MAST CELL MEDIATOR RELEASE AS A FUNCTION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVATION*

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Cross-linking of IgE-Fc receptors of purified rat mast cells produces a transient elevation in cyclic 3′,5′-adenosine monophosphate (cyclic AMP) at 15 s, which is associated with the onset of secretion of granule constituents (1, 2). By analogy to the mechanism by which pharmacologic receptors are transmembrane-linked to adenylate cyclase (3, 4), recent studies have indicated that the IgE receptor is also linked to a subpopulation of adenylate cyclase through a coupling protein termed the G/F protein because of its biologic activation by guanosine triphosphate (GