Temporal regulation of the IgE-dependent 1,2-diacylglycerol production by tyrosine kinase activation in a rat (RBL 2H3) mast-cell line

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We explored the possible role of tyrosine kinases in the IgE-dependent regulation of 1,2-diacylglycerol (DAG) production in RBL 2H3 cells. When triggered via their high-affinity IgE receptors (Fc_eRI), there was a rapid phosphorylation of tyrosine residues on a number of proteins. The phosphorylation of these proteins and ultimately histamine release were inhibited in a concentration-dependent manner by the tyrosine kinase inhibitor, tyrphostin. In cells labelled with [^{3}H]myristic acid, we observed a characteristic biphasic increase in [^{3}H]DAG production. In the presence of tyrosine kinase inhibitor, the initial increase in DAG was still observed, but the secondary increase, which was dependent on phosphatidylcholine-specific phospholipase D (PC-PLD) activation, was completely abolished. Tyrphostin significantly inhibited IgE-dependent activation of PC-PLD, suggesting that PC-PLD activation was regulated by tyrosine phosphorylation. Furthermore, when proteins from RBL 2H3 cells were immunoprecipitated with an anti-phosphotyrosine antibody, PC-PLD activity was recovered from the immunoprecipitated fraction. These results demonstrate that the secondary, but not the initial, phase of 1,2-DAG production in response to Fc_eRI aggregation is regulated by the initial activation of tyrosine kinases and that PC-PLD may be regulated directly by this mechanism.

INTRODUCTION

Aggregation of high-affinity IgE receptors (Fc_eRI) on the surface of mast cells results in the initiation of a number of specific signal-transduction events, including the activation of *src*-related tyrosine kinases (Eiseman and Bolen, 1992), elevation of intracellular free Ca^{2+} levels (Beaven et al., 1984; Beaven and Cunha-Melo, 1988), phospholipase C-catalysed phosphoinositide hydrolysis (Cunha-Melo et al., 1987), phospholipase D (PLD)-dependent phosphatidylcholine (PC) hydrolysis (Kennerly, 1987; Gruchalla et al., 1990; Lin et al., 1991, 1992a), and an increase in intracellular diacylglycerol (DAG) levels (Kennerly et al., 1979; Rando, 1988; Lin et al., 1991, 1992a) with resulting activation of protein kinase C (White et al., 1985; Nagao et al., 1987; Lin and Gilfillan, 1992; Lin et al., 1992a). In the rat mast-cell line RBL 2H3, we previously demonstrated that the IgE-dependent increase in DAG production is biphasic in nature: an initial transient increase, which maximizes within 30–60 s, followed by a more prolonged increase, which maximizes within 5 min (Lin et al., 1992a). The second phase of DAG production is primarily dependent on the activation of PC-specific PLD (PC-PLD) (Lin et al., 1992a), but the initial phase appears to be the result of activation of other phospholipase(s) (Lin et al., 1992b). Our previous studies have indicated that PC-PLD activation, and thus DAG production, in RBL 2H3 cells is regulated by Ca^{2+} and protein kinase C (Lin and Gilfillan, 1992) activation. There is increasing evidence, however, for an important role of tyrosine kinases in IgE-dependent mast-cell degranulation (Yu et al., 1991; Park et al., 1991; Benhamou and Siraganian, 1992; Eiseman and Bolen, 1992; Kawakami et al., 1992; Li et al., 1992; Stephan et al., 1992).

A number of proteins have been reported to be phosphorylated on their tyrosine residues following receptor aggregation (Li et al., 1992; Benhamou et al., 1990). Several of these proteins have been identified, including the 140 kDa phospholipase C γ (Park et al., 1991), a proto-oncogene *vav* product (Margolis et al., 1992), a 72 kDa tyrosine kinase (Hutchcroft et al., 1991) and the β (33 kDa) and γ (9–11 kDa) chains of the IgE receptor (Li et al., 1992). Tyrosine kinase inhibitors block IgE-dependent histamine release (Yu et al., 1991; Kawakami et al., 1992; Stephan et al., 1992), suggesting that the IgE-dependent degranulation of mast cells appears to depend on this mechanism. In addition, it has recently been reported that genistein blocks IgE-dependent PC-PLD activation (Kumada et al., 1993). The aim of this study was therefore to examine the role of tyrosine kinases in the signal-transduction cascade leading to the production of DAG following Fc_eRI aggregation. The initial results from these studies have been previously presented in abstract form (Gilfillan et al., 1992).

METHODS

RBL 2H3 cell culture and histamine release studies

RBL 2H3 cells were maintained in 175 cm^2 tissue-culture flasks as previously described (Lin et al., 1991). The cells were grown as a monolayer, then dislodged with trypsin (0.05%, v/v)/EDTA (0.02%, v/v) solution (Gibco, Grand Island, NY, U.S.A.). After rinsing, the cells were re-plated at a density of 1×10^6 cells in Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal-bovine serum (Hyclone, Logan, UT, U.S.A.), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco) in 35 mm-diameter tissue-culture dishes (Falcon; Becton Dickinson, Lincoln Park, NJ, U.S.A.) which were maintained at 37 °C in a humidified atmosphere of air/CO_2 (19:1). The cells were sensitized overnight by the inclusion of mouse monoclonal

Abbreviations used: FC_eRI, high-affinity IgE receptors; PC, phosphatidylcholine; PLD, phospholipase, D; PC-PLD, PC-specific PLD; DAG, diacylglycerol; TNP, trinitrophenol; OVA, ovalbumin; EGF, epidermal growth factor.

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trinitrophenol (TNP)-specific IgE (anti-TNP IgE) (0.5 μg/ml) in the incubation medium. The cells were then rinsed thoroughly with Hepes buffer (10 mM Hepes, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 5.6 mM glucose, 1.8 mM $CaCl_2$ and 1.3 mM $MgSO_4$), followed by addition of 1 ml of this buffer to each dish. After a 5–10 min preincubation, the cells were triggered with TNP–ovalbumin (OVA) (routinely 10 ng/ml). Inhibitors were added during the preincubation period. Appropriate volumes of the carrier solutions were added to the control dishes. These additions did not affect mediator release. The cells were routinely incubated for 30 min, after which time the incubation medium was carefully removed and centrifuged at 200 *g* for 5 min to remove any free floating cells. Distilled water was added to the cells remaining attached to the dish, and the cellular material was removed by freeze–thawing and scraping with a rubber policeman.

To assay for histamine content, the supernatants and lysates were diluted with distilled water, then mixed with equal volumes of $HClO_4$ (0.8 %, v/v). The samples were then centrifuged at 500 *g* for 10 min. The histamine content of the resulting supernatants was analysed by an automated spectrofluorimetric assay (Siraganian, 1974).

1,2-DAG assay

The cells were plated and sensitized overnight with anti-TNP IgE as described above. The cells were then incubated in MEM containing [^{3}H]myristic acid (1 μCi/ml) for 1 h at 37 °C to label the cellular lipids. The cells were rinsed twice, fresh medium was added, and the cells were incubated for another 1 h. The cells were rinsed again and the medium was replaced with 1 ml of the Hepes buffer described above. At pre-determined times after triggering with TNP–OVA (10 ng/ml), the experiments were terminated by rapidly removing the Hepes buffer, and then adding 1 ml of methanol. The cells were scraped from the plates, and then the methanol cell extracts were placed in separate extraction tubes. The lipids were extracted by the Bligh and Dyer (1959) procedure and the DAG was separated from the other labelled lipids by t.l.c. (Lin et al., 1991). After it was located by exposure to iodine vapour, the area corresponding to the 1,2-DAG standard was scraped into scintillation vials and the radioactivity determined by scintillation counting in Aquasol containing 3 % (v/v) water. We have previously demonstrated that monitoring the time course of 1,2-DAG production utilizing [^{3}H]myristic acid to label the cellular lipids provides data identical with those obtained when total 1,2-DAG mass is assayed (Lin et al., 1992a).

PC-PLD assays

Method A

PC-PLD activity *in vivo* was assayed by utilizing its ability to catalyse a transphosphatidylation reaction (Anthes et al., 1989). The cells were plated and labelled with [^{3}H]myristic acid as described above. During the preincubation period (5 min), ethanol (0.5 %, v/v) was added in the absence or presence of inhibitor to the incubation medium. The cells were triggered, and after 30 min the lipids were extracted (Bligh and Dyer, 1959). The extracted lipids were spotted on to Si250 silica-gel t.l.c. plates, and the plates were developed in the organic phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10, by vol.) as described by Anthes et al. (1989). After it was located by exposure to iodine vapour, the area corresponding to a phosphatidylethanol standard was scraped into scintillation vials and the radioactivity determined.

Method B

For some experiments, PC-PLD activity was measured after cells were lysed (see below). PC-PLD activity was measured in samples that had been immunoprecipitated with an anti-phosphotyrosine antibody (see below), by determining the amount of [^{3}H]choline released from [^{3}H]choline-labelled dipalmitoyl PC. Typically, the samples were incubated with the substrate (10^6 c.p.m.) in 1 ml of Buffer A (Hepes buffer containing 1 mM Na_3VO_4 and 0.1 mM dithiothreitol) at 37 °C for 4 h. The reaction mixture was aspirated and extracted by the method of Bligh and Dyer (1959). The water-soluble radiolabelled products were separated by t.l.c. (Martin and Mechaelis, 1989), and the area corresponding to choline standard was scraped, and then quantified by scintillation counting (Martin and Mechaelis, 1989).

Method C

To confirm the results obtained with the above assay, we utilized a PC-PLD-catalysed transphosphatidylation reaction *in vitro*. Immunoprecipitation of the cytosol and membrane proteins was allowed to proceed overnight at 4 °C (see below), and then the beads were rinsed three times with Buffer A. The beads were then resuspended in Buffer A (200 μl) containing EGTA (1.8 mM), ethanol (2 %, v/v) and substrate (dipalmitoyl PC, 2.5 mM/0.75 μCi; sodium oleate, 5 mM) which was prepared by the method described by Inamori et al. (1993). The reaction was allowed to proceed on the beads for 4 h at 37 °C, and then the lipids were extracted in chloroform/methanol/0.1 M HCl (final ratio 10:10:9, by vol.). The labelled phosphatidylethanol was then separated from the other labelled lipids by the t.l.c. technique described for Method A.

Protein tyrosine phosphorylation assay

RBL 2H3 cells were plated, sensitized and triggered with TNP–OVA as described above. At predetermined times, the cells were lysed by aspiration of the incubation buffer and addition of 500 μl of boiling SDS-sample buffer (60 mM Tris/HCl, pH 6.8, 0.72 M β-mercaptoethanol, 3 % SDS, 1 mM Na_3VO_4 and 5 mM dithiothreitol). Portions of the samples were boiled for 5 min; the proteins were fractionated by SDS/PAGE on 8–16 %-acrylamide gradient gels in Tris/glycine (Novex, San Diego, CA, U.S.A.) under reducing conditions (Laemmli, 1970) and then transferred to nitrocellulose by standard procedures. The blots were blocked for 2 h in 20 mM Tris (pH 8)/0.5 M NaCl/0.05 % (v/v) Tween-20 (TTBS) and 4 % (w/v) BSA (blocking buffer). Phosphotyrosyl-proteins were detected after overnight incubation of the blots with anti-phosphotyrosine monoclonal antibody PY20 (ICN, Costa Mesa, CA, U.S.A.) at 1 μg/ml in blocking buffer containing 1 μCi/ml ^{125}I-labelled sheep anti-mouse IgG F(ab′)$_2$ fragment (Amersham, Arlington Heights, IL, U.S.A.). After extensive washing with TTBS and a rinse in 20 mM Tris (pH 8)/0.5 M NaCl, the blots were autoradiographed.

Immunoprecipitation with anti-phosphotyrosine antibody

The cells (8×10^7) were plated as described for the histamine-release studies and then triggered with TNP–OVA for 30 min. The cells were harvested and then sonicated in Buffer A. The resulting sonicated material was centrifuged at 100000 *g* for 1 h to separate the membrane and cytosolic fractions. Membrane-

bound PC-PLD was extracted with Buffer A containing 2 M NaCl. A 10 μg portion of either anti-phosphotyrosine monoclonal antibody 4G10 (UBI, Lake Placid, NY, U.S.A.) or control antibody IE5 (a monoclonal antibody against E-selectin) was preincubated for 2 h at room temperature with 100 μl of affinity-purified goat anti-mouse IgG–agarose in Buffer A containing BSA (1 mg/ml). The resin was washed with Buffer A and then incubated at 4 °C overnight with either the cytosolic fraction or the membrane extract that had been diluted with Buffer A. The resin was washed three times with Buffer A. PC-PLD activity was determined with [^{3}H]choline-labelled dipalmitoyl PC substrate as described for Method B. To confirm that the observed activity was a consequence of PC-PLD activation, in some experiments we utilized Method C.

Viability studies

To assess the effect of tyrphostins on cell viability, the cells were triggered as described above for secretion experiments and the rate of lactate dehydrogenase release in these cultures was compared with non-treated cells (Wroblewski and La Due, 1955).

Statistics and data analysis

All data were normalized for cell number. Unless otherwise indicated, the results are presented as means $\pm$ S.E.M. and were analysed statistically by Student's two-tailed t test for paired samples.

Materials

Mouse anti-TNP IgE and TNP–OVA were obtained from Dr. J. Hakimi, Hoffmann–La Roche (Nutley, NJ, U.S.A.). [^{3}H]Myristic acid and [^{3}H]choline-labelled dipalmitoyl PC were purchased from New England Nuclear (Boston, MA, U.S.A.). The tyrosine kinase inhibitors tyrphostins 8, 11 and 12 were obtained from Dr. C.-K. Huang, University of Connecticut Health Science Center (Farmington, CT, U.S.A.). These compounds were first synthesized by Yaish et al. (1988). Unless specified in the text, other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Effect of tyrosine kinase inhibitors on histamine release from RBL 2H3 cells

To examine the potential role of tyrosine kinases in the regulation of the IgE-dependent production of DAG leading to histamine release in RBL 2H3 cells, we initially examined the effect of three tyrosine kinase inhibitors on IgE-dependent histamine release. These tyrosine kinase inhibitors, tyrphostins 8, 11 and 12, are structural analogues of tyrosine (Yaish et al., 1988) and have a higher degree of selectivity towards tyrosine kinases compared with either protein kinase A or protein kinase C (Enright and Booth, 1991). They have previously been shown to be specific EGF (epidermal growth factor)-receptor tyrosine kinase inhibitors that block EGF-dependent proliferation of A431 cells (Yaish et al., 1988). When these compounds were added before triggering the cells with TNP–OVA, there was a concentration-dependent inhibition of histamine release (Figure 1). The tyrphostins had no detrimental effects on cell viability, as assessed by lactate dehydrogenase release (results not shown). The potency of these compounds on histamine release was comparable with that reported for the inhibition of EGF-dependent cell proliferation of A431 cells (Yaish et al., 1988) and of tyrosine kinase-dependent interleukin-2 secretion from murine T-cells (Stanley et al., 1990). Tyrphostin 8 was the most potent compound tested (IC_{50} 20 μM). Therefore, for subsequent studies we used tyrphostin 8.

Effect of tyrphostin on tyrosine kinase activation in RBL 2H3 cells

To asses the relative kinetics of tyrosine phosphorylation and DAG production in response to $Fc_\epsilon RI$ aggregation, we initially examined the nature of the time-dependent increase in tyrosine phosphorylation in the absence and presence of tyrphostin 8.

(*a*) Time course of tyrosine kinase activation

Sensitized RBL 2H3 cells were triggered with a concentration (10 ng/ml) of TNP–OVA that produced maximal histamine release. Under these conditions, there was a time-dependent increase in the phosphorylation of tyrosine residues on a number of proteins. The most noticeable increases were on proteins of approx. 72 kDa and 110 kDa (Figure 2a). Onset of phosphorylation of these proteins was generally observed within 10–30 s, and maximal phosphorylation was observed by 5–10 min after stimulation. The proteins remained phosphorylated for the duration of the experiments (30 min). These results were similar to those reported previously (Stephan et al., 1992). When cells were preincubated for 5 min with tyrphostin 8 (0.1 mM) before triggering with TNP–OVA, the increase in tyrosine phosphorylation was inhibited at all time points (Figure 2b).

(*b*) Concentration-dependent inhibition of tyrosine kinases by tyrphostin

When cells were triggered with TNP–OVA in the presence of increasing concentrations of tyrphostin 8, there was a concentration-dependent inhibition of the tyrosine phosphorylation of the proteins observed (Figure 3). Taken together with

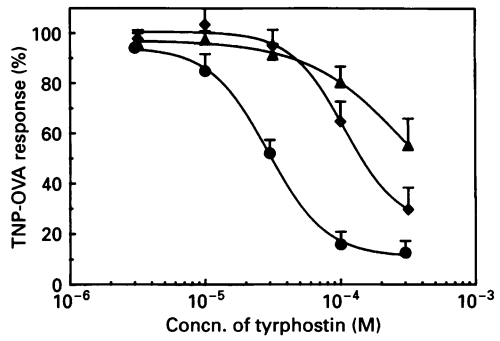

Figure 1 Effect of tyrphostins on IgE-dependent histamine release

RBL 2H3 cells were sensitized overnight with anti-TNP IgE (0.5 μg/ml), then preincubated for 5 min with tyrphostins 8 (●), 11 (◆) or 12 (▲) before triggering with TNP–OVA (10 ng/ml) for 30 min. Histamine release was then determined as described in the Methods section. The values represent the percentage of the release obtained in the absence of tyrphostins. The results are means of 4–5 experiments. When the effect of the tyrphostins at 300 μM were analysed statistically by Student's t test for paired samples, they were all significantly lower than the responses of TNP–OVA observed in their absence ($P < 0.01$). The release from the stimulated cells in the absence of tyrphostins was 48.8 $\pm$ 5.4% of the total cellular content, compared with a basal release of 4.5 $\pm$ 1.2% ($n = 5$).

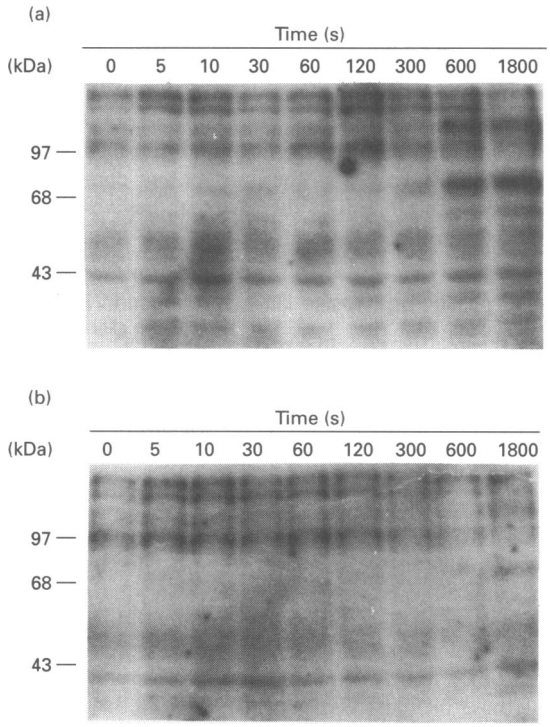

Figure 2 Time course of tyrosine phosphorylation after aggregation of $Fc_\epsilon RI$ in the absence (a) or presence (b) of tyrphostin 8

RBL 2H3 cells were sensitized as described in the text, then incubated for 5 min in the absence (**a**) or presence (**b**) of tyrphostin 8 (0.1 mM) before triggering with TNP–OVA (10 ng/ml). At the designated times, cells were lysed and the phosphorylated proteins analysed by immunoblotting as described in the Methods section. The ordinate numbers represent the relative positions of molecular-mass markers (kDa). The abscissa numbers represent the time after stimulation with TNP–OVA. The results are typical of several such observations.

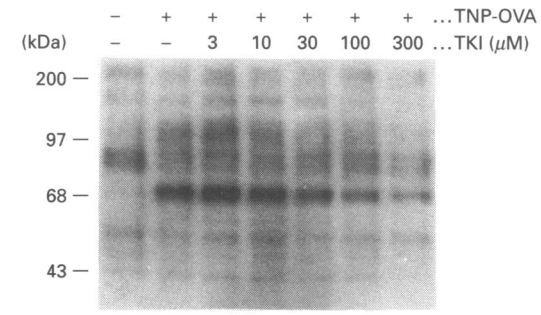

Figure 3 Concentration-dependent effect of tyrphostin on IgE-dependent tyrosine kinase activation

RBL 2H3 cells were sensitized and triggered in the absence or presence of increasing concentrations of tyrphostin 8 (TKI). The experiments were terminated 10 min later, and the tyrosine-phosphorylated proteins were analysed as described in Figure 2. The Figure is typical of four such observations. The ordinate numbers represent the relative positions of molecular-mass markers (kDa) (not shown).

the results of tyrphostin on tyrosine phosphorylation described above, these data support previous studies suggesting a role for tyrosine phosphorylation in IgE-dependent histamine release (Benhamou and Siraganian, 1992).

