FcεRI-induced Protein Tyrosine Phosphorylation of pp72 in Rat Basophilic Leukemia Cells (RBL-2H3)

EVIDENCE FOR A NOVEL SIGNAL TRANSDUCTION PATHWAY UNRELATED TO G PROTEIN ACTIVATION AND PHOSPHATIDYLINOSITOL HYDROLYSIS*

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Recently, we demonstrated that aggregation of the high affinity IgE receptor in rat basophilic leukemia (RBL-2H3) cells results in rapid tyrosine phosphorylation of a 72-kDa protein (pp72). Here we investigated the relationship of pp72 phosphorylation to guanine nucleotide-binding protein (G protein) activation and phosphatidylinositol hydrolysis. The activation of G proteins by NaF in intact cells or by guanosine 5'-O-(3-thiotriphosphate) in streptolysin O-permeabilized cells induced both phosphatidylinositol hydrolysis and histamine release without tyrosine phosphorylation of pp72. Similarly, in RBL-2H3 cells expressing the G protein-coupled muscarinic acetylcholine receptor, carbachol activated phospholipase C and induced secretion without concomitant pp72 phosphorylation. Therefore, pp72 phosphorylation was not induced by G protein activation or as a consequence of phosphatidylinositol hydrolysis. To investigate whether pp72 tyrosine phosphorylation precedes the activation of phospholipase C, we studied the effect of the tyrosine kinase inhibitor genistein. Preincubation of cells with genistein decreased, in parallel, antigen-induced tyrosine phosphorylation of pp72 (IC50 = 34 μg/ml) and histamine release (IC50 = 31 μg/ml). However, genistein at concentrations of up to 60 μg/ml did not inhibit phosphatidylinositol hydrolysis nor did it change the amount of the secondary messenger inositol (1,4,5)-triphosphate. Previous observations showed that there was no pp72 tyrosine phosphorylation after activation of protein kinase C or after an increase in intracellular calcium. Taken together, these results suggest that pp72 tyrosine phosphorylation represents a distinct, independent signaling pathway induced specifically by aggregation of the FcεRI.

Aggregation of the high affinity receptor (FcεRI)† for immunoglobulin E on mast cells, basophils, and related cultured cell lines such as rat basophilic leukemia (RBL-2H3) cells, initiates a number of biochemical events leading to degranulation (1-5). These include increased phospholipase C (PI-PLC) activity resulting in phosphatidylinositol (PtdIns) hydrolysis, stimulation of phospholipase A2 and D, a rise in intracellular free calcium concentration, increased calcium influx into the cell, and membrane depolarization. The hydrolysis of PtdIns correlates with the number of aggregated receptors and the nature of the cross-linking agent (4, 5). Activation of PI-PLC results in generation of inositol 1,4,5-triphosphate and 1,2-diacylglycerol, that induce, respectively, the release of intracellular calcium from endocytosomal stores and the activation of protein kinase C (6, 7). These secondary messengers are thought to initiate a cascade of biochemical events leading to the release of histamine and other mediators from RBL-2H3 cells.

Similar to other transmembrane receptors, FcεRI activation may regulate intracellular second messengers through intermediate guanine nucleotide-binding (G) proteins (8). Some G, subunits are substrates for ADP-ribosylation by cholera toxin or pertussis toxin which modify receptor-G protein or G protein-effector coupling (9). In RBL-2H3 cells, pertussis toxin does not inhibit secretion whereas cholera toxin enhances the FcεRI-induced formation of inositol phosphates, influx of calcium, and histamine release (10, 11). A G protein is also probably involved in the phospholipase A2 stimulation in RBL-2H3 cells (12). In rat mast cells, patch-clamp studies suggest that the G protein that activates PI-PLC and results in a calcium response is different from the G protein that mediates degranulation (13). The use of nonhydrolyzable GTP analogues such as guanosine 5'-O-(3-thiotriphosphate) (GTPγS) has suggested that the coupling of receptors to PI-PLC involves a G protein (14). In RBL-2H3 cells stimulation of G proteins by GTPγS or aluminum fluoride complexes results in PtdIns hydrolysis and histamine release. In addition, blocking of G proteins by guanosine 5'-O-(2-thiodiphosphate) (GDPβS) or depletion of GTP by mycophenolic acid inhibit FcεRI-mediated cell activation (15, 16).

Recently, we have shown that aggregation of the FcεRI in RBL-2H3 cells induces rapid protein tyrosine phosphorylations, the most prominent being that of a 72-kDa protein (pp72) (17). This phosphorylation correlated with both the...
time course and antigen dose for histamine release. Protein kinase C activation by phorbol ester or an increase of the intracellular calcium concentration by ionophore A23187 did not induce pp72 tyrosine phosphorylation. Furthermore, in the absence of extracellular calcium there was FcRI-mediated pp72 phosphorylation but not dephosphorylation.

The purpose of the present study was to investigate relationships among FcRI-mediated protein-tyrosine phosphorylation of pp72, activation of G proteins, and PtdIns hydrolysis. Our findings revealed that activation of G proteins by several means resulted in PtdIns hydrolysis and secretion or the formation of inositol 1,4,5-triphosphate. Taken together with previous observations, our results suggest that pp72 phosphorylation represents a distinct, independent signaling pathway induced specifically by aggregation of the FcRI.

EXPERIMENTAL PROCEDURES

Materials—Pipes, carbocerh, GTP-S, sodium fluoride, aluminum fluoride, trichlorotrifluoroethane, and tri-n-octylamine were obtained from Sigma. The tyrosine kinase inhibitor genistein was purchased from Calbiochem (La Jolla, CA). Materials for polycrylamide electrophoresis were from Novex (Las Encinitas, CA). Dowex AG 1-X8 resin was purchased from Bio-Rad. All other materials were obtained as described previously (17).

Expression of Human Subtype 3 Muscarinic Acetylcholine Receptors (m3-mAcR) in RBL-2H3 Cells—A retrovirus vector approach was utilized to transfer the human m3-mAcR gene into RBL-2H3 cells. Briefly, NIH/3T3 cells were transfected with DNA constructs in which the m3-mAcR cDNA sequence would be expressed in eukaryotic cells, at the direction of the Moloney leukemia virus long terminal repeat (18). Isolation and characterization of such transfectants have been described (19). Transfectants were infected with AP129 retrovirus (20). Cell culture supernatants were titered for recombinant virus by colony formation in NIH/3T3 cells. RBL-2H3 cells were infected with 105 colony-forming units of viral stocks in the presence of 5 μg/ml of polybrene. Individual colonies selected on the basis of their resistance to the neomycin analogue G418 (Geneticin) (200 μg/ml) were isolated with the aid of cloning cylinders and examined for receptor expression by binding of a labeled nonselective mAChR agonist as described (19). Cells, Antibodies, and Antigens—RBL-2H3 cloned subline of rat basophilic leukemia cells and the 2H3-7 cell clone that expressed 600 pmol of N-methyisocapolin-binding sites/mg protein were maintained in Eagle's minimum essential medium with Earle's salts (EMEM) (GIBCO) supplemented with 15% heat-inactivated fetal calf serum (Biofluids Inc., Rockville, MD), penicillin at 100 units/ml, streptomycin at 100 μg/ml, gentamicin at 0.25 μg/ml (GIBCO), and 2 mM glutamine (Biofluids Inc.) in 5% CO2 at 37 °C in 150 cm2 tissue culture flasks (21). Anti-trinitrophenyl (TNP) monoclonal mouse IgE was isolated as described previously (22). 2,4-Dinitrophenyl conjugated to human serum albumin (DNPA-HSA) was purchased from Calbiochem.

Cell Stimulation and Histamine Release Assay—Cell stimulation experiments were performed in 6-well cluster plates (Costar, Cambridge, MA). Cells were plated at 1 × 106/35-mm diameter well in 2 ml of culture medium containing mouse monoclonal anti-TNP IgE ascites (1:15,000 dilution) where indicated. After overnight culture at 37 °C and 5% CO2, cell monolayers were washed twice with Pipes-buffered saline (Pipes, 25 mM; NaCl, 125 mM; KCl, 2.7 mM; glucose, 5.5 mM; CaCl2, 1 mM; bovine serum albumin (BSA), 0.1%; pH 7.4). The cells were then stimulated with either antigen or various agents at the indicated concentrations. After stimulation with NaF cells were incubated with 5 μM AIFC in Pipes-buffered saline for 10 min, and NaF was added subsequently to give the stated final concentrations. 2H3-m7 cells, that expressed the gene for the human m3-mAcR, were also stimulated with the stable acetycholin agonist carbascarb. In experiments with the protein tyrosine kinase inhibitor genistein, cells were cultured overnight, washed, and incubated with either genistein or its solvent (dimethyl sulfoxide, MeSO) at the indicated concentrations. After 30 min at 37 °C, supernatants were discarded, and cells were treated with anti-genistein or MeSO at the indicated final concentration. All reactions were in triplicate. Total histamine content was measured by the addition of perchloric acid to the washed cell layer in some wells. Histamine release as well as total histamine was measured by fluorometric assay (23). Percent histamine release is expressed as a percent of the total amount of histamine present in unstimulated cultures. Corresponding cell pellets were analyzed for phosphotyrosine-containing proteins.

