized cells are similarly rounded (Figure 2A) and that the addition of antigen causes no ruffling and very little spreading (Figure 2B). Incubation of lovastatin-treated cells for 4 h with MVA before permeabilization restores the normal flattened morphology to resting cells (Figure 2D) and permits ruffling and spreading in antigen-treated cells (Figure 2E).

In contrast with their inability to ruffle and spread in response to antigen, lovastatin-treated cells form cell surface lamellae in response to GTPγS (Figure 2C). However, the spreading response of lovastatin-treated cells is significantly reduced in comparison with the membrane responses of either control (see Figure 1F) or lovastatin- and mevalonate-treated cells (Figure 2F) to GTPγS.

The effect of reagents that modulate GTP-binding protein- and tyrosine kinase-mediated signaling pathways on stimulated secretion in permeabilized RBL-2H3 cells

Two hypotheses were developed to explain the preferential inhibition by lovastatin of antigen-induced responses in permeabilized RBL-2H3 cells. The first hypothesis assumes that antigen and GTPγS both activate RBL-2H3 cells via a GTP-binding protein-coupled signaling pathway. In this case, we postulated that lovastatin may inhibit receptor-mediated GTP-binding protein activation without impairing the ability of directly activated GTP-binding proteins to elicit functional responses. The second hypothesis proposes that antigen and GTPγS activate separate pathways leading to transmembrane responses. In this case, we postulated that lovastatin inhibits only the antigen-stimulated pathway. Additional pharmacological studies were conducted to distinguish between these two explanations.

The results in Table 3 report the effect on antigen and GTPγS-induced signaling of the β-thio derivative of GDP (GDPβS), an inhibitor of GTP-binding protein-mediated signaling pathways. As expected for a GTP-binding protein-mediated pathway, secretion induced by GTPγS (20 μM) is 25% inhibited by 0.1 mM GDPβS, almost 50% inhibited by 0.2 mM GDPβS and >90% inhibited by 0.8 mM GDPβS. In contrast, antigen-induced secretion is essentially unaffected by 0.1 and 0.2 mM GDPβS and is <35% inhibited by 0.8 mM GDPβS. These results suggest either that antigen-induced secretion from permeabilized cells occurs by a pathway that is independent of GTP-binding proteins or that GDPβS cannot access the nucleotide-binding site of the receptor-coupled GTP-binding protein.

The effects of the tyrosine kinase inhibitors, genistein (4,5,7-trihydroxyisoflavone) (Akiyama

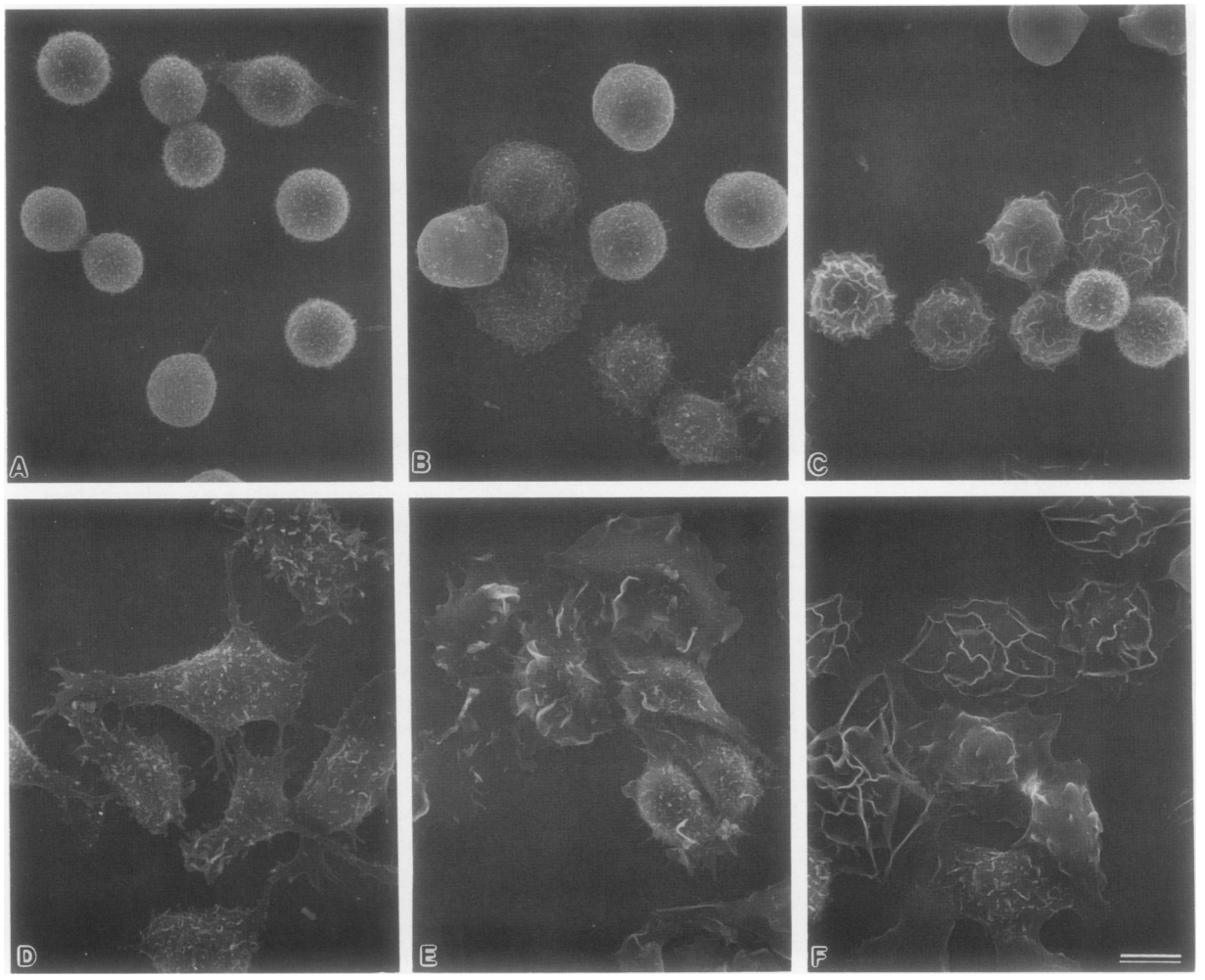


Figure 2. **Effects of lovastatin on the morphology of permeabilized RBL-2H3 cells.** RBL-2H3 cell monolayers were incubated with IgE and 10 μM lovastatin for 20 h. Where indicated, MVA (0.1 mM) was present during the last 4 h of incubation. The cells were rinsed in PG buffer and incubated for 10 min at 37°C in PG-SLO buffer containing no added stimulus (left column), 2 μg/ml DNP-BSA (center column), or 20 μM GTPγS (right column). Unstimulated lovastatin-treated cells are rounded (A). They show essentially no morphological response to antigen (B), but they ruffle in response to GTPγS (C). The presence of 0.1 mM MVA for 4 h restores the normal shape and surface topography of unstimulated, lovastatin-treated cells (D) and permits normal ruffling and spreading responses to antigen (E) and GTPγS (F). Bar = 10 μm.

et al., 1987), and the benzylidene malononitrile compounds, tyrphostins 8 and 11 (Yaish *et al.*, 1988; Levitski, 1990), on antigen- and GTPγS-induced secretion from SLO-permeabilized cells are reported in Table 4. Antigen-induced secretion is ~50% inhibited by 4 μM genistein and is essentially abolished by 20 μM genistein. In contrast, genistein has no effect on G protein-dependent secretion elicited by GTPγS. These results raise the possibility that antigen-induced secretion in permeabilized RBL-2H3 cells depends on the activation of a tyrosine kinase-coupled pathway. Consistent with this interpretation, tyrphostins 8 and 11 (25 μM) also inhibit antigen-induced secretion by >50% without affecting GTPγS-induced secretion. Higher tyrphostin concentrations were not used because of their unacceptably high quenching of ^{3}H-serotonin radioactivity.

In tyrosine kinase-coupled pathways, the effects of ligand-receptor interaction can often be mimicked by sodium orthovanadate (Na_3VO_4), whose activities include the inhibition of protein tyrosine phosphatases (Hunter, 1989). The results in Table 5 show that Na_3VO_4 causes a dose-dependent secretory response from RBL-2H3 cells. These results are consistent with the hypothesis that protein tyrosine phosphorylation may play an important role in the control of antigen-stimulated secretion in RBL-2H3 cells.

Table 3. GDPβS inhibits GTPγS- but not antigen- induced secretion from permeabilized cells

GDPβS concentration (mM)	% ^{3}H-Serotonin released in 20 min in response to: DNP-BSA (2 μg/ml)	GTPγS (20 μM)
0	24.3 ± 1.1	29.2 ± 1.2
0.02	23.9 ± 1.3	26.8 ± 0.9
0.05	23.1 ± 0.8	24.3 ± 1.1
0.10	23.8 ± 1.0	21.9 ± 0.8
0.20	20.0 ± 1.7	17.0 ± 0.4
0.40	16.9 ± 0.8	4.6 ± 0.09
0.80	16.5 ± 0.9	2.4 ± 0.1

IgE-primed, ^{3}H-serotonin-loaded cells were incubated for 20 min at 37°C in PG-SLO buffer with 0.6 μM $CaCl_2$ and various concentrations of GDPβS. Results, corrected for spontaneous release (20.0 ± 1.7% for 20 min), are the average ± SEM of 3 experiments, each performed in duplicate.

The effects of reagents that modulate GTP-binding protein- and tyrosine kinase-mediated signaling pathways on stimulated membrane responses in permeabilized RBL-2H3 cells

Figures 3 and 4 illustrate the effects of inhibitors of GTP-binding protein, tyrosine kinase, and tyrosine phosphatase activities on the ruffling and spreading responses of permeabilized cells to antigen and GTPγS. GDPβS and genistein alone do not alter the shape or surface topography of permeabilized cells (Figure 3, D and G). The micrographs in Figure 3, B and C confirm the ruffling and spreading responses of permeabilized cells to antigen and to GTPγS. It is also shown (Figure 3F) that GDPβS prevents the membrane responses to GTPγS, as expected for a GTP-binding protein-coupled pathway. In contrast, GDPβS does not prevent the membrane responses to antigen (Figure 3E). Conversely, genistein prevents the membrane responses to antigen (Figure 3H), as expected for a tyrosine kinase-coupled pathway, without affecting the responses to GTPγS (Figure 3I). In addition, the tyrosine phosphatase inhibitor, Na_3VO_4, stimulates a marked spreading response in permeabilized RBL-2H3 cells (Figure 4B), and it enhances the flattening and the formation of fewer, large ruffles on antigen-treated cells (compare Figure 4C with Figures 1D and 3B). These results support the hypothesis that antigen-induced membrane responses are controlled through a tyrosine kinase-coupled pathway that is separate from the GTP-binding protein-coupled pathway activated by GTPγS.

Table 4. Genistein and tyrphostins inhibit antigen- but not GTPγS-induced secretion from permeabilized cells

Inhibitor concentration (μM)	% ^{3}H-serotonin released in 20 min in response to: DNP-BSA (2 μg/ml)	GTPγS (20 μM)
A. Genistein		
0	19.6 ± 0.9	20.4 ± 1.1
4	9.9 ± 0.3	19.5 ± 0.8
20	1.2 ± 0.06	21.4 ± 1.3
40	0.5 ± 0.01	20.9 ± 0.6
B. Tyrphostin 8		
0	20.2 ± 0.4	24.2 ± 0.8
5	14.6 ± 1.2	25.8 ± 1.1
10	12.6 ± 0.4	24.7 ± 0.4
25	8.3 ± 0.4	25.6 ± 0.9
C. Tyrphostin 11		
0	20.6 ± 1.0	23.8 ± 2.0
5	15.6 ± 0.9	27.2 ± 1.9
10	12.5 ± 0.5	25.5 ± 1.8
25	8.7 ± 0.2	21.3 ± 0.9

The experiments were performed as described in the legend to Table 3. Results are corrected for spontaneous release (20.3 ± 2.1% in 20 min).

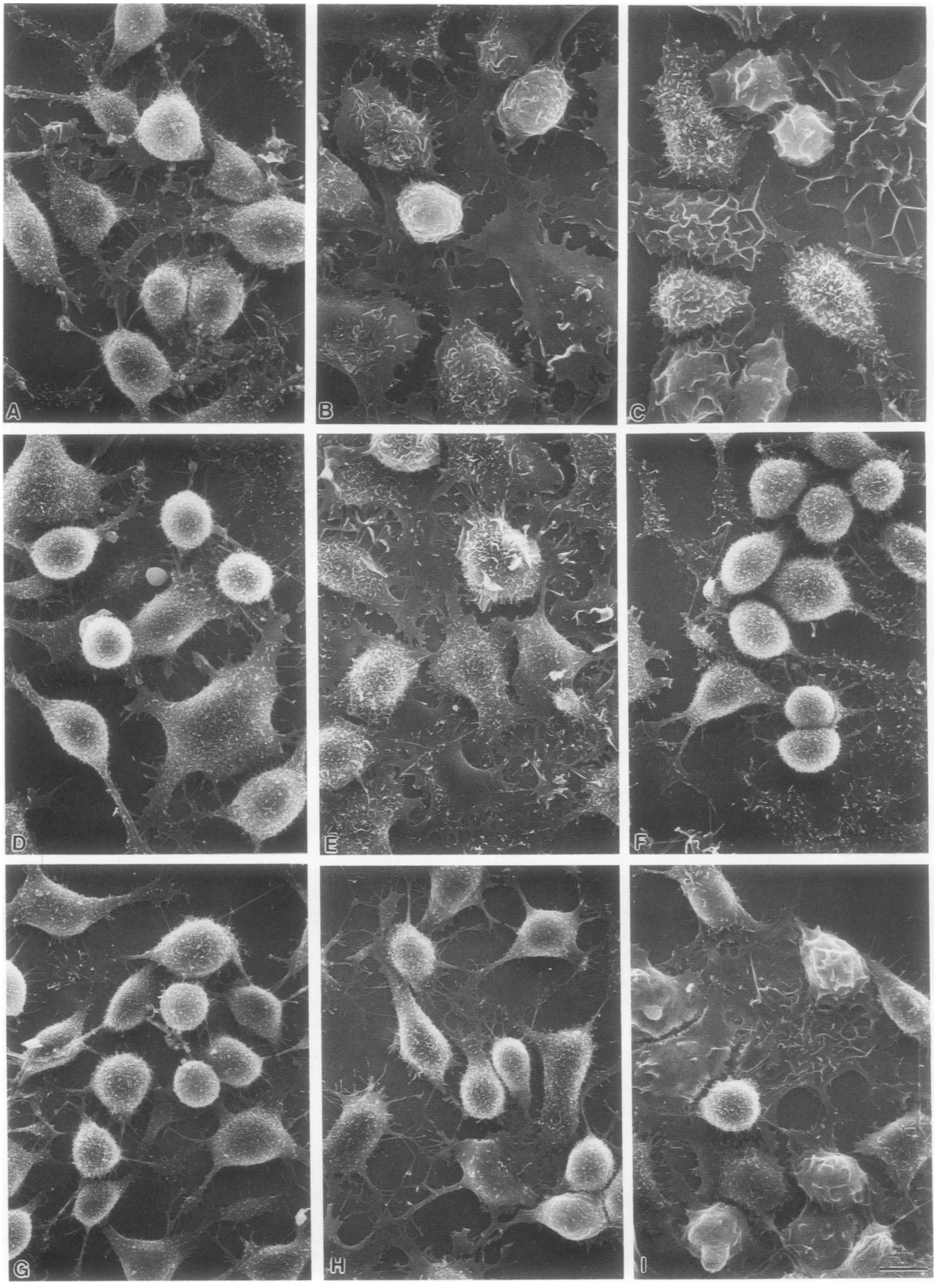
Discussion

We have proposed that the integrity of the isoprenoid pathway is essential for IgE receptor-mediated transmembrane signaling in intact RBL-2H3 cells (Deanin *et al.*, 1991). This hypothesis was based on evidence that blocking HMG CoA reductase with lovastatin inhibits antigen-stimulated Ca^{2+} influx, 1,4,5-IP_3 production, secretion, and membrane responses. We speculated that the inhibition occurred because isoprenoid metabolism is required for a post-synthetic modification of proteins, their isoprenylation, that typically occurs by the covalent

Table 5. Sodium orthovanadate stimulates secretion from permeabilized RBL-2H3 cells

Na_3VO_4 concentration (μM)	% ^{3}H-serotonin released in 20 min
25	2.3 ± 1.1
50	9.0 ± 0.9
100	13.8 ± 2.1
250	25.1 ± 1.7
500	29.5 ± 1.3

The experiments were performed as described in the legend to Table 3. Results are corrected for spontaneous release (21.6 ± 1.5% in 20 min.)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binding of farnesyl or geranylgeranyl groups to C-terminal cysteine residues on specific proteins. The known substrates for this post-synthetic modification include, but are not restricted to, the $p21^{ras}$ family of small GTP-binding proteins, the non-*ras* small GTP-binding protein, G25K, the γ subunit of the heterotrimeric GTP-binding proteins, and the nuclear envelope proteins, lamins A and B (reviewed in Glomset *et al.,* 1990; Goldstein and Brown, 1990; Maltese, 1990). Because antigen-induced Ca^{2+} influx occurs by a GTP-dependent pathway (Wilson *et al.,* 1989), we proposed that a GTP-binding protein linked to Ca^{2+} transport is one likely substrate for isoprenylation in intact cells. We speculated that lovastatin may inhibit PLC activation and other antigen-activated responses at least in part by inhibiting the isoprenylation and function of additional proteins involved in receptor-activated transmembrane signaling.

In the present study, we used the signaling-competent permeabilized cell system developed by Beaven and colleagues (Maeyama *et al.,* 1988; Ali *et al.,* 1989a,b; Ludowyke *et al.,* 1989) to explore further the role of isoprenoid pathway intermediates in IgE receptor-mediated signaling. Preliminary studies to characterize the antigen-induced secretory response of permeabilized cells revealed a higher rate of spontaneous ^{3}H-serotonin release and a lower rate of antigen-stimulated release than is observed in intact cells. We also confirmed that GTPγS induces secretion from permeabilized RBL-2H3 cells. In addition, we showed for the first time that permeabilization does not significantly alter RBL-2H3 cell shape or surface topography and that both the antigen-induced transition of the cell surface from a microvillous to a lamellar topography and the antigen-induced cell spreading response described in intact cells (Pfeiffer *et al.,* 1985) are reproduced in permeabilized RBL-2H3 cells. It was established that GTPγS elicits ruffling and spreading responses in permeabilized cells. Thus, the direct activation of GTP-binding proteins can stimulate membrane as well as secretory responses in SLO-permeabilized RBL-2H3 cells.

Despite the overall similarity of the functional responses of permeabilized RBL-2H3 cells to antigen and GTPγS, the effects of lovastatin were different for the two stimuli. Specifically, lovastatin inhibits antigen-stimulated secretion and membrane responses in SLO-permeabilized cells. In contrast, lovastatin-treated, permeabilized cells secrete at control levels in response to GTPγS. Lovastatin-treated cells are also capable of ruffling in response to GTPγS, although their spreading responses are reduced in comparison with control permeabilized cells, very likely because lovastatin has an additional target of action among the cytoskeletal proteins that maintain cell shape (discussed in Deanin *et al.,* 1991). Two hypotheses were developed to explain the persistence of the GTPγS-activated responses in lovastatin-treated, permeabilized RBL-2H3 cells. The first assumed that antigen and GTPγS both activate permeabilized RBL-2H3 cells by GTP-binding protein-coupled pathways. In this case, we supposed that isoprenoid metabolism may be required for receptor-mediated GTP-binding protein activation but not for directly activated GTP-binding proteins to elicit functional responses. This hypothesis was attractive because the known protein substrates for isoprenylation include a range of GTP-binding proteins and GTP-binding protein subunits. The second hypothesis was that GTPγS and antigen may activate separate pathways leading to functional responses in permeabilized RBL-2H3 cells.

The results of studies with the GDP analog, GDPβS, that stabilizes GTP-binding proteins in their inactive form, supported the hypothesis that GTPγS and antigen use separate signaling pathways. We first established that concentrations of GDPβS that strongly inhibit GTPγS-induced secretion cause only a small inhibition of antigen-induced secretion. We also established that concentrations of GDPβS that abolish GTPγS-induced membrane responses have little effect on antigen-induced ruffling and spreading. The simplest explanation for these data is that antigen-induced signal transduction in permeabilized RBL-2H3 is not dependent on GTP-binding protein activation. Our results are

Figure 3. **The effect of GTP-binding protein and tyrosine kinase inhibitors on the morphology of resting and stimulated permeabilized cells.** IgE-primed cell monolayers were incubated for 10 min in PG-SLO medium in the absence of added stimuli or in the presence of 2 μg/ml DNP-BSA or 20 μM GTPγS. Inhibitors (0.2 mM GDPβS and 40 μM genistein) were present as indicated. Permeabilized cells have a microvillous surface topography before stimulation (A). They ruffle and spread in response to 10 min incubation with antigen (B) or GTPγS (C). GDPβS does not alter the morphology of unstimulated cells (D) or affect the membrane responses to antigen (E). However, GDPβS abolishes GTPγS-induced ruffling and spreading (F). The addition of genistein also has no effect on the morphology of resting cells (G). Genistein inhibits antigen-induced ruffling and spreading (H), but does not impair the ruffling and spreading responses to GTPγS (I). Bar = 10 μm.

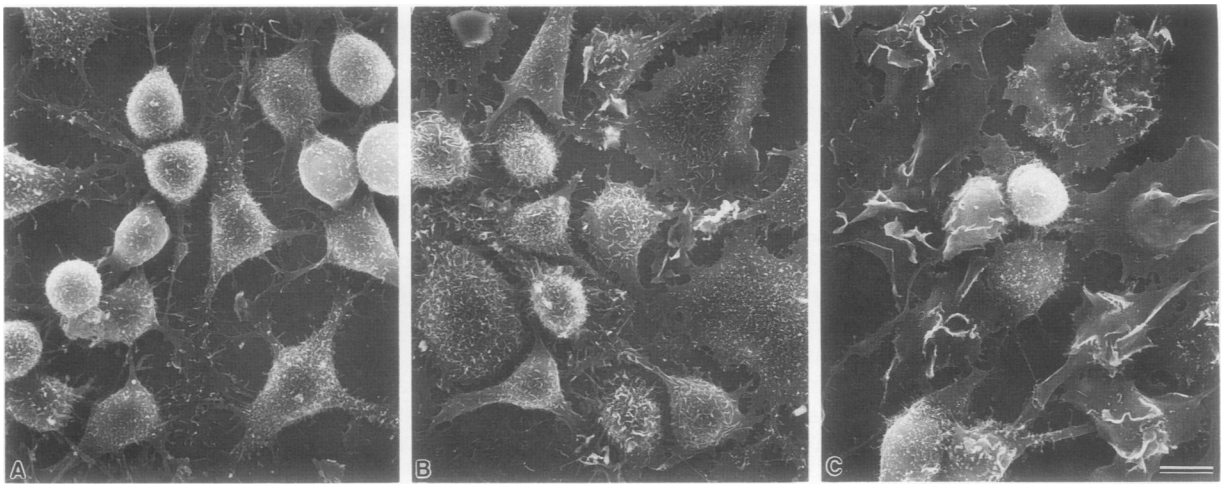

Figure 4. **The effect of sodium orthovanadate on cell morphology.** IgE-primed, permeabilized cells were incubated for 10 min in PG-SLO medium with no additions (A), with 200 μM Na_3VO_4 (B), and with Na_3VO_4 plus 2 μg/ml DNP-BSA (C). Na_3VO_4 causes a marked increase in cell spreading and it potentiates the flattening of antigen-treated cells. Bar = 10 μm.

not in agreement with Ali *et al.* (1989b), who reported that antigen-induced PLC activation and secretion in RBL-2H3 cells are more sensitive to inhibition with GDPβS than GTPγS-induced responses. However, they are in complete agreement with Saito *et al.* (1989), who found that concentrations of high-pressure liquid chromatography-purified GDPβS that significantly impair GTPγS-induced secretion from ATP-permeabilized primary mast cells have little effect on antigen-induced secretion.

Previously, Quarto and Metzger (1986) showed that purified IgE-receptor complexes contain an associated, soluble tyrosine kinase activity that can phosphorylate tyrosine residues on the β and γ chains of the receptor. In addition, Benhamou *et al.* (1990) demonstrated recently that antigen binding stimulates the phosphorylation on tyrosine residues of several RBL-2H3 cell proteins, the most prominent having a mass of 72 kDa. We therefore tested the ability of tyrosine kinase and tyrosine phosphatase inhibitors to alter antigen- and GTPγS-induced responses in SLO-permeabilized RBL-2H3 cells. It was found that the tyrosine kinase inhibitor, genistein, abolishes both antigen-stimulated secretion and antigen-induced membrane responses in SLO-permeabilized RBL-2H3 cells at concentrations that had no inhibitory effect on GTPγS-induced responses. Concentrations of the tyrosine kinase inhibitors, tyrphostins 8 and 11, that reduce antigen-induced secretion by ~50% in permeabilized cells also have no inhibitory effect on GTPγS-induced secretion. In addition, it was found that Na_3VO_4, whose activities include the inhibition of tyrosine phosphatase activity, stimulates secretion and spreading in RBL-2H3 cells. Although genistein, tyrphostins, and Na_3VO_4 may each affect cellular functions unrelated to tyrosine phosphorylation, the sum of these results are consistent with the hypothesis that the activation of a tyrosine kinase-coupled pathway is an essential event leading to antigen-stimulated secretion and membrane responses in SLO-permeabilized RBL-2H3 cells. This pathway appears to be separate from the GTPγS-activated (GTP-binding protein-dependent) pathway that also results in secretion and morphological responses in RBL-3H3 cells. Strikingly similar results were presented by Verheijden *et al.* (1990) based on studies of the regulation of epidermal growth factor (EGF)-induced PLC activation in permeabilized 3T3 cells. In this study, GTPγS enhanced PLC activation in response to the mitogenic phospholipid, lysophosphatidic acid, but not in response to EGF. Conversely, tyrosine kinase inhibitors blocked EGF-induced PLC activation without altering the response to lysophosphatidic acid and GTPγS. It was concluded that a GTP-binding protein-coupled pathway to PLC activation exists in 3T3 cells but that EGF activates PLC through the activation of a separate tyrosine kinase-coupled pathway.

When the present work was begun, it was known that GTP-binding proteins and GTP-binding protein subunits are important substrates for isoprenylation and that their localization and function is impaired in lovastatin-treated cells (reviewed in Glomset *et al.*,

1990; Goldstein and Brown, 1990; Maltese, 1990). It was also known that lovastatin inhibits antigen-induced responses in intact cells at least in part by inhibiting the GTP-binding protein-mediated Ca^{2+} influx pathway (Deanin *et al.*, 1991). These results led us to expect that the IgE receptor-mediated activation of permeabilized RBL-2H3 cells would be mediated through additional GTP-binding proteins that depended on isoprenylation for function. We found instead that antigen activates secretory and membrane responses in permeabilized cells by a tyrosine kinase-coupled signaling pathway. The inhibition of this pathway by lovastatin provides the first indication that protein isoprenylation may play an essential role in tyrosine kinase-mediated signaling. We also found that lovastatin does not inhibit the GTP-binding protein-dependent signaling pathway that is activated by GTPγS in permeabilized cells. This indicates either that isoprenylation is not essential to elicit functional responses to directly activated GTP-binding proteins or that the turnover time of these GTP-binding proteins exceeds 20 h, so that the lovastatin treatment used here is insufficient to inhibit their function.