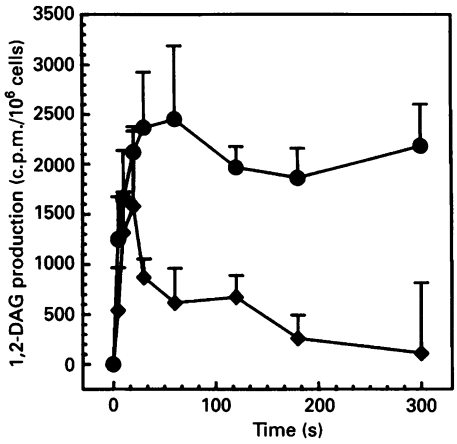

Figure 4 Effect of tyrphostin on the IgE-dependent increase in 1,2-DAG formation

RBL 2H3 cells were sensitized and labelled with [^{3}H]myristic acid, and then preincubated for 5 min in the absence (●) or presence (◆) of tyrphostin (0.1 mM) before triggering with TNP–OVA (10 ng/ml) for the time period indicated. The 1,2-[^{3}H]DAG was then extracted and separated from the other lipids as described in the Methods section. The values represent the increase in DAG levels above those of the cells which were not treated with TNP–OVA. These values were 4036.4 ± 282.7 c.p.m./10^6 cells for cells incubated in the absence of tyrphostin and 3221.3 ± 182.7 c.p.m./10^6 cells for cells incubated in the presence of tyrphostin. The results are means $\pm$ S.E.M. ($n = 4$–5).

Effect of tyrphostin on the accumulation of DAG

We have previously demonstrated that, when sensitized RBL 2H3 cells are triggered with TNP–OVA (10 ng/ml), there is a characteristic biphasic increase in DAG production, with an initial increase maximizing within 30–60 s, followed by a more prolonged increase. To study whether or not both phases of DAG production were regulated by tyrosine kinases, cells were treated with tyrphostin 8 and the time course of DAG production was monitored. In the presence of tyrphostin, the initial increase in DAG was still observed, but the secondary response was completely inhibited (Figure 4). This suggests that both phases of DAG production may be independently regulated, and that only the second phase depends on tyrosine kinase activation.

Role of tyrosine kinases in the IgG-dependent activation of PC-PLD

As the second phase of DAG production appears to be dependent on the activation of PC-PLD (Lin et al., 1991) and tyrosine kinase(s), we explored the possibility that tyrosine kinases regulated PC-PLD activation upon $Fc_\epsilon RI$ aggregation. We initially examined the ability of tyrphostin 8 to inhibit the IgE-dependent activation of PC-PLD. PC-PLD catalyses a trans-phosphatidylation reaction, and therefore the catalytic action of PC-PLD *in vivo* can be assessed by measuring the extent of phosphatidylethanol production in cells incubated with media containing ethanol (Anthes et al., 1989). When the cells were preincubated with tyrphostin before triggering with TNP–OVA, there was a concentration-dependent inhibition of PC-PLD activity (Figure 5) over a concentration range similar to that described for histamine release and tyrosine kinase activity. These results suggest that the activity of PC-PLD was indeed dependent on tyrosine kinase activation.

To support this conclusion, we examined whether PC-PLD activity could be recovered by immunoprecipitation using an

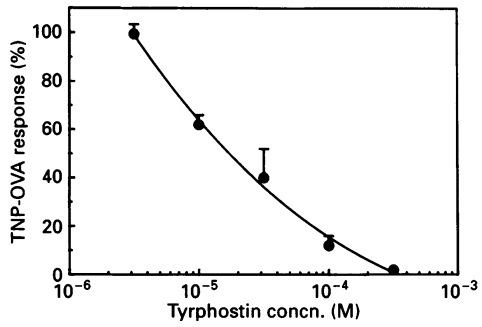

Figure 5 Effect of tyrphostin on the IgE-dependent activation of PC-PLD

RBL 2H3 cells were sensitized and then preincubated for 5 min with various concentrations of tyrphostin before triggering with TNP–OVA (10 ng/ml) for 30 min. PC-PLD activity was measured by the transphosphatidylation reaction using ethanol (0.5%, v/v) as described in the Methods section. The values represent percentages of the results observed in response to TNP–OVA in the absence of tyrphostin (2850.6 ± 449.1 c.p.m./10^6 cells) compared with the production in unstimulated cells (457.7 ± 37.3 c.p.m./10^6 cells). The results are means ± S.E.M. ($n = 4$). When the effect of tyrphostin at 300 μM was compared with that of TNP–OVA alone, it was significantly lower ($P < 0.05$, Student's two-tailed t test for paired samples).

