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PHOSPHORYLATION OF THE IgE RECEPTOR FROM IONOPHORE A23187 STIMULATED INTACT RAT MAST CELLS

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SUMMARY: Purified rat serosal mast cells were labeled either with [^{32}P]orthophosphate or [^{35}S]methionine and their receptors for immunoglobulin E were isolated by repetitive affinity chromatography. In ^{35}S-labeled receptor preparations SDS polyacrylamide gels revealed a broad receptor band, M_r 45,000 to 53,000, and two other bands, M_r 30,000 and 16,000, which apparently represent receptor-associated proteins. Only the receptor band was labeled by ^{32}P. Phosphorylation of receptor was markedly stimulated by the divalent cation ionophore A23187, a known stimulator of histamine release, with changes occuring as early as 15 seconds. This early increase in receptor phosphorylation may be involved in the control of mediator secretion.

Mast cells and basophils bear cell surface receptors specific for immunoglobulin E (IgE)[1]. Crosslinking of IgE receptors initiates the noncytotoxic release of a variety of chemical mediators from the cells (1). A number of non-immunologic stimuli, including the divalent cation ionophore A23187, produce a similar response. Cellular activation and the coupled release reaction are rapid events, with histamine release occurring within 15 to 45 seconds after the addition of the stimulating agent (2). Multiple cellular events occur in stimulated mast cells before or during mediator release including increased levels of diacylglycerol (3), altered phospholipid methylation (4), an early rise in

[1]The abbreviations used are: IgE, immunoglobulin E; BSA, bovine serum albumin; Pipes, piperazine-N-N bis 2-ethane sulfonic acid; Buffer A, 0.15 M NaCl, 0.01 M phosphate, pH 7.4; NP-40, Nonidet P-40; Buffer B, 150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 5.55 mM glucose, 5 mM sodium phosphate, 2 mM Pipes, 1 mg/ml BSA, 1 unit/ml heparin, pH 6.8; RBL-1, rat basophilic leukemia cell line; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Me_2SO, dimethyl sulfoxide.

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cyclic AMP levels (5), and a rapid influx of calcium ions (4). In this communication we describe the rapid phosphorylation of the IgE receptor in intact rat mast cells stimulated with A23187.

MATERIALS AND METHODS

Materials. Bovine serum albumin, Fraction V (BSA), piperazine-N-N bis 2-ethane sulfonic acid (Pipes), (Sigma); divalent cation ionophore A23187 (Calbiochem); Sprague-Dawley male rats (400-450 g) (Camm Research Institute, Wayne, NJ); L-[^{35}S]methionine (600-1400 Ci/mmol) (Amersham); [^{32}P]ortho-phosphoric acid (carrier-free in water) (New England Nuclear); RPMI 1640 Select-amine media (Grand Island Biological Co.).

Rat myeloma IgE was purified from LOU/M/Wsl rats bearing the IgE-producing immunocytoma IR162 (6). Rat IgE or rabbit gamma globulin (Miles) was coupled to Sepharose 2B with cyanogen bromide (7). Immediately before use, the Sepharose conjugates were preincubated with 0.15 M NaCl, 0.01 M phosphate, pH 7.4 (Buffer A) containing 10 mg/ml rabbit gamma globulin and washed twice with Buffer A containing 1% Nonidet P-40 (NP-40).

Mast cell suspensions. Mast cells were isolated from the peritoneal and thoracic cavities of rats lavaged with Buffer B (150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 5.55 mM glucose, 5 mM sodium phosphate, 2 mM Pipes, 1 mg/ml BSA and 1 unit/ml heparin, pH 6.8), utilizing 35 to 60 rats per experiment. The cells were purified on a BSA density gradient (8) and were 87 to 95% pure, by crystal violet staining, and 98% viable, by trypan blue dye exclusion.

To obtain ^{32}P-labeled cells, cells were depleted of phosphate (9) by washing twice in Buffer B without phosphate and by incubating at 2 x 10^6 cells/ml for 1 h at 37°C in the same medium. The cells were centrifuged and resuspended at 1 x 10^7 cells/ml in fresh Buffer B without phosphate containing 1.1 to 2.0 mCi/ml ^{32}P. Control studies indicated that the phosphate depletion did not interfere with subsequent histamine release. After 1 h at 37°C, the cells were centrifuged, resuspended at 1 x 10^7 cells/ml in Buffer B containing 20 μg/ml phosphatidylserine and distributed among replicate tubes. As a rule 1.2 to 10 x 10^7 cells were used for each experimental condition, equivalent to 6 to 40 rats, so that the number of experimental conditions was severely curtailed. Following a 4 min incubation at 37°C, the divalent cation ionophore A23187 in dimethyl sulfoxide (Me_2SO) was added to a final concentration of 4 μg/ml, (Me_2SO final concentration of 0.1%) a concentration of A23187 found previously to produce marked release of histamine from the cells without overt cytotoxicity. As a control, otherwise identical cell suspensions received an equal volume of Me_2SO. After various time periods at 37°C an equal volume of ice cold cell disruption medium (100 mM Tris-HCl, 100 mM NaF, 10 mM sodium pyrophosphate, 4 mM EDTA, 20 mM diisopropyl fluorophosphate, 1% NP-40, pH 7.3) was added. This medium was selected on the basis its ability to effectively inhibit protein phosphorylation and dephosphorylation (10) and the ability of NP-40 to solubilize active IgE receptor (7). The cell suspension was incubated at 4°C for 10 min, centrifuged at 30,000 x g for 20 min and the supernatants were used immediately for receptor purification.

Receptor purification. IgE receptor was purified from the NP-40 cell extract at 4°C by repetitive affinity chromatography using successive rabbit gamma globulin-Sepharose, rat IgE-Sepharose, and a second rat IgE-Sepharose column with extensive washing over a 2 day period. Specifically bound receptor was recovered from the IgE columns by rapid elution with acetic acid followed by immediate neutralization (7). The eluted material from the final IgE column was lyophilized, precipitated with acetone (11), and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (12) using a linear 6 to 18% acrylamide gradient slab gel (11). Gels were stained, destained, dried and calibrated using molecular

weight standards (11), and autoradiography was performed with Kodak XR-5 medical x-ray film using Cronex (Dupont) light intensifying screens. Phosphorylation was quantitated by densitometric scans of radioautographs or by liquid scintillation counting. The absolute estimate of the phosphorylation response is somewhat greater in densitometric scans due to better resolution of the receptor (small amounts of radioactivity migrate outside the receptor area) and possibly also due to the nonlinearity of autoradiographic sensitivity.

RESULTS

Biosynthetic labeling of the IgE receptor from rat mast cells. Based on estimates in rat basophilic leukemia (RBL-1) cells, the IgE receptor probably represents less than 0.02% of total cellular protein (7). It thus appeared that investigation of IgE receptor phosphorylation in rat mast cells would require extensive receptor purification. We utilized a method of repetitive affinity chromatography, previously described by this laboratory (7), to obtain highly purified receptor preparations from rat mast cells. When analyzed by SDS-PAGE only three bands were evident in purified receptor preparations from [^{35}S]methionine-labeled mast cells (Fig. 1, track A). The 47,000 to 53,000 M_r protein corresponded in apparent size and breadth to the previously identified IgE receptor molecules obtained from radioiodinated rat mast cells (13), rat basophilic leukemia (RBL-1) cells (13,14,15), and human basophils (11). The 30,000 M_r band was similar to a receptor-associated protein previously identified only from RBL-1 cells biosynthetically labeled with radioactive amino acids (7,16). This band was apparently absent from receptor preparations labeled with radioiodine or carbohydrate precursors (7,16). The third band, a 16,000 M_r protein, might correspond to the ^{125}I-labeled band which copurified with IgE receptor from rat mast cells but not from RBL-1 cells (17).

Phosphorylation of the IgE receptor in intact rat mast cells. The IgE receptor was purified from ^{32}P-labeled mast cells stimulated with the ionophore A23187 for 1 min, or from unstimulated mast cells, and analyzed by SDS-PAGE. In 5 determinations a single labeled band with an apparent M_r of approximately 47,000 to 53,000, corresponding very closely to the ^{35}S-

