

Evidence for Two Distinct Phosphorylation Pathways Activated by High Affinity Immunoglobulin E Receptors*

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The high affinity receptor for immunoglobulin (Ig) E on mast cells, along with the antigen receptors on T and B cells and Fc receptors for IgG, belongs to a class of receptors which lack intrinsic kinase activity, but activate non-receptor tyrosine and serine/threonine kinases. Receptor engagement triggers a chain of signaling events leading from protein phosphorylation to activation of phosphatidylinositol-specific phospholipase C, an increase in intracellular calcium levels, and ultimately the activation of more specialized functions. IgE receptor disengagement leads to reversal of phosphorylation by undefined phosphatases and to inhibition of activation pathways. Here we show that phenylarsine oxide, a chemical which reacts with thiol groups and has been reported to inhibit tyrosine phosphatases, uncouples the IgE receptor-mediated phosphorylation signal from activation of phosphatidylinositol metabolism, the increase in intracellular calcium levels, and serotonin release. Phenylarsine oxide inhibits neither the kinases (tyrosine and serine/threonine) phosphorylating the receptor and various cellular substrates nor, unexpectedly, the phosphatases responsible for the dephosphorylation following receptor disengagement. By contrast, it abolishes the receptor-mediated phosphorylation of phospholipase C- γ 1, but not phospholipase C activity *in vitro*. Therefore the phosphorylation and activation of phospholipase C likely requires a phenylarsine oxide-sensitive element. Receptor aggregation thus activates at least two distinct phosphorylation pathways: a phenylarsine oxide-insensitive pathway leading to phosphorylation/dephosphorylation of the receptor and of various substrates and a sensitive pathway leading to phospholipase C- γ 1 phosphorylation.

Phosphorylation of receptors and other proteins is a feature of cell activation in a variety of systems. One class of such receptors, exemplified by the growth factor receptors, is defined by an intrinsic kinase activity. Receptors of the other class lack intrinsic kinase activity and mediate phosphorylation by activating non-receptor kinases. Among the members of this latter class are the antigen receptors on T and B cells,

and the Fc receptors for immunoglobulin (Ig)¹ E (high affinity Fc ϵ RI) and IgG (low affinity Fc γ RII and -III). These receptors share similarities in structural elements and in their signaling mechanisms. For example, Fc ϵ RI, Fc γ RIII, and some forms of the T cell antigen receptor have a common subunit identified as Fc ϵ RI- γ (Blank *et al.*, 1989; Ra *et al.*, 1989; Hibbs *et al.*, 1989; Kurosaki and Ravetch, 1989; Anderson *et al.*, 1990; Lanier *et al.*, 1989; Orloff *et al.*, 1990). This subunit and the homologous T cell antigen receptor ζ and η subunits (Weissman *et al.*, 1988; Jin *et al.*, 1990) contain amino acid motifs which function as transducing elements (Reth, 1989; Romeo and Seed, 1991; Chan *et al.*, 1991; Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Koyasu *et al.*, 1992; Wegener *et al.*, 1992).

Following receptor engagement, different members of this family of receptors physically associate with and activate a variety of *src*-related tyrosine kinases. Such an interaction has been demonstrated for the T cell antigen receptor and *fyn* kinase (Samelson *et al.*, 1990), the B cell antigen receptor and *fyn*, *lyn*, and *blk* kinases (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991) and Fc ϵ RI and *lyn* and *yes* (Eiseman and Bolen, 1992). These and possibly other kinases phosphorylate a number of cellular substrates (Hsi *et al.*, 1989; June *et al.*, 1990a; Benhamou *et al.*, 1990), among them receptor subunits and the γ 1 isoform of phosphatidylinositol-specific phospholipase C (PLC- γ 1). PLC- γ 1 is activated by phosphorylation to hydrolyze phosphoinositides such as phosphatidylinositol (PI) to inositolphosphates (Kim *et al.*, 1991; Weiss *et al.*, 1991; Park *et al.*, 1991; Mustelin *et al.*, 1990; Nishibe *et al.*, 1990). Products of phosphoinositide hydrolysis cause the increase in intracellular Ca²⁺ that ultimately leads to cell type-specific effector functions (Penner *et al.*, 1988). For the antigen receptor on T and B cells, the obligatory involvement of a protein tyrosine phosphatase, leucocyte common antigen (CD45), in signal transduction has been demonstrated (Koretzky *et al.*, 1990, 1991; Justement *et al.*, 1991). Antibodies against CD45 also inhibit Fc ϵ RI-mediated activation of human basophils (Hook *et al.*, 1991). However, the role of CD45, and in particular its position in the signaling cascade, is not yet understood. (Trowbridge, 1991; Klausner and Samelson, 1991).

One way to study the involvement of particular reactions in signal transduction is the use of inhibitors. Studies using genistein and herbimycin A, inhibitors of tyrosine kinases, were among the first to argue for a role of tyrosine phos-

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¹ The abbreviations used are: Ig, immunoglobulin; DNP, dinitrophenyl; HSA, human serum albumin; Fc ϵ RI, high affinity receptor for immunoglobulin E; Fc γ RII, Fc γ RIII, low affinity receptors for immunoglobulin G; PAO, phenylarsine oxide; PI, phosphatidylinositol; PLC, phospholipase C; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenedibis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

phorylation in signaling by the T cell antigen receptor and Fc ϵ RI (Mustelin *et al.*, 1990; Trevillyan *et al.*, 1990; June *et al.*, 1990b; Deanin *et al.*, 1991; Park *et al.*, 1991). Recently, it has been shown that the thiol-reactive chemical phenylarsine oxide (PAO) inhibits signaling by the insulin receptor (Bernier *et al.*, 1988; Medema *et al.*, 1991). PAO in micromolar concentrations is a potent inhibitor of tyrosine-specific phosphatases, including CD45 (Garcia-Morales *et al.*, 1990), although at millimolar concentrations it also affects numerous other enzymes (reviewed in Webb, 1966). PAO-induced changes in the level and pattern of protein tyrosine-phosphorylation are observed not only for insulin receptor signaling, which involves a receptor tyrosine kinase, but also in T cells (Levenson and Blackshear, 1989; Garcia-Morales *et al.*, 1990).