In some experiments, 2H3-m3.7 cells were stimulated in the absence of extracellular calcium. Cells were washed twice in calcium-free, spinner-modified minimum essential medium with Earle's salts (Biofluids Inc., Rockville, MD) containing 10 mM Hepes, 0.1% BSA, and 4 mM EDTA or in EMEM containing 10 mM Hepes and 0.1% BSA. The cells were then stimulated, respectively, in spinner-modified minimum essential medium containing 10 mM Hepes, 0.1% BSA, and 50 μM EDTA or in EMEM containing 10 mM Hepes and 0.1% BSA for 30 min. The phosphotyrosine-containing proteins were then analyzed as described.

Immunoblotting of Proteins with Anti-Phosphotyrosine Monoclonal Antibodies—After stimulation the supernatants were removed for histamine measurements and cells were lysed by the addition of 200 μl of lysis buffer/106 cells (phosphate buffer containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 50 μg/ml leupeptin (Boehringer Mannheim), 0.5 unit/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% NaN3). Insoluble material was removed by centrifugation at 12,000 rmp for 20 min. Protein concentration of the supernatants was determined by the Micro-BCA assay (Pierce Chemical Co.). After addition of 25 μg of mouse polyacrylamide gel-separated lysate proteins to a 10% polyacrylamide gel (40 μg/lane) and electrotransferred onto nitrocellulose membrane as previously described (17). The free binding sites were blocked by incubating the membrane for 4 h with Tris-buffered saline, 0.05% Tween 20, and 5% BSA. Then, the nitrocellulose membranes were incubated with 20 ng/ml of the antiphosphotyrosine monoclonal antibody PY 20 coupled to horseradish peroxidase (ICN Immunobiologicals, Lisle, IL) diluted in Tris-buffered saline, 0.05% Tween 20, and 0.5% BSA for 2 h at room temperature. After extensive washing of the membranes, the phosphoproteins were visualized using the Enhanced Chemiluminescence kit (Amersham Corp.) according to the manufacturer's recommendations. The nitrocellulose membranes were exposed to Kodak X-AR film for 15 s to 1 h. This immunoblotting technique proved to be more sensitive than the one we used previously (17) allowing the detection of a number of additional tyrosine phosphoprotein bands that had not been observed formerly following cell stimulation (see "Results" and footnote 2). However, as had been observed previously pp72 was the most prominent band phosphorylated specifically after FcRI stimulation. For quantitative analysis, we used as an internal standard a cell lysate prepared from antigen-stimulated cells which was included in each immunoblot. Densitometric analysis of the autoradiographs was with an LKB Ultrascan XL densitometer.

Measurement of PtdIns Hydrolysis by Anion-exchange Chromatography—Cells were plated in culture medium containing 5 μCi of myo-[3H]inositol/106 cells/35-mm diameter well and anti-TNP monoclonal IgE. After overnight culture (18 h), the cells were washed twice with Pipes-buffered saline and resuspended in 1 ml of 10 mM LiCl, and 1.0 ml of the secretagogue was added. After 30 min
the reaction was stopped by addition of 3 ml of chloroform, methanol, 4 N HCl (100:200:2, v/v/v). The reaction mixture was added to 1.0 ml of chloroform and vortexed vigorously 3 × 1 min. After centrifugation for 5 min at 1500 × g, the aqueous phase containing [3H]inositol phosphates was removed and applied to a column containing 250 mg of Dowex AG 1-X8 resin, 100–200 mesh. The columns were washed with 4 ml of 5 mM myo-inositol (Fluka, Switzerland), and inositol phosphates were eluted with 1.5 ml of 1 M sodium formate, 0.1 M formic acid. Corresponding phospholipids were recovered from the organic phase and radioactivity determined after sample evaporation. Percent PtdIns hydrolysis are calculated from the radioactivity in [3H]inositol phosphates and [3H]phospholipids.

Preparation of Samples for High Performance Liquid Chromatography (HPLC) Analysis—The samples were prepared according to Ref. 25. RBL-2H3 cells were plated at 1 × 10⁶ cells/35-mm diameter well in 2.0 ml of inositol-free EMEM supplemented with 15% dialyzed fetal calf serum, 4 μL of myo-[3H]inositol, and anti-TNP monoclonal IgE. After 24 h of culture, monolayers were washed twice with Pipes-buffered saline containing 10 mM LiCl and genistein or its solvent (Me2SO). After removal of the supernatant for histamine analysis, reactions were stopped by addition of 400 μl of ice-cold 5% (w/v) perchloric acid. The cell pellet was centrifuged at 1000 g, the aqueous phase containing [3H]inositol phosphates and [3H]phospholipids were stored at −70 °C.

Separation of Inositol Phosphates by HPLC—Immediately prior to analysis, samples were neutralized with 1 μl NaOH and [3H]inositol phosphates were resolved by strong anion-exchange chromatography on a SynChropak Q 100 250 × 4.6-mm HPLC column (SynChrom, IN). Elution was performed at a constant flow rate of 1.0 ml/min with a stepwise gradient of 0–1.7 M NH₄H₂PO₄; pH 3.5, using the following protocol: 0–5 min 100% H₂O; 5–35 min linear gradient to 0.6 M NH₄H₂PO₄; 35–125-min linear gradient to 0.9 M NH₄H₂PO₄; 125–140-min linear gradient to 1.7 M NH₄H₂PO₄. Between applications the column was washed with H₂O for 25 min. Column effluent was continuously measured by using the on-line radioactivity detector Flo-One\Beta A 140 (Radiomatic Instruments, Meriden, CT). Identification of the respective peaks was based on comigration with the radiolabeled standard compounds, that were kindly synthesized and provided by Drs. T. Baillie and A. Baukal (National Institute of Child Health and Human Development, Bethesda, MD).

RESULTS

Time Course of FcεRI-induced pp72 Tyrosine Phosphorylation—A previous study demonstrated that protein tyrosine phosphorylation of a 72-kDa protein (pp72) followed aggregation of FcεRI in RBL-2H3 cells by 60 s (17). Using a technique involving chemiluminescence, we were able to show that FcεRI aggregation-induced tyrosine phosphorylation of pp72 was detected as early as 15 s after the addition of antigen (Fig. 1). The band intensity peaked at 10–15 min and remained unchanged for another 30 min (17 and data not shown). Taken together with the previous results that phosphorylation of pp72 is independent of the calcium signal (17) and is specific for activation for FcεRI, these findings emphasize that phosphorylation of this protein is an early event. We therefore investigated the relationship of this phosphorylation to other early metabolic events in RBL-2H3 cells, namely its relationship to the activation of G proteins and the hydrolysis of PtdIns by phospholipase C.

Activation of PtdIns Hydrolysis by G Proteins Does Not Induce Tyrosine Phosphorylation of pp72—Three different approaches were employed to stimulate G proteins in RBL-2H3 cells. In intact cells, AlF₃ complexes were utilized to activate phospholipase C and to release histamine. GTPγS was similarly utilized in permeabilized cells (24, 26). In addition, we investigated G protein activation in RBL-2H3 cells expressing the human m3-mAchR, which can elicit PtdIns hydrolysis through a G protein-coupled mechanism (27). RBL-2H3 cells were stimulated with NaF after 10 min of preincubation with 5 μM AlCl₃, and histamine release as well as tyrosine phosphorylations were measured. At optimal NaF concentrations (10–15 mM), 15–63% of cellular histamine was released (Fig. 2). By immunoblot analysis, stimulation of cells with NaF did not result in detectable phosphorylation of pp72. However, as with antigen stimulation, there was consistently an increase in tyrosine phosphorylation of a 110-kDa protein, and more variably, of phosphoproteins at 40–42 and 57 kDa. Similar bands have been observed after calcium ionophore stimulation. In parallel experiments, there was tyrosine phosphorylation of pp72 following FcεRI stimulation that resulted in histamine release comparable to that induced by NaF (Fig. 2).

In permeabilized RBL-2H3 and rat mast cells, GTPγS is thought to stimulate G proteins and to increase phosphoinositide hydrolysis and release of histamine (24, 28). Permeabilization of RBL-2H3 cell membranes with steptolysin O was used to introduce GTPγS into the cells. Because streptolysin O treatment is associated with leakage of intracellular proteins it was important to establish that pp72 tyrosine phosphorylation was not affected by this procedure. In permeabilized cells, FcεRI-mediated cell activation resulted in an antigen dose-dependent increase of PtdIns hydrolysis and histamine secretion as well as tyrosine phosphorylation of pp72 (Fig. 3A). Using these conditions we investigated the effect of GTPγS on protein tyrosine phosphorylation. Stim-

Tyrosine Phosphorylation of pp72 in RBL-2H3 Cells

RBL-2H3 cells. Cells were cultured for 18 h in the presence or absence of anti-TNP IgE. Parallel cultures were incubated for 18 h in medium containing 3 μCi of myo-[3H]inositol for determination of PtdIns hydrolysis. After two washes with permeabilization buffer, antigen (DNPHSA, panel A) or GTPγS (panel B) were added at the indicated concentrations simultaneously with 0.2 unit/ml of reduced streptolysin O. Cell cultures in triplicate were stimulated for 30 min. All washing and stimulation buffers contained 10 mM LiCl. Histamine release (HR) and PtdIns hydrolysis is indicated below each lane. Results shown are one of four similar experiments.

Fig. 3. FcRI aggregation or activation with GTPγS stimulated protein tyrosine phosphorylation in permeabilized RBL-2H3 cells. Cells were cultured for 18 h in the presence or absence of anti-TNP IgE. Parallel cultures were incubated for 18 h in medium containing 3 μCi of myo-[3H]inositol for determination of PtdIns hydrolysis. After two washes with permeabilization buffer, antigen (DNPHSA, panel A) or GTPγS (panel B) were added at the indicated concentrations simultaneously with 0.2 unit/ml of reduced streptolysin O. Cell cultures in triplicate were stimulated for 30 min. All washing and stimulation buffers contained 10 mM LiCl. Histamine release (HR) and PtdIns hydrolysis is indicated below each lane. Results shown are one of four similar experiments.

The third type of experimental approach we investigated G protein activation and protein tyrosine phosphorylation in RBL-2H3 cells expressing the human m3-mAchR. Activation of the m3-mAchR is known to induce PtdIns hydrolysis by coupling to a stimulatory G protein (27). Native RBL-2H3 cells do not express muscarinic receptors and do not secrete upon challenge with the acetylcholine agonist carbachol. By using a retrovirus vector, two different cloned RBL-2H3 cell lines were isolated each expressing m3-AchR. Cross-linking of FcRI with antigen or activation of the m3-mAchR by carbachol induced dose-dependent PtdIns breakdown and histamine release (Fig. 4A). The array of tyrosine-phosphorylated proteins was nearly identical in antigen-stimulated 2H3-m3.7 and parental RBL-2H3 cells. The most consistent bands represented pp72 and 38- and 110-kDa phosphoproteins. By contrast, the only tyrosine phosphoprotein consistently induced by carbachol was the 110-kDa protein, although in some experiments, 66–71-kDa tyrosine phosphoproteins were also detected. Unlike the pp72, the 66–71-kDa proteins were not phosphorylated when cells were stimulated in the absence of extracellular calcium (Fig. 4B). Therefore, the experimental evidence indicates that the tyrosine phosphorylation of these 66–71-kDa proteins is activated through a different pathway than that leading to pp72 tyrosine phosphorylation. Thus, activation of G proteins by a variety of different means resulted in increased PtdIns hydrolysis and the release of histamine without inducing tyrosine phosphorylation of pp72. Taken together, these findings strongly suggest that G protein activation and G protein-mediated PtdIns hydrolysis were insufficient to induce pp72 tyrosine phosphorylation.

Genistein Inhibits pp72 Tyrosine Phosphorylation and Histamine Release without Affecting PtdIns Hydrolysis—To determine whether PtdIns hydrolysis was secondary to tyrosine phosphorylation of pp72, we investigated the effect of the tyrosine kinase inhibitor genistein (4',5,7-trihydroxyisoflavone) on pp72 phosphorylation, hydrolysis of PtdIns, and histamine release. This isoflavone compound inhibits the tyrosine kinase activity of epidermal growth factor receptor, pp60c-src, pp110c-yes, and pp56eck, but it has only minor effects on several serine and threonine kinases (29). To maximize the inhibition of tyrosine phosphorylation, RBL-2H3 cells were incubated for 30 min with genistein prior to the addition of antigen. When RBL-2H3 cells were treated with increasing concentrations of genistein (Fig. 5A), we observed a dose-dependent inhibition of all tyrosine-phosphorylated proteins including pp72 (IC50 = 34 μg of genistein/ml). The drug also inhibited histamine release up to 88% at a concentration of 100 μg/ml (IC50 = 31 μg of genistein/ml). There was parallel inhibition of pp72 phosphorylation and histamine release by genistein (Fig. 5B).

The next series of experiments investigated the effect of genistein on both histamine release and PtdIns hydrolysis. Duplicate cultures were stimulated with 10 ng/ml DNPHSA in the presence of increasing concentrations of genistein: one culture was used for histamine assay and the other for PtdIns measurements (Fig. 6). Again there was dose-dependent inhibition of histamine release (Fig. 6A). However, total PtdIns hydrolysis as measured by anion-exchange chromatography was minimally affected by incubation with genistein (Fig. 6B). Only at the highest concentration of genistein (100 μg/ml) did we find a moderate decrease of PtdIns breakdown (18%), probably due to nonspecific effects. Similar results were obtained with genistein in antigen dose-response experiments (Fig. 6, C and D). A genistein concentration of 60 μg/ml was selected to minimize nonspecific drug toxicity while maintaining significant inhibition of histamine release. Histamine release was inhibited at all antigen concentrations, with the strongest inhibition at suboptimal antigen levels (78% inhibition at 10 ng/ml DNPHSA) (Fig. 6C). In contrast, PtdIns hydrolysis was essentially unaffected by genistein (Fig. 6D).

To further study the relationship of the tyrosine phos-
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**A**

- + + + + +

DNP-HSA (10 ng/ml)

- 10 30 100 Genistein (pg/ml)

**B**

% of Control

Genistein (µg/ml)

FIG. 5. Effect of genistein on FeRI-induced protein tyrosine phosphorylation and histamine release in RBL-2H3 cells. A, after culture for 18 h with anti-TNP IgE, cells were washed and genistein or vehicle (0.15% Me₂SO) was added. After incubation for 30 min at 37°C, the supernatant was discarded and 10 ng/ml DNP₃₅-HSA was added together with genistein or Me₂SO at the indicated final concentrations. After 30 min, the supernatants were removed for measurement of histamine release, and reactions were stopped by addition of lysis buffer and used for immunoblotting as described in the legend of Fig. 1. Histamine released (HR) into the supernatant is indicated below each lane. B, inhibition of pp72 tyrosine phosphorylation and histamine release by genistein. The pp72 protein tyrosine phosphorylation was quantitated by densitometry and compared to histamine release determined from the identical cell cultures. Results shown are mean ± S.E. from four different experiments.

FIG. 6. Effect of genistein on FeRI-induced phosphatidylinositol hydrolysis and histamine release. RBL-2H3 cells were sensitized with anti-TNP IgE and stimulated with antigen (DNP₃₅-HSA) in the absence or presence of genistein as indicated in the legend for Fig. 5. Cell cultures incubated in medium containing 3 µCi of myo-[³H]inositol were stimulated in parallel for determination of PtdIns hydrolysis. A and B, genistein dose-response curve in cells stimulated with 10 ng/ml of DNP₃₅-HSA. C and D, antigen dose-response curve in cells stimulated in the absence (●) or presence (○) of 60 µg/ml genistein. A and C, histamine release, B and D, PtdIns hydrolysis. Results shown are mean ± S.E. from 5 to 12 different experiments.

FIG. 7. The effect of genistein on FeRI-induced pp72 tyrosine phosphorylation and PtdIns hydrolysis. RBL-2H3 cells were sensitized with anti-TNP IgE and stimulated with DNP₃₅-HSA (1–100 ng/ml) in the absence (○) or presence (●) of genistein (30, 60, or 100 pg/ml) as indicated in the legend to Fig. 5. Tyrosine phosphorylation of pp72 was measured by densitometry and plotted against PtdIns hydrolysis determined from the parallel cell cultures. Error bars indicate the mean ± S.D. there was a mean phosphorylation index of 1.41 ± 0.51 arbitrary units (mean ± S.D.) and an average PtdIns hydrolysis of 30.1 ± 10.2%. In the corresponding cultures stimulated in the presence of various concentrations of genistein (30–100 µg/ml), pp72 phosphorylation was significantly reduced while PtdIns breakdown was essentially unchanged (0.4 ± 0.3 arbitrary units and 28.3 ± 11.4%, respectively; mean ± S.D.). Activation of PI-PLC results in the generation of inositol 1,4,5-trisphosphate, which both acts as a secondary messenger to release intracellular calcium and plays a role in the influx of extracellular calcium (30). To investigate a possible effect of genistein on the formation of inositol 1,4,5-trisphosphate and other inositol phosphate metabolites, samples from RBL-2H3 cells stimulated with antigen in the presence or absence of genistein were analyzed by high performance liquid chromatography (representative HPLC patterns are shown in Fig. 8). Confirming results obtained by anion-exchange chromatography, the amount of total inositol phosphate metabolites was not significantly affected by genistein treatment of the