Further work is required to identify the tyrosine kinase that is activated by IgE receptor cross-linking in RBL-2H3 cells. None of the subunits of the IgE receptor contains a tyrosine kinase domain (Blank *et al.*, 1989). However, this is also true of receptors on T and B cells that nevertheless appear to activate tyrosine kinase-coupled pathways (Veillette *et al.*, 1989a; Gold *et al.*, 1990; June *et al.*, 1990; Mustelin *et al.*, 1990; Lane *et al.*, 1991). In the case of T cells, it has been shown that two members of the *src* family of soluble kinases, *lck* and *lyn* are associated with specific receptors (CD4, CD8, and CD3) and are activated by receptor cross-linking (Mustelin *et al.*, 1989; Veillette *et al.*, 1989b; Samelson *et al.*, 1990). In B cells, there is evidence that the *lyn* gene product is a tyrosine kinase that is physically associated with membrane immunoglobulin (mIg) and is activated in response to mIg cross-linking (Yamanashi *et al.*, 1991). We speculate that the soluble tyrosine kinase activity that copurified with IgE-receptor complexes in the study by Quarto and Metzger (1986) may also be a *src* family kinase that interacts with a component of the IgE receptor of RBL-2H3 cells and may be responsible for the antigen-stimulated protein tyrosine phosphorylation reported by Benhamou *et al.* (1990).

Work is also needed to determine how lovastatin inhibits tyrosine kinase-mediated cell activation. The results of recent experiments (unpublished studies with Drs. W. Li, B. Margolis, and J. Schlessinger, Department of Pharmacology, New York University Medical Center) have indicated that lovastatin treatment does not abolish the antigen-stimulated tyrosine phosphorylation of a group of at least 10 RBL-2H3 cell proteins, including the 72-kDa protein described by Benhamou et al. (1991). We are therefore testing two hypotheses: first, that isoprenylation is required for the phosphorylation of one or a few tyrosine kinase substrates that are not among the more prominent protein bands and second, that signal transduction may depend on both the isoprenylation and phosphorylation of certain proteins. The known substrates for tyrosine kinase-mediated phosphorylation include PLCγ, phosphatidylinositol 3-kinase, the $p74^{raf}$ serine/threonine kinase GTPase-activating protein (GAP), the protein that controls the activity of $p21^{ras}$, and the non-*ras* GTP-binding protein, G25K (Anderson *et al.*, 1990; Hart *et al.*, 1990; Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). At least two of these proteins, G25K (Maltese and Sheridan, 1990) and $p21^{ras}$ (Hancock *et al.*, 1989) are isoprenylated in vivo.

In summary, the results of pharmacological studies have indicated that permeabilized RBL-2H3 cells can be activated via at least two signaling pathways; one, linked to the IgE receptor that is coupled to tyrosine kinase activation and another, stimulated by GTPγS, that involves GTP-binding protein activation. The isoprenoid pathway inhibitor, lovastatin, preferentially inhibits functional responses mediated through the tyrosine kinase-coupled pathway. Experiments are in progress to determine the effect of lovastatin on antigen-induced protein tyrosine phosphorylation and to identify receptor-associated tyrosine kinases and isoprenylated proteins in RBL-2H3 cells. Experiments are also in progress to determine the relationship between the lovastatin-sensitive tyrosine kinase-activated signaling events revealed here and the lovastatin-sensitive GTP-binding protein-dependent Ca^{2+} influx pathway revealed before by studies in intact cells (Wilson *et al.*, 1989; Deanin *et al.*, 1991).

Methods

Reagents

Monoclonal anti-DNP IgE was prepared from the ascites of mice bearing the HI-DNP-ε-26-82 hybridoma (Liu *et al.*, 1980) using the methods of Holowka and Metzger, 1982. ^{3}H-serotonin (5-[1,2-^{3}H(N)]hydroxytryptamine binoxalate) (24.1 Ci/mmol) was from Dupont/NEN, Boston, MA. DNP-BSA and rhodamine-phalloidin were from Molecular Probes Inc.,

Junction City, OR; GTPγS, GDPβS, MVA lactone, and Na_3VO_4 were from Sigma Chemicals, St. Louis, MO. SLO was from Wellcome Reagents Ltd., Beckenham, U.K. Genistein was from ICN, Cleveland, OH. Yaish *et al.* (1988) described the synthesis of a range of inhibitors of the mitogen receptor tyrosine kinases (tyrphostins); the inhibitors used here, tyrphostin 8 (K_i for EGFRK = 11 μM) and tyrphostin 11 (K_i for EGFRK = 2.3 μM), were generously provided by Dr. C-K Huang, University of Connecticut Health Center. Genistein and tyrphostins were dissolved in dimethyl sulfoxide (DMSO). Before use, they were diluted in incubation buffers so that the final DMSO concentration during assay was no >0.5%; this amount of DMSO did not affect the secretory or membrane responses to antigen or GTPγS. Lovastatin was a generous gift from Mr. Alfred Alberts of Merck, Sharp and Dohme, Rahway, NJ. It was solubilized as described by Kita *et al.* (1980) and added to cell cultures at 10 μM for 20 h before harvest and functional analyses. All aqueous solutions were prepared in ultrafiltered water containing between 1 and 2 μM Ca^{2+} as determined by direct measurement using a Ca^{2+} electrode.

Cells

RBL-2H3 cells were grown at 37°C in a 5% CO_2 incubator in Eagle's minimal essential medium (MEM) plus penicillin, streptomycin, and supplementary L-glutamine (Gibco, Grand Island, NY) with 15% Hybrimax CPSR-3 serum replacement (Sigma Chemical Co). The cells were passaged twice weekly by trypsinization, with selection for adherent cells. Because secretory activity typically decreases with passage, cells were discarded and new stocks thawed after 10–12 passages. This variability is demonstrated in the somewhat different values for % ^{3}H-serotonin release measured in 20 min between the groups of experimental results reported in Tables 1–5.

For experiments, cells were replated onto bacterial grade Petri plates in MEM with 15% fetal calf serum (Hyclone, Logan, UT), incubated overnight with 1 μg/ml anti-DNP IgE, and harvested by scraping with a rubber policeman. Glass-adherent cell monolayers were prepared by plating cells into Petri plates containing 15-mm glass coverslips and incubating overnight with IgE. For secretion assays, ^{3}H-serotonin (1 μCi/ml) was also added to the medium overnight.

Permeabilization

Permeabilized cells were prepared by a modification of methods developed by Beaven and colleagues (Maeyama *et al.*, 1988; Ali *et al.*, 1989a,b; Ludowyke *et al.*, 1989). Briefly, cell suspensions or cell monolayers were washed in buffer containing 20 mM Pipes, pH 7.0, 138 mM potassium glutamate, 4.8 mM glucose, 7.0 mM $MgCl_2$, and 5 mM ATP (PG buffer). Cell suspensions (~1.5×10^6 cells/ml) were permeabilized by the addition of 0.2 U/ml SLO (PG-SLO) plus sufficient $CaCl_2$ to increase the final Ca^{2+} concentration by 0.6 μM (unless otherwise indicated). Cell monolayers were permeabilized by the addition of 100 μl of PG buffer containing 0.1–0.15 U/ml SLO with or without 0.6 μM $CaCl_2$. Although high SLO doses can make large (up to 30 nm diameter) pores in erythrocyte membranes (Bhakdi *et al.*, 1985), the lesions formed in response to the small amounts of SLO used here were barely permeable to rhodamine-phalloidin (mw 1–1.2 kDa) and were not visible by either scanning or transmission electron microscopy at magnifications up to 20,000×. Beaven and colleagues remove the SLO after 5 min incubation; we obtain a constant rate of spontaneous secretion and more antigen-induced secretion over 20 min in the continuous presence of SLO.

Secretion assays

Secretion from intact cells was measured as previously described (Pfeiffer *et al.*, 1985; Wilson *et al.*, 1989). IgE-primed, ^{3}H-serotonin-loaded cells were washed once in modified Hanks' buffer (Becker, 1972) with 0.05% (Hanks'-BSA), and resuspended to a concentration of 1.75×10^6 cells/ml in Hanks'-BSA buffer. Duplicate portions (0.2 ml) of the cells were incubated with or without antigen (0.1 μg/ml DNP-BSA) at 37°C for times varying from 0 min (the blank value) to 20 min. Secretion was stopped by the addition of 0.5 ml cold phosphate-buffered saline, the samples were centrifuged to pellet the cells, and the radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting. The data are reported as percent of the total radioactivity, determined by lysing an aliquot of the cells with Triton-X-100 detergent. 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.

Secretion from permeabilized cells was measured by the same assay except that the cells were washed in PG buffer and incubated in PG-SLO plus $CaCl_2$ (0.6 μM, except as noted), with and without antigen (2 μg/ml DNP-BSA) or 20 μM GTPγS.

Scanning electron microscopy

IgE-primed RBL-2H3 cell monolayers were incubated at 37°C in Hanks'-BSA medium (intact cells) or PG-SLO buffer (permeabilized cells) plus antigen or GTPγS for various times. They were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and processed for scanning electron microscopy as described in Pfeiffer *et al.* (1985). The surface morphology of carbon-coated cells was observed with an Hitachi S800 SEM equipped with a field emission tip. The routine accelerating voltage was 15 kV. Conclusions about cell morphology were based on examination of ≥500 cells per experimental condition, where >90% of cells shared the same general shape and cell surface architecture. Results are presented in the form of representative micrographs.

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