Mast cells are especially suitable for studying signal transduction involving non-receptor kinases and phosphatases because aggregation of the physiological receptor leads to rapid and easily measured activation reactions. Further, a unique feature of the system allows a direct visualization of phosphatase activity: when monomeric hapten is used to interrupt stimulation by polymeric antigen, immediate dephosphorylation of the receptor and of cellular proteins is observed (Pardini *et al.*, 1991). We were interested in the role of phosphatases in mast cell activation and the effect of PAO on dephosphorylation and signal transduction. We report here that PAO uncouples the receptor from later events in cell activation by inhibiting phosphorylation and activation of PLC- γ 1, but not phosphorylation/dephosphorylation of the receptor and other cellular substrates.

EXPERIMENTAL PROCEDURES

Materials—Media and sera were purchased from Biofluids, Rockville, MD, dinitrophenyl[30]human serum albumin (DNP-HSA, 30 molecules of DNP/molecule of HSA) and ϵ -dinitrophenyl-lysine from Sigma, phenylarsine oxide from Aldrich, and Dowex AG X1-8 resin from Bio-Rad. Radiochemicals were purchased either from Amersham Corp. ([^{32} P]orthophosphate, cat. no. PBS.13A) or Du Pont-New England Nuclear ([^{3} H]serotonin, cat. no. NEC-225, [^{3} H]phosphatidylinositol, cat. no. NET 862, [^{3} H]-myo-D-inositol, cat. no. NET-114A). The anti-phosphotyrosine antibody 4G10 and mixed monoclonal antibodies against phospholipase C- γ 1 were from UBI, Lake Placid, NY; anti-Fc ϵ RI β subunit antibody JRK (Rivera *et al.*, 1988) was described previously. DNP-specific monoclonal mouse IgE Hi-DNP ϵ 26.82 (Liu *et al.*, 1980) was purified from supernatant of hybridoma cells grown in a mini flo-path bioreactor (Amicon) by ion-exchange chromatography on diethylaminoethyl-Trisacryl (IBF). The chemiluminescent substrate for Western blotting, AMPPD, was purchased from Tropix, Boston, MA.

Cell Culture—A subline of RBL-2H3 with high ability to release serotonin was obtained from B. Rivnay, Procept, Boston, MA. Cells were maintained as adherently growing cells in minimal essential medium with Earle's salts, 16% fetal calf serum in a humidified atmosphere of 5% CO $_2$, 95% air. For passaging and where required by experiments, cells were detached with trypsin-EDTA for 5–6 min at 37 °C. The mouse mast cell line MC C57 (Young *et al.*, 1987) was obtained from S. J. Galli, Beth Israel Hospital, Boston, MA. These cells were maintained in suspension culture in Iscove's modified minimal essential medium with 10% fetal calf serum in a humidified atmosphere of 5% CO $_2$, 95% air.

Serotonin Release Experiments—For RBL cells, 0.5 ml of a suspension containing 4 $\times$ 10 ^{6} cells and 1 μ Ci of [^{3} H]serotonin/ml was plated into each well of a 24-well plate. Plates were incubated in a humidified atmosphere of 5% CO $_2$, 95% air for 18 h, for the last 2 h additionally with 2 μ g/ml of IgE. Prior to triggering, wells were washed twice with 0.5 ml of assay buffer (25 mM Na $_2$ PIPES, pH 7.1, 100 mM NaCl, 5 mM KCl, 0.4 mM MgCl $_2$, 1 mM CaCl $_2$, 5 mM D-glucose, and 0.1% BSA). The assay volume was 0.2 ml/well. Cells were incubated at 37 °C for 2 min with phenylarsine oxide or vehicle and then triggered with DNP-HSA or ionomycin in the amount indicated for individual experiments. After 20 min at 37 °C (or as indicated for the time course), the reaction was stopped by removing the supernatant and

centrifuging it for 15 s in a microfuge to remove any detached cells. The radioactivity in the supernatant was measured to determine released serotonin. Residual serotonin in cells was measured by lysing cells with 1% Triton X-100 in phosphate-buffered saline, washing wells twice, and determining radioactivity in the combined lysate and wash fluid. The fraction of serotonin that was released was calculated by dividing the amount of radioactivity in the supernatant by the amount of total radioactivity. For MC C57, the assay was modified slightly to accommodate cells growing in suspension.

Cytoplasmic Ca $^{2+}$ Measurements—RBL cells, which previously had been trypsinized and then saturated with 2 μ g/ml IgE for 2 h, were loaded at 5 $\times$ 10 ^{6} cells/ml for 45 min at 37 °C with the acetoxymethyl ester of fura-2 (2 μ M). The buffer was identical to the one used for serotonin release except for the addition of 2.5 mM Probenecid. Cytoplasmic free Ca $^{2+}$ was measured on a Photon Technology International Deltascan 4000 spectrofluorimeter (cuvette system) by comparing the fluorescence emission at 500 nm of a cell suspension alternately excited at 340 and 380 nm. Cells were preincubated with 10 μ M phenylarsine oxide or vehicle for 2 min and then triggered with 100 ng/ml DNP-HSA.

Phosphoinositide Metabolism in Cells—4 $\times$ 10 ^{7} adherently growing RBL cells were labeled with 0.4 μ Ci/ml of [^{3} H]-myo-D-inositol for 18 h. Cells were detached with trypsin-EDTA as described above, washed twice in complete medium, and incubated with 2 μ g/ml IgE for 2 h. Prior to triggering, wells were washed twice with 0.5 ml of assay buffer which was the same as for serotonin release experiments except for the further addition of 10 mM LiCl (25 mM diNaPIPES, pH 7.1, 100 mM NaCl, 5 mM KCl, 0.4 mM MgCl $_2$, 1 mM CaCl $_2$, 10 mM LiCl, 5 mM D-glucose, and 0.1% BSA). Cells were then resuspended at 1 $\times$ 10 ^{6} /ml in assay buffer. The assay volume was 200 μ l/sample. Cells were preincubated with phenylarsine oxide or vehicle at 37 °C for 2 min before triggering with 100 ng/ml DNP-HSA. At different time points after triggering, the reaction was stopped by the sequential addition of 750 μ l of chloroform/methanol/4 N HCl (100:200:4), 250 μ l of water, and 250 μ l of chloroform. The resulting phases were separated, and radioactivity in the organic phase was determined directly. The aqueous phase was subjected to Dowex anion-exchange chromatography essentially as described (Maeyama *et al.*, 1986), to separate myo-inositol from inositolphosphates. This assay measures total PI hydrolysis as opposed to specific isomers and has been shown to be a direct measurement of Fc ϵ RI-mediated activation of RBL cells (Maeyama *et al.*, 1986, 1988). PI hydrolysis was calculated by dividing the amount of radioactivity of inositolphosphates in the aqueous phase by the total amount of radioactivity in both the aqueous and the organic phase.

Assay of Phospholipase C Activity in Vitro—RBL cells were detached with trypsin-EDTA as described, washed once with phosphate-buffered saline, and once with 50 mM HEPES, pH 7.3, 1 mM EGTA, and resuspended in this buffer at 4 $\times$ 10 ^{6} /ml. They were then homogenized with 10 strokes in a Dounce homogenizer. The resulting whole cell extract was preincubated for 2 min with PAO or vehicle. To start the enzymatic reaction, 50 μ l of this cell extract was added to a reaction mixture containing the substrate, buffer, and PAO or vehicle. The volume was 200 μ l/assay, the final concentration was 1 $\times$ 10 ^{6} cell equivalents/ml, 50 mM HEPES, pH 7.3, 1 mM EGTA, 1.5 mM CaCl $_2$, 1 mg/ml deoxycholate, 100 μ g/ml phosphatidylinositol, and 2 μ l/ml [^{3} H]phosphatidylinositol. The assay was stopped after different timespans by the sequential addition of 750 μ l of chloroform/methanol/4 N HCl (100:200:4), 250 μ l of water, and 250 μ l of chloroform. The resulting phases were separated, and radioactivity in each phase was measured separately. Percent product was calculated by dividing the amount of radioactivity of inositolphosphates in the aqueous phase by the total amount of radioactivity in both the aqueous and the organic phase.

Receptor Phosphorylation Experiments—RBL-2H3 cells were incubated in phosphate free medium for 4 h at 37 °C before addition of 2 μ g/ml of monomeric monoclonal anti-DNP IgE for 1 h at 37 °C, then labeled at 3 $\times$ 10 ^{7} cells/ml with 37 MBq/ml [^{32} P]orthophosphate for 2 h at 37 °C. As indicated for individual experiments, some samples (5 $\times$ 10 ^{7} cells/sample) were incubated for 2 min with phenylarsine oxide and then for 1 min with 100 ng/ml of DNP-HSA, followed by 50 μ M DNP-lysine for 30 s. The cells were immediately washed with 50 ml of ice-cold PBS containing 1 mM sodium vanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, and 5 mM EDTA. Cells were lysed in a buffer, pH 8.0, containing 200 mM boric acid, 160 mM NaCl, 0.5% Triton X-100, 1% BSA, 10 mM iodoacetamide, phosphatase inhibitors as above, and protease inhibitors: 10 μ g/ml aprotinin, 4 μ g/ml leupeptin, 10 μ g/ml pepstatin, 1 mM phenylmethylsulfonyl

fluoride. The postnuclear supernatants were precleared with protein A-Sepharose beads and sequentially immunoprecipitated first with nonspecific rabbit IgG and then with rabbit polyclonal anti-IgE bound to protein A-Sepharose. The beads were washed with lysis buffer and the immunoprecipitates were analyzed on 13.5% SDS-gel electrophoresis under reducing conditions before autoradiography. Radioactivity in the regions of the gel corresponding to FcεRI-β and -γ was also quantitated directly using a radioanalytic imaging system (AMBIS Systems, San Diego, CA). For Western blotting, the postnuclear supernatants from $1.5 \cdot 10^7$ cells were immunoprecipitated and analyzed by SDS-gel electrophoresis as above, and the proteins were transferred electrophoretically to Immobilon-P membranes for 6 h at 50 V. The membrane was incubated overnight in blocking buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% BSA) before a 2-h incubation in the same buffer containing 15 μg/ml anti-β or 6 μg/ml anti-phosphotyrosine antibody. After two washes in washing buffer (as above but with 0.1% Triton X-100 and 2.5% BSA), the membranes were incubated with a 1:3000 dilution of rabbit anti-mouse antibody conjugated to alkaline phosphatase and developed with the chemiluminescent substrate AMPPD.