anti-phosphotyrosine antibody. Both cytosolic and membrane extracts from stimulated and unstimulated cells were incubated with anti-phosphotyrosine antibody 4G10, and PC-PLD activities associated with the immunoprecipitates were measured by assessing the amount of free choline release from PC. In both cytosol and membrane extracts (Table 1), significantly higher amounts of PC-PLD activities were immunoprecipitated from TNP–OVA-stimulated cells than from unstimulated cells. As a control, no significant amounts of PC-PLD activity were immunoprecipitated by an unrelated antibody, IE5 (results not shown). To confirm that the observed activity was indeed PC-PLD, we assayed the immunoprecipitates in the above fractions by determination of phosphatidylethanol formation *in vitro*. Under these conditions, we again observed higher PC-PLD activities in the immunoprecipitates from the TNP–OVA-stimulated cells compared with unstimulated cells (control cytosol 46.9 c.p.m./10^7 cells; treated cytosol 696.8 ± 174.9 c.p.m./10^7 cells; control membrane 132.8 ± 17.2 c.p.m./10^7 cells; treated membrane 685.0 ± 144.4 c.p.m./10^7 cells; means ± S.E.M. of duplicate experiments). These results strongly suggest that PC-PLD was either directly tyrosine-phosphorylated or tightly bound to a protein that was phosphorylated upon stimulation. Thus both cytosolic and membrane-bound PC-PLD activities appear to be regulated by tyrosine phosphorylation upon $Fc_\epsilon RI$ aggregation.

DISCUSSION

We have examined the possibility that the signal-transduction cascade leading to the production of DAG in RBL2H3 cells following $Fc_\epsilon RI$ aggregation is dependent on the initial activation of tyrosine kinases. In order to correlate the time course of tyrosine phosphorylation with DAG production and histamine release, we selected a concentration of TNP–OVA (10 ng/ml) that was optimal for histamine release (Lin et al., 1991). We have demonstrated, in the current and previous studies (Lin et al., 1991, 1992a), that there is a rapid biphasic increase in DAG production. The initial peak increase is observed within 5 s after receptor aggregation and maximizes within 30 s, whereas the second increase maximizes after 5 min and then remains elevated for at least 30 min.

When the RBL 2H3 cells were triggered with TNP–OVA, we observed an increase in tyrosine phosphorylation mainly on 72 and 110 kDa proteins. It was first detected within 10–30 s, reached a plateau between 5 and 10 min, and then remained elevated for the duration of the experiments (up to 30 min). This time course of tyrosine phosphorylation agrees well with previous studies (Benhamou and Siraganian, 1992). Thus, during the first phase of DAG production, the extent of tyrosine phosphorylation is small. However, it is possible that some proteins not readily detected by SDS/PAGE are tyrosine phosphorylated (Li et al., 1992a) at these early time points. Tyrosine phosphorylation appears just to precede or to coincide with the secondary increase in DAG production.

Data obtained with the tyrosine kinase inhibitor tyrphostin 8 support differential regulation of IgE-dependent DAG production by tyrosine kinases. In the presence of tyrphostin, the second phase of DAG production was completely inhibited. In contrast, the initial phase of DAG production was still observed. As analysed by immunoblots using an anti-phosphotyrosine antibody, tyrphostin produced a concentration-dependent inhibition of all phosphorylated proteins following receptor aggregation. The results were consistent with the report by Yu et al. (1991) that tyrphostin 12 (RG 50864)) inhibits all the antigen-stimulated tyrosine-phosphorylated proteins. Results observed with other tyrosine kinase inhibitors, such as genistein (Kawakami et al., 1992; Stephan et al., 1992) and lavandustin A (Kawakami et al., 1992), were also similar. Taken together, these data suggest that tyrosine phosphorylation may influence the second, but not the initial, increase in DAG production. The mechanism of the initial production of DAG following receptor aggregation is unclear. It is possible, however, that this takes place as a consequence of phospholipase C activation. In this context, it is noteworthy that the tyrosine kinase inhibitor genistein failed to prevent the IgE-dependent activation of phosphatidylinositol-specific phospholipase C (Stephan et al., 1992; Kumada et al., 1993). Initial data from our studies have also suggested a potential role for glycosylphosphatidylinositol hydrolysis in the initial phase of DAG production (Lin et al., 1992b).