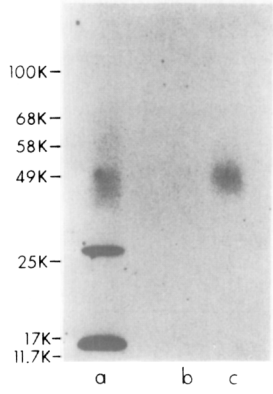


FIGURE 1. IgE receptor preparations from mast cells labeled with [^{35}S]methionine or [^{32}P]orthophosphate. Highly purified receptor preparations were precipitated with acetone and analyzed by SDS-PAGE. Track a; Receptor preparation from ^{35}S-labeled cells. Mast cells were washed twice and resuspended at 2 x 10^6 cells/ml in methionine-free RPMI 1640. [^{35}S]methionine, 0.23 mCi/ml, and fetal bovine serum, 20% final concentration, were added to the cell suspension for 8 hours, after which the cells were washed twice in Buffer A, treated with Buffer A containing 1% NP-40, 2 mM phenylmethylsulfonylfluoride at 4°C for 10 min, centrifuged and used immediately for receptor purification. Track b: Receptor from ^{32}P-labeled cells, unstimulated. Track c: Receptor from ^{32}P-labeled cells, stimulated with A23187 for 1 min. Exposure time was 9 days.

labeled receptor, was present (Fig. 1, track c, and Fig. 2, panel B). In each experiment this band was obtained from, but was much less prominent in, unstimulated ^{32}P-labeled mast cells (Fig. 1, track b and Fig. 2, panel B). By liquid scintillation counting the increase in receptor phosphorylation was 2 to 7 fold higher than in control cells (4 determinations). In contrast to the ^{35}S-labeled receptor preparations, very little radioactivity was present in the 16,000 or 30,000 M_r regions, and no increase in labeling was evident with A23187 stimulation.

Identification of the receptor-phosphate bond as phosphoserine.

Preparations of highly purified ^{32}P-labeled IgE receptor (each from 40 rats) were subjected to limited acid hydrolysis. In high voltage paper electrophoresis of two receptor preparations, 30% and 22% of the total hydrolyzed radioactivity (40% and 35% of recovered counts, respectively) migrated as phosphoserine (Fig. 3). Six and four percent, respectively, of the total radioactivity migrated as phosphothreonine.

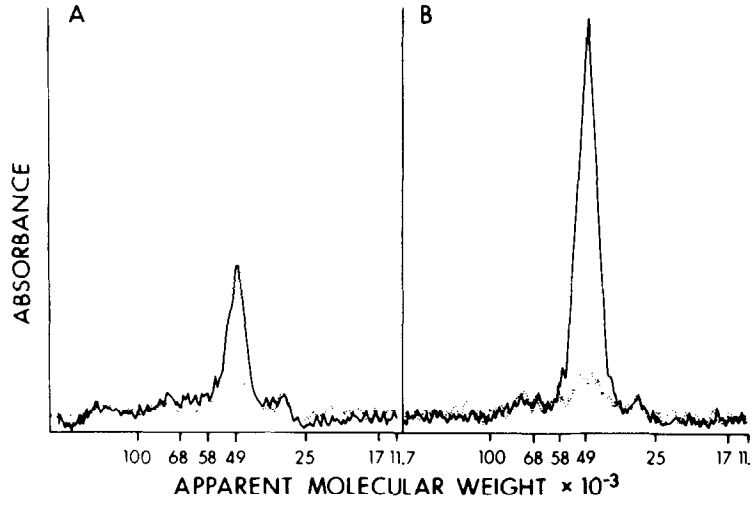
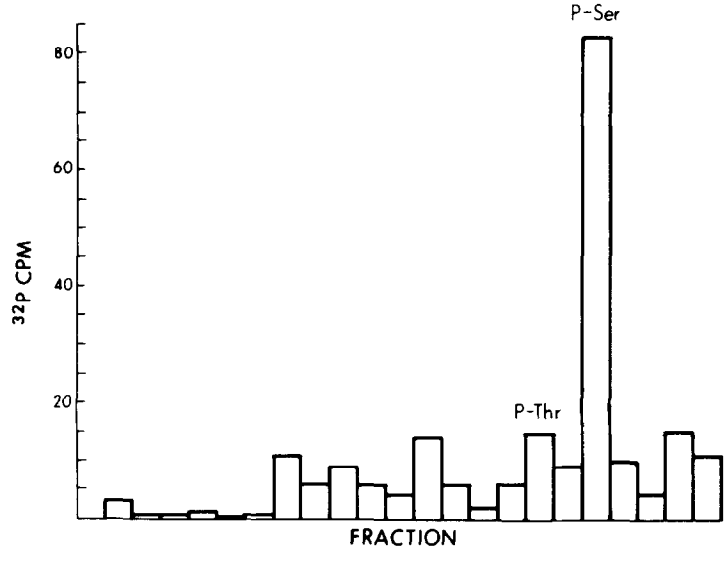