Tyrosine Phosphorylation of Phospholipase C-γ1 and Other Substrates—RBL cells ($1 \cdot 10^8$ cells/sample) were incubated with phenylarsine oxide, antigen, and hapten as described for receptor phosphorylation experiments and lysed in the same lysis buffer but with 1% Triton X-100. Tyrosine-phosphorylated proteins were precipitated with monoclonal anti-phosphotyrosine 4G10 on protein A beads and recovered after washing with lysis buffer by incubating the beads with 10 mM phenylphosphate for 10 min. From this PLC-γ1 was precipitated with mixed monoclonal antibodies to bovine PLC-γ1 bound to protein G beads. The supernatant of this precipitation was analyzed on a 9% reducing SDS-gel electrophoresis, the precipitate on a 6% gel. Transfer to membranes and detection of total tyrosine-phosphorylated proteins with 4G10 and PLC-γ1 with anti-bovine PLCγ1 were as described above.

Representation of Data—The error bars in the graphs represent the standard deviations of triplicate experiments.

RESULTS

Inhibition of Receptor-mediated Serotonin Release from Mast Cell Lines—We first investigated the effect of PAO on mediator release from mast cells. RBL or MC C57 cells were incubated with saturating amounts of DNP-specific monoclonal mouse IgE. Receptors were then cross-linked via IgE with multivalent antigen DNP-HSA. When IgE-saturated RBL cells were preincubated with PAO for 2 min before triggering with antigen in the continued presence of PAO, serotonin release was inhibited in a dose-dependent way (Fig. 1c). This inhibition was observed over a wide range of antigen concentrations including concentrations suboptimal for serotonin release (Fig. 1a). The viability of cells, as assessed by trypan blue dye exclusion, did not change during the experiment. Washing the cells twice with reaction buffer without PAO and conducting the experiment in the absence of PAO did not reverse the effects of the initial preincubation. MC C57 cells, which were treated analogously and triggered with 1 ng/ml of DNP-HSA or less, displayed greater sensitivity to PAO (Fig. 2, a and c).

Kinetics of Inhibition—We then determined the minimum time required for PAO to take effect by adding the inhibitor to an ongoing serotonin release reaction (Fig. 3). RBL cells, which had been saturated with IgE, were triggered with 100 ng/ml antigen, and release was allowed to proceed for 2 min. Upon addition of PAO, further serotonin release was instantaneously reduced almost to the level of spontaneous release, whereas control cells continued to release.

Effect of Phenylarsine Oxide on Ionomycin-mediated Release—In order to localize the site of action of PAO in the signaling cascade, we examined whether the cells' ability to respond to increased intracellular Ca^{2+} was affected by the inhibitor. Both RBL and MC C57 were triggered by ionomycin, an ion channel that allows the influx of Ca^{2+} from the

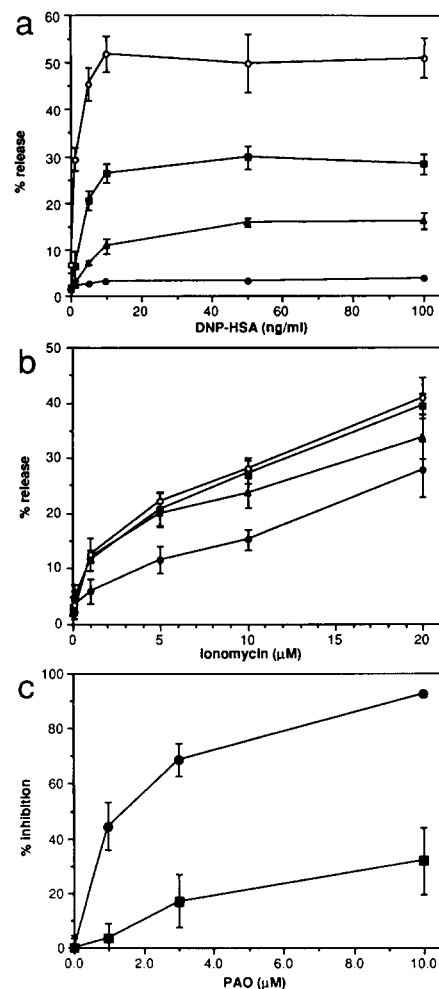


FIG. 1. Inhibition of serotonin release from RBL cells. Cells were labeled with [^3H]serotonin and saturated with 2 μg/ml DNP-specific IgE. Before triggering with DNP-HSA (panel a) or ionomycin (panel b) at the concentrations indicated, they were preincubated for 2 min with PAO or vehicle (open circle, no PAO; filled square, 1 μM; filled triangle, 3 μM; filled circle, 10 μM). The amount of [^3H]serotonin released into the media is displayed as percentage of total [^3H]serotonin. Panel c, data from panels a and b were replotted to show concentration dependence of inhibition. Filled circle, triggering by 100 ng/ml DNP-HSA; filled square, triggering by 20 μM ionomycin.

media, bypassing receptor activation. Cells were still able to respond to Ca^{2+} at all concentrations of PAO and ionomycin employed, but some inhibition was observed (Figs. 1b and 2b). In both cell lines this inhibition was significantly lower than that of receptor-mediated release (Figs. 1c and 2c). The inhibition of the cellular response to ionomycin was thus insufficient to explain the inhibition of receptor-mediated release, indicating that PAO inhibited an element upstream of the Ca^{2+} signal in the signaling cascade.

Effect of Phenylarsine Oxide on Receptor-mediated Ca^{2+} -Signal and PI Turnover—Concentrating our efforts on the examination of the receptor-specific inhibition in RBL cells, we measured the effect of PAO on the Ca^{2+} response and PI turnover. RBL cells were saturated with IgE, loaded with the Ca^{2+} -sensitive fluorochrome fura-2 and analyzed spectrofluorimetrically. PAO (10 μM) or vehicle was added for 2 min before triggering of the cells with 100 ng/ml DNP-HSA. The FcεRI-mediated increase in intracellular Ca^{2+} was almost completely abolished by PAO (Fig. 4). To test whether phosphoinositide metabolism was similarly affected, RBL cells, which had been grown in medium containing [^3H]inositol,

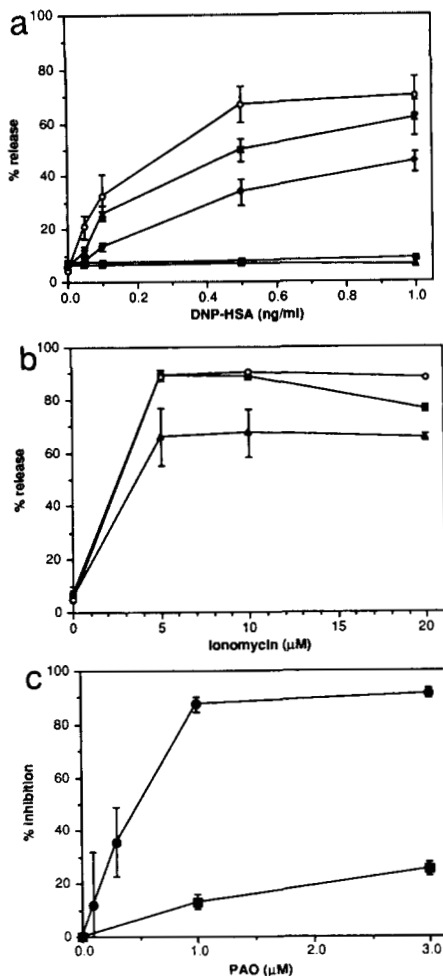


FIG. 2. Inhibition of serotonin release from MC C57 cells. Cells were labeled with [^{3} H]serotonin and saturated with 2 μ g/ml DNP-specific IgE. Before triggering with DNP-HSA (panel a) or ionomycin (panel b) in the concentrations indicated, they were preincubated for 2 min with PAO or vehicle (open circle, no PAO; $\times$, 0.1 μ M; filled diamond, 0.3 μ M; filled square, 1 μ M; filled triangle, 3 μ M). The amount of [^{3} H]serotonin released into the media is displayed as percentage of total [^{3} H]serotonin. Panel c, data from panels a and b were replotted to show concentration dependence of inhibition. Filled circle, triggering by 1.0 ng/ml DNP-HSA; filled square, triggering by 20 μ M ionomycin.

were preincubated with PAO and stimulated in the conditions used for the Ca^{2+} response. The production of water soluble [^{3} H]inositol phosphates in intact cells was monitored. PAO inhibited phosphoinositide metabolism almost completely at 10 μ M and partially at lower concentrations (Fig. 5a).

Phospholipase C Activity in Vitro Is Not Inhibited by Phenylarsine Oxide—To rule out a direct effect of PAO on PI-specific phospholipase C, the effect of the inhibitor on PLC activity was tested *in vitro*. For these assays a whole-cell extract of non-activated RBL cells was used as enzyme source. The extract was preincubated for 2 min with PAO at the concentrations used in the previous experiments or with vehicle. [^{3} H]PI was used as substrate. No significant inhibition at any of the inhibitor concentrations could be observed (Fig. 5b).

Effect of Phenylarsine Oxide on Receptor Phosphorylation and Dephosphorylation—To test whether PAO affected the phosphorylation or dephosphorylation of receptor β and γ subunits, we saturated RBL cells with IgE and labeled them *in vivo* with [^{32} P]orthophosphate. With or without preincu-

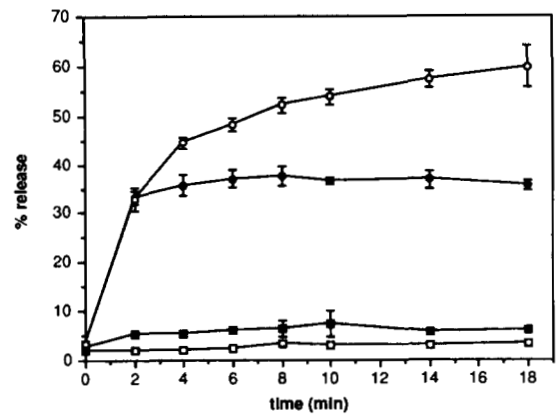


FIG. 3. Rapid action of PAO. Cells were labeled with [^{3} H]serotonin and saturated with 2 μ g/ml DNP-specific IgE. They were triggered at 0 min with 100 ng/ml DNP-HSA (open circles). PAO (10 μ M) was added to other samples at 2 min (closed circles) or 2 min before triggering (closed squares). Spontaneous release (open squares). The amount of [^{3} H]serotonin released into the media is displayed as percentage of total [^{3} H]serotonin.

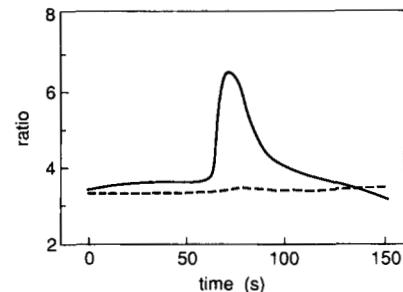


FIG. 4. Inhibition of receptor-stimulated increase in free cytoplasmic Ca^{2+} . RBL cells, which previously had been saturated with IgE, were loaded with fura-2 (2 μ M). Cytoplasmic free Ca^{2+} was measured on a Photon Technology International Deltascan 4000 spectrofluorimeter (cuvette system). Cells were preincubated with 10 μ M PAO (dotted line) or vehicle (solid line) for 2 min before triggering with 100 ng/ml DNP-HSA at 60 s of data acquisition. Data are presented as the ratio of fluorescence emitted upon alternate excitation at 340 and 380 nm.

bation with PAO, cells were stimulated with multivalent antigen DNP-HSA for 1 min, and some samples were then treated with monovalent hapten DNP-lysine for 30 s to disengage receptors. The receptor subunits were coprecipitated with an anti-IgE antiserum and separated in reducing SDS-gel electrophoresis. Gels were analyzed by autoradiography (Fig. 6a). Radioactivity in the regions of the gel corresponding to Fc ϵ RI- β and - γ was also quantitated directly using a radioanalytic imaging system (Fig. 6d). Aliquots of the same immunoprecipitates were transferred to membranes after SDS-gel electrophoresis and probed with antibodies to phosphotyrosine to determine tyrosine-specific phosphorylation of the β subunit (Fig. 6b). Under these conditions tyrosine phosphorylation on γ subunits cannot be detected with anti-phosphotyrosine antibodies. The same blot was also probed with antibodies to Fc ϵ RI β to demonstrate loading of equal amounts of protein (Fig. 6c). Compared to the corresponding samples from untreated cells, the level of total and tyrosine-specific phosphorylation on β and of total phosphorylation on γ were elevated in PAO-treated cells. This is the case for the basal level in resting cells and for the increase in specific activity upon stimulation. However, the particular phosphatase activity responsible for the return of receptor subunit phosphorylation to resting levels after receptor disengagement was undiminished.

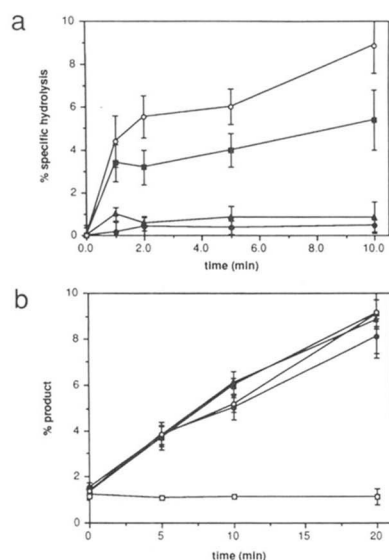


FIG. 5. Inhibition of receptor-stimulated PI turnover, but not PLC activity. *Panel a*, PI turnover in RBL cells. Cells were labeled with [^{3} H]myo-inositol and saturated with 2 μ g/ml DNP-specific IgE. Before triggering with DNP-HSA (100 ng/ml), they were preincubated for 2 min with PAO or vehicle (open circle, no PAO; filled square, 1 μ M; filled triangle, 3 μ M; filled circle, 10 μ M). The amount of water-soluble [^{3} H]inositol phosphates is displayed as percentage of total [^{3} H] activity in the cells. *Panel b*, effect of PAO on basal PLC activity *in vitro*. A whole cell extract of RBL cells was preincubated for 2 min with PAO or vehicle (open circle, no PAO; filled square, 1 μ M; filled triangle, 3 μ M; filled circle, 10 μ M) and then incubated with [^{3} H]PI. Production of water-soluble [^{3} H]inositol phosphates was monitored.

Effect of Phenylarsine Oxide on Tyrosine Phosphorylation of Various Substrates—Although phosphorylation of the receptor itself is not inhibited, there was the possibility that phosphorylation of cellular substrates was impaired. In preliminary experiments, we compared substrate phosphorylation in RBL cells by precipitating all tyrosine-phosphorylated cellular proteins with anti-phosphotyrosine antibody, separating them by SDS-gel electrophoresis, and revealing them on Western blots with the same antibody. In the presence of PAO, there was increased tyrosine phosphorylation of a number of substrates including pp72 and Fc ϵ RI- β both before and after receptor engagement, which returned to base levels after receptor disengagement (data not shown). However, under these conditions PLC- γ 1 phosphorylation could not be observed because of the very low abundance of the enzyme in RBL cells (Park *et al.*, 1991²). To increase the sensitivity of detection, total tyrosine phosphorylated proteins were immunoprecipitated from lysates of triggered and non-triggered cells with anti-phosphotyrosine monoclonal antibody and recovered by elution with phenylphosphate. Tyrosine-phosphorylated PLC- γ 1 was precipitated from this preparation with anti-PLC- γ 1 monoclonal antibody. PLC- γ 1 and the remaining supernatant were separated by SDS-gel electrophoresis on separate gels and analyzed in Western blot. As in the preliminary experiments, in PAO-treated samples the level of total tyrosine phosphorylation in resting cells and the increase upon stimulation were slightly elevated (Fig. 7a). However, PLC- γ 1 from the same samples showed the opposite pattern: its tyrosine-phosphorylation upon activation was inhibited in PAO-treated cells (Fig. 7b).

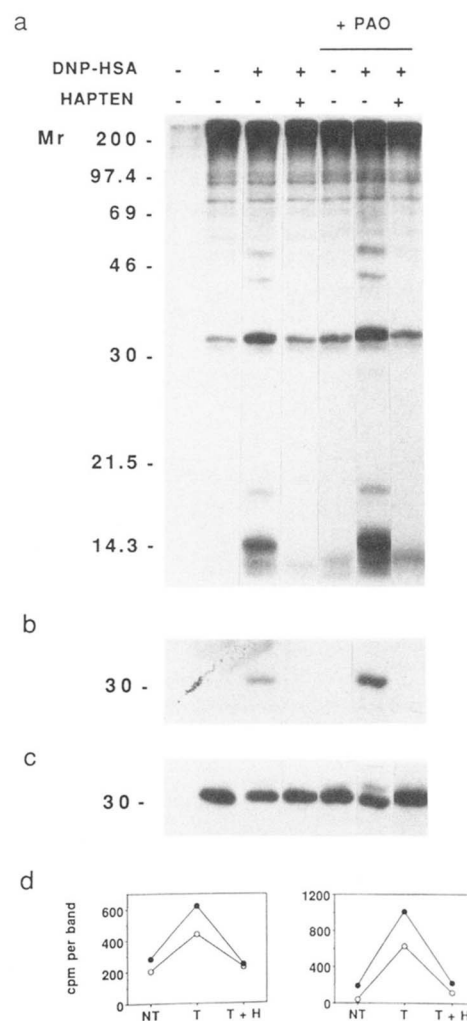


FIG. 6. Effect of PAO on phosphorylation of β and γ chains. *Panel a*, autoradiograph of *in vivo* phosphorylated, immunoprecipitated β and γ . RBL-2H3 cells were incubated with IgE and labeled with [^{32} P]orthophosphate. As indicated for individual lanes, samples ($5 \cdot 10^7$ cells/sample) were incubated for 2 min with 10 μ M PAO and then for 1 min with 100 ng/ml of DNP-HSA, followed by 50 μ M DNP-lysine for 30 s. Receptor subunits were immunoprecipitated from cell lysates with rabbit polyclonal anti-IgE, as control (leftmost lane) with nonspecific rabbit IgG. Immunoprecipitates were analyzed on 13.5% SDS-gel electrophoresis under reducing conditions before autoradiography. *Panel b*, Western blot of tyrosine phosphorylation on β subunits from the same immunoprecipitates as above. For Western blotting, the postnuclear supernatants from $1.5 \cdot 10^7$ cells were transferred to membranes after SDS-gel electrophoresis. Tyrosine phosphorylation on β was revealed with monoclonal anti-phosphotyrosine and a secondary antibody coupled to alkaline phosphatase. *Panel c*, Western blot demonstrating equal amounts of β subunits. The membrane described above was stripped and reincubated with monoclonal anti- β antibody JRK. *Panel d*, results of the quantitative analysis of phosphorylation levels of β (left panel) and γ (right panel). The gel shown under panel a was analyzed with a radioanalytic imaging system (open circles, no PAO; filled circles, 10 μ M PAO).

DISCUSSION

We have shown previously that the phosphorylation status of receptor β (serine and tyrosine) and γ (threonine and tyrosine) subunits reflects the balance of kinase and phosphatase activities: receptors are phosphorylated in response to engagement and dephosphorylated upon disengagement (Paolini *et al.*, 1991). The simplest explanation of this assigns phosphatases the role of returning the system to its non-activated state. This is in apparent contradiction to the fact

² M. Adamczewski, R. Paolini, and J.-P. Kinet, unpublished observations.

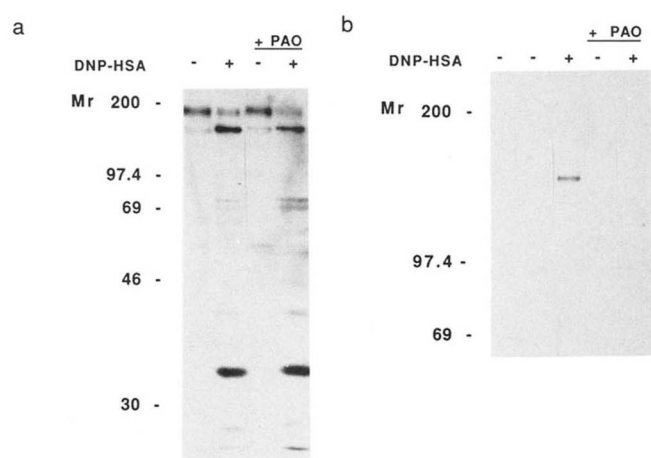


FIG. 7. Effect of PAO on phosphorylation of cellular substrates and PLC- γ 1. RBL cells ($1 \cdot 10^8$ cells/sample) were incubated with PAO, antigen, and hapten as described for receptor phosphorylation experiments. Tyrosine-phosphorylated proteins were recovered from anti-phosphotyrosine immunoprecipitates with phenylphosphate. PLC- γ 1 was immunoprecipitated from the eluate. *Panel a*, Western blot of the supernatant of this precipitation (containing all tyrosine-phosphorylated proteins except for PLC- γ 1) separated on a 9% reducing SDS-gel electrophoresis, transferred to membranes, and detected with anti-phosphotyrosine 4G10. In different experiments there is consistently an increase in the phosphorylation of β (prominent band at 33 kDa) and pp72, however, the magnitude of the increase varies slightly. *Panel b*, Western blot of precipitates of PLC- γ 1 from the eluates described above separated on a 6% gel and detected with mixed monoclonal antibodies to bovine PLC- γ 1. The left most lane represents a control precipitation with nonspecific IgG.

that various receptors coupled to non-receptor kinases seem to require CD45 for cell activation. (Koretzky *et al.*, 1990, 1991; Justement *et al.*, 1991; Hook *et al.*, 1991).

The original intent of our study was to analyze the role of receptor dephosphorylation and to ask whether phosphatases are required for mast cell signaling. We decided to use PAO, a thiol-reactive chemical which inhibits signaling by the insulin receptor (Bernier *et al.*, 1988; Medema *et al.*, 1991) and has been shown to be an inhibitor of tyrosine-specific phosphatases, among them CD45 (Garcia-Morales *et al.*, 1990).

When we initially observed that PAO inhibited mediator release from RBL cells, our first goal was to ensure that PAO did not simply poison the cells or deprive them nonspecifically of some function required for mediator release. This concern was allayed by the following observations: 1) the cells' response to the increase in intracellular Ca^{2+} caused by ionomycin is only slightly inhibited (Figs. 1b and 2b). 2) PAO stops an ongoing release reaction almost instantly (Fig. 3). This permits us to use the shortest feasible preincubation time (2 min) in our experiments and thus minimize exposure to PAO.

The ionophore treatment also allowed us to separate the overall inhibition into two components; one that is specific to receptor-mediated release, is upstream of the Ca^{2+} signal and accounts for most of the inhibition; and one that is common to both receptor- and ionophore-mediated release and is relatively minor. (Figs. 1c and 2c). Indeed, a direct examination of the effect of PAO on early events of the signal transduction chain shows that both the activation-induced increase in PI turnover (Fig. 5a) and the increase in free Ca^{2+} (Fig. 4), which follows it, are inhibited in PAO-treated cells. However, the activation-induced phosphorylation not only of receptor β and γ chains but, surprisingly, also of all substrates detected in anti-phosphotyrosine immunoprecipitates seems not inhibited by PAO.

The second surprising finding is that, while levels of serine/threonine and tyrosine phosphorylation in unstimulated as well as stimulated cells are increased slightly (Figs. 6, a and b, and 7a), the particular phosphatase responsible for hapten-induced receptor dephosphorylation is not inhibited by PAO (Fig. 6 a and d). This observation could be explained by the action of at least two phosphatases, one sensitive to PAO, the other not. Inhibition of the sensitive activity leads to the apparent increase of phosphorylation in resting and stimulated cells, however, the insensitive activity is sufficient to completely dephosphorylate receptor subunits within the time of the experiment.

The third and most surprising finding of the study is that PAO abolishes the phosphorylation of PLC- γ 1. This contrasts with the absence of inhibition of the phosphorylation of the receptor and other substrates. All available evidence points to the γ 1 isoform of PLC being the enzyme responsible for Fc ϵ RI-mediated PI turnover in RBL cells. In these cells it is the only isoform detected by Western blot so far, it is phosphorylated upon IgE-receptor engagement, and inhibitors of tyrosine kinases abolish this phosphorylation and the consequent cell activation (Park *et al.*, 1991). Because PI-specific PLC activity *in vitro* is not affected by PAO (Fig. 5b), the activation of PLC- γ 1 (as opposed to its activity) or an even earlier step in the signal transduction chain emerges as the likely site of action of PAO.

In an analogous study of T cells stimulated with anti-Thy 1, PAO increased both the basal level and the activation-induced increase of phosphorylation of the most prominent substrates (Garcia-Morales *et al.*, 1990). However, neither the phosphorylation and function of PLC nor any later activation parameters were investigated, so it is not possible to say whether PAO inhibited signal transduction in these cells. In our case, the astonishing discrepancy between the number of proteins whose phosphorylation is not inhibited and the complete inhibition of all activation reactions leading to degranulation raises the question whether phosphorylation of all these molecules is indeed necessary for or even involved in mast cell degranulation. Furthermore, it is evidence that at least two distinct phosphorylation pathways are involved in mast cell activation: one that leads to phosphorylation of the receptor subunits and the majority of cellular substrates and is not inhibited by PAO, and the other, either completely independent or branching off from the first, leading to the phosphorylation of PLC- γ 1, but required for all the activation events commonly measured, including degranulation.

The technique of anti-phosphotyrosine immunoprecipitation and blotting detects primarily the most abundant and most heavily tyrosine-phosphorylated proteins. Of course there may be other substrates whose phosphorylation is inhibited by PAO, but, as in the case of PLC- γ 1, it may be necessary to use specific antibodies to detect these other proteins. Such proteins might even include as yet unidentified links in the phosphorylation pathway leading to PLC- γ 1.

It is tempting to speculate that the PAO-sensitive element in the second pathway is a phosphatase such as CD45, since CD45 is known to be affected by PAO and probably essential to signal transduction (Garcia-Morales *et al.*, 1990; Koretzky *et al.*, 1990, 1991; Justement *et al.*, 1991; Hook *et al.*, 1991). However, we have no proof for this and the effects of other inhibitors of phosphatases, such as H_2O_2 /vanadate, on RBL cells differ markedly from those of PAO; treatment with H_2O_2 /vanadate stimulates PLC activation and PI turnover and induces degranulation independent of receptor engagement or other stimuli (Zick and Sagi-Eisenberg, 1990). Furthermore, the engagement of Fc ϵ RI does not affect the acti-

vation of PLC induced by H_2O_2 /vanadate, so there is no inhibition of the pathway to PLC- γ 1 as we observe it.³ An alternative hypothesis is that the interaction between two early elements of the signal transduction chain is sensitive to PAO. In fact, it has been shown that the interaction of CD4 and CD8 with pp56^{lck} depends on interaction between thiol groups (Turner *et al.*, 1990) and that this interaction is sensitive to *N*-ethylmaleimide (Kanner *et al.*, 1992), which like PAO reacts with thiol groups (Webb, 1966). It will be important to distinguish between these hypotheses to better understand the inhibitory mechanism of PAO and the signaling pathways involving non-receptor kinases.

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