It has recently been shown in RBL 2H3 cells that a *src*-protein-related tyrosine kinase, $p56^{lyn}$, can associate with $Fc_\epsilon RI$ and

Table 1 Immunoprecipitation of PC-PLD activity with an anti-phosphotyrosine antibody

RBL 2H3 cells were sensitized with anti-TNP IgE, incubated with or without TNP–OVA (10 ng/ml) for 30 min, and then lysed and fractionated. PC-PLD activities in cytosolic and membrane fractions were then determined after immunoprecipitation with an anti-phosphotyrosine antibody (4G10) as described in the Methods section. The results are means ± S.E.M. ($n = 4$). For both fractions the PC-PLD activity recovered from the immunoprecipitate of the TNP–OVA-treated cells was significantly higher than the activity recovered from the control cells ($P < 0.05$, Student's two-tailed t test for paired samples). There was no significant difference between control and treated activities when cellular proteins were similarly treated with a control antibody (IE5, anti-E selection antibody) (results not shown).

	Choline liberated (c.p.m./10^7 cells)	
Fraction	Control	TNP–OVA
Membrane	1263.0 ± 289.2	3464.5 ± 530.4
Cytosol	2061.3 ± 551.0	4575.8 ± 875.5

become activated after receptor engagement (Eiseman and Bolen, 1992). In BALB/c 3T3 cells, it has been shown that v-*src* tyrosine kinase increases DAG levels via PC-PLD activation (Song et al., 1991). PC-PLD may also be regulated by tyrosine phosphorylation in neutrophils and HL-60 cells (Uings et al., 1992; Bourgoin and Grinstein, 1992). Our previous studies have suggested that PC-PLD is responsible for the secondary increase in DAG following $Fc_\epsilon RI$ aggregation in RBL 2H3 cells. We therefore explored the possibility that PC-PLD activation is dependent on the activation of tyrosine kinases. As previously described (Lin et al., 1991, 1992a; Lin and Gilfillan, 1992), PC-PLD was rapidly activated after $Fc_\epsilon RI$ aggregation. The activation could be inhibited by tyrphostin over a concentration range similar to that observed for inhibition of tyrosine phosphorylation. Although the recent study by Kumada et al. (1993) suggests that PC-PLD activation is dependent on tyrosine phosphorylation, these results may be open to other interpretations, as the pharmacological agents used may not be entirely specific for tyrosine kinases. For this reason, we extended our studies by determining whether PC-PLD activity could be recovered from RBL 2H3 protein after immunoprecipitation by an anti-phosphotyrosine antibody. Utilizing this approach, we could demonstrate that the PC-PLD activation produced by IgE-receptor aggregation could be recovered from both the membrane and the cytosolic fractions after immunoprecipitation of the tyrosine-phosphorylated proteins. Wang et al. (1991) have reported that mammalian PC-PLD exists in multiple forms in the cytosol, as well as in association with membranes. Although the identities of these different forms of PC-PLD are currently unknown, both cytosolic and membrane-bound PC-PLDs appear to be regulated by tyrosine phosphorylation upon stimulation. Taken together, the above results demonstrate that the PC-PLD activated upon IgE-receptor aggregation in RBL 2H3 cells is a consequence of direct tyrosine-phosphorylation of the enzyme or of phosphorylation of another protein that is tightly bound to the enzyme.

In conclusion, the results of this study provide evidence that production of DAG upon $Fc_\epsilon RI$ aggregation is, in part, regulated by the activation of tyrosine kinases. Although the initial phase of DAG production appears to be independent of tyrosine kinase activation, the secondary phase of DAG production, which is a consequence of PC-PLD activation, depends on initial activation of tyrosine kinases. This, and supporting data, suggest that PC-PLD may be activated by tyrosine phosphorylation. However, as our previous studies suggest that intracellular [Ca^{2+}] and protein kinase C may also influence cellular PC-PLD activity (Lin et al., 1992a; Lin and Gilfillan, 1992), it is likely that more than one pathway exists for regulating PC-PLD activity in RBL 2H3 cells.

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