FIGURE 2. Stimulation of receptor phosphorylation by A23187 at 15 and 60 seconds. Receptor preparations purified from ^{32}P-labeled cells were analyzed by SDS-PAGE. The autoradiograph was scanned by a Joyce Loebl double beam recording microdensitometer. Panel A; cells incubated with A23187 in Me_2SO (————), or with Me_2SO alone (·····) for 15 seconds. Panel B; cells incubated with A23187 in Me_2SO (————) or with Me_2SO alone (·····) for 60 seconds. Autoradiograph exposure time was 13 days.



IGURE 3. Determination of phosphoaminoacids. Receptor purified by repetitive affinity chromatography from ^{32}P-labeled samples was lyophilized, precipitated with acetone, and hydrolyzed in 6 N HCl as described (24) for 2.5 to 3.5 h. Samples were dried under N_2 and dissolved in pyridine: glacial acetic acid: water; 8:83:2409; pH 3.5. Unlabeled phosphoserine and phosphothreonine were added and high voltage electrophoresis was performed for 75 min at 3000 V. After internal markers were detected by staining with ninhydrin, the chromatograph was cut into 2 cm slices and radioactivity in each sample determined by liquid scintillation counting for at least 30 min.

Phosphorylation of the IgE receptor is an early event in the release reaction. In 4 determinations, receptor phosphorylation was increased 2 to 3 fold at 15 seconds in ionophore stimulated cells compared to unstimulated cells as determined by liquid scintillation counting. The increase in receptor phosphorylation at 15 seconds can also be seen in microdensitometric tracings of SDS polyacrylamide gels of receptor preparations (Fig. 2, panel A). While the response was considerably greater at 60 than at 15 seconds in this experiment (compare Fig. 2B and 2A), in other experiments the 15 and 60 second responses were nearly equal.

DISCUSSION

The results of the present study conclusively establish that the IgE receptor is phosphorylated in intact rat serosal mast cells exposed to the divalent cation ionophore A23187. This response was not seen in a previous study of phosphorylation in A23187 stimulated rat mast cells (18), presumably because unpurified cell lysates were examined. The phosphorylation is apparently selective since the 16,000 and 30,000 M_r proteins which copurify with the receptor from [^{35}S]methionine-labeled cells contain very little demonstrable ^{32}P. However, the possibility is not excluded that phosphorylated receptor interacts less effectively with these receptor associated proteins leading to their dissociation during purification.

The present study is concerned only with the IgE receptor and its associated proteins and the possible participation of other proteins in the phosphorylation response, as described in the previous study (18), is obviously not excluded. Since the increase in phosphorylation with A23187 begins within 15 seconds and since IgE receptors are critically involved in IgE mediated responses, it seems possible that the increase in receptor phorphorylation is part of the secretory stimulus. While the evidence that cell surface receptors are phosphorylated is limited, the results of the present study, taken together with recent observations that proteins similar

in molecular weight to epidermal growth factor receptor are phosphorylated when stimulated by epidermal growth factor (19) and recent studies with acetylcholine receptor proteins (20), suggest that receptor phosphorylation may be an important general mechanism for the transmission of extracellular signals into the cell.

While the basis for the increased receptor phosphorylation will require further study, the incorporation of ^{32}P into phosphoserine indicates that a protein kinase is involved. Possible types of kinases include the cAMP dependent protein kinases, which are present in virtually all mammalian cells including mast cells (21), and a non-cyclic AMP dependent protein kinase termed kinase C (22,23), which has recently been isolated from brain and platelets. Kinase C is of special interest because it is activated by calcium in combination with diacylglycerol and phospholipids; A23187 and several of the other stimulators of mast cell secretion including antibody to IgE promote both calcium uptake (2,4) and very rapid increases in intracellular diacylglycerol in mast cells (3, Kennerly, D.A., Sullivan, T.J., and Parker, C.W., personal communication). It is therefore attractive to hypothesize that a similar kinase might be present and might mediate the phosphorylation response.

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