phorylation of pp72 to phospholipase C activation, data obtained from parallel cultures and collected over a period of 9 months were pooled. To allow the comparison of a large number of samples, pp72 tyrosine phosphorylation was quantified by densitometry and calculated with respect to an internal standard included with each anti-phosphotyrosine immunoblot (Fig. 7). Thus, in experiments performed with different antigen concentrations (1–100 ng/ml DNP₃₅-HSA)
FIG. 8. HPLC elution profile of [3H]inositol phosphate metabolites after FcεRI activation in the absence or presence of genistein. RBL-2H3 cells were cultured for 24 h in inositol-free medium supplemented with 15% dialyzed fetal calf serum, 4 μCi of myo-[3H]inositol, and anti-TNP IgE. After washing and a 30-min preincubation with 60 μg/ml genistein or 0.15% Me2SO, cells were stimulated by addition of 100 ng/ml of DNP38-HSA in the absence (top panel) or presence of 60 μg/ml genistein (bottom panel). After 30 min the reaction was stopped, samples were extracted, and analyzed as described under "Experimental Procedures." GPZ indicates the position of glycerophosphoinositol; Ins-1-P, inositol 1-monophosphate; Ins-4-P, inositol 4-monophosphate; Ins-1,4-P2, inositol 1,4-bisphosphate; Ins-3,4-P2, inositol 3,4-bisphosphate; Ins-1,3,4-P3, inositol 1,3,4-trisphosphate; Ins-1,4,5-P3, inositol 1,4,5-trisphosphate; Ins-3,4,5,6-P4, inositol 3,4,5,6-tetrakisphosphate; Ins-1,3,4,6,5-P5, inositol 1,3,4,6,5-hexakisphosphate. Histamine release was 38.6 and 5.7% in the absence and presence of genistein, respectively. One experiment representative of five.

FIG. 9. Time course of appearance of inositol 1,4,5-trisphosphate in FcεRI-stimulated RBL-2H3 cells in the absence and presence of genistein. Cells were cultured and stimulated with 100 ng/ml of DNP38-HSA in the absence (C) or presence of 60 μg/ml genistein (●) as indicated in the legend of Fig. 8. Reactions were terminated at the indicated times by addition of 5% perchloric acid and [3H]inositol phosphates from cell extracts were separated by high performance anion-exchange chromatography. Inhibition of histamine secretion determined from identical cultures was (mean ± S.E.) 98% ± 2, 91% ± 4, and 87% ± 3 at 3, 10, and 30 min, respectively (mean control histamine release was 7.5, 35.6, and 40.6%). Values are mean ± S.E. from two to four experiments.

DISCUSSION

There is indirect evidence that activation of PI-PLC by FcεRI aggregation in basophils and mast cells is mediated by G proteins (14, 32). Stimulation of G proteins by either AlF3 complexes or GTPγS in RBL-2H3 cells has been shown to induce both PtdIns hydrolysis and histamine release (24, 26). Furthermore, GDPβS, an inhibitor of G protein activation, decreases both PtdIns hydrolysis and histamine secretion induced by either FcεRI cross-linking or by GTPγS (16). However, to date, no G protein responsible for coupling the FcεRI to PI-PLC has been identified. By contrast, studies with bone marrow-derived cultured mouse mast cells have suggested that the PI-PLC activation is independent of G protein coupling (33). Studies with bacterial toxins in RBL-2H3 cells indicate the FcεRI activation is pertussis toxin-sensitive (10). Therefore, the coupling of FcεRI to PI-PLC must require a pertussis toxin-resistant Ga subunit. At least two such subunits, 9G, Gα3 (34) and Gα4 are found in RBL-2H3 cells and are possible candidates as signal transducers of FcεRI activation. In the present study, PtdIns hydrolysis induced by carbachol in m3-mAchR expressing cells was comparable to that produced by FcεRI aggregation. Three different approaches to G protein stimulation resulted in activation of signal transduction pathways that overlapped with FcεRI-induced biochemical events, i.e. generation of inositol phosphates, tyrosine phosphorylation of 110-kDa proteins, and histamine secretion. Therefore, although G protein activation resulted in increased tyrosine phosphorylation of a number of substrates, it did not result in tyrosine phosphoryl-

2 J. S. Gutkind, unpublished observations.
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...of pp72. Taken together, these findings indicate that pp72 tyrosine phosphorylation is not coupled to G protein activation and cannot be induced by G protein-dependent PtdIns hydrolysis.

The generation of inositol phosphates may be due to the activation of PI-PLC by tyrosine phosphorylation in several receptor systems. Phosphoinositide-specific PLC-γ1 is phosphorylated on tyrosine residues by receptors with cytoplasmic tyrosine kinase domains (e.g. epidermal growth factor and platelet-derived growth factor receptor) (35) and probably by non-receptor kinases of the src family following activation of the interleukin-2 receptor and T-cell and B-cell antigen receptors (38-39). Accordingly, T-cell or platelet-derived growth factor receptor stimulated PI-PLC activation and PtdIns hydrolysis are blocked by genistein and other tyrosine kinase inhibitors (39-42) although contradictory results have been reported (43). However, in RBL-2H3 cells genistein blocked FcRI-induced pp72 tyrosine phosphorylation without quantitative changes in PtdIns hydrolysis. Therefore, our present observations suggest that unlike T-cell or platelet-derived growth factor receptors-mediated activation, FcRI-mediated stimulation of PI-PLC is independent of the major cellular tyrosine phosphorylations. Thus, there appears to be a fundamental difference between T-cells and RBL-2H3 cells in tyrosine phosphorylation signaling of PtdIns hydrolysis.

Separation by high performance liquid chromatography of inositol phosphates from FcRI-activated cells showed no significant changes in the amount of inositol 1,4,5-trisphosphate after incubation with genistein. Furthermore, there were no consistent changes in its immediate metabolites, i.e. in inositol, 1,4-bisphosphate and inositol 1,3,4,5-tetrakisphosphate. However, two inositol phosphate metabolites, inositol 3,4-bisphosphate and inositol 1,3,4,5-tetrakisphosphate were found to be decreased by 24-70%. These decreases might be due to the inhibition of the enzyme inositol 1,3,4,5-tetrakisphosphate phosphatase which converts inositol 1,3,4,5-tetrakisphosphate to inositol 1,3,4,5-trisphosphate. This, however, seems unlikely since the level of inositol 1,3,4,5-tetrakisphosphate was essentially unaltered upon genistein treatment of the cells. Alternatively, the inhibition of the tyrosine phosphorylation of phosphatidylinositol-3-kinase by genistein might play a role in the observed decrease of 3-phosphorylated inositol phosphates.

The tyrosine kinase inhibitor genistein blocked histamine release in RBL-2H3 cells in parallel to the inhibition of pp72 tyrosine phosphorylation. Genistein also inhibited histamine release induced by ionophore and carbachol in RBL-2H3 cells expressing the muscarinic receptor, two secretagogues that do not induce pp72 tyrosine phosphorylation. This effect of genistein suggests that tyrosine phosphorylations of proteins other than that of pp72 play a role in histamine release. Interestingly, we have observed that antigen, ionophore, as well as carbachol in m3-expressing cells induce tyrosine phosphorylations of substrates late after stimulation (e.g. proteins of 110 kDa). Some of these tyrosine-phosphorylated proteins appear to be involved in modulation of the secretory process. Therefore, the inhibition of histamine release by genistein suggests that secretion requires tyrosine phosphorylation of a number of different substrates.

The mechanism of the FcRI-mediated tyrosine phosphorylation of pp72 and other substrates has not been identified. The FcRI has no intrinsic tyrosine kinase activity, but three members of the src family of non-receptor kinases have been identified in RBL-2H3 cells (17, 44). Association of the aggregated receptor with one of these or with an unidentified tyrosine kinase could initiate tyrosine phosphorylation signals. In this regard it has been suggested that lyn associated with the FcRI in RBL-2H3 cells and could be involved in signal transduction (44).

The present study provides a model for the events that are initiated by the cross-linking of the FcRI (Fig. 10). Aggregation of the receptor results in the increased tyrosine phosphorylation of pp72 as an early signal; in parallel there is activation of a PI-PLC pathway that results in the release of inositol phosphates, a rise in intracellular free calcium concentration, the influx of extracellular calcium, and the activation of protein kinase C. Tyrosine phosphorylation of pp72 is an FcRI-specific, distinct pathway that is independent of previously described metabolic events. This event is important for FcRI-mediated histamine secretion as demonstrated by the parallel inhibition by genistein of protein tyrosine phosphorylation and cell degranulation. However, cell stimulation in the absence of extracellular calcium established that activation of the tyrosine phosphorylation pathway alone is not sufficient to induce degranulation. Therefore, tyrosine phosphorylation of pp72 is another component of the complex network of biochemical events initiated by aggregation of FcRI that contribute to intracellular signaling.

REFERENCES


4. V. Stephan, unpublished observation.
Tyrosine Phosphorylation of pp72 in RBL-2H3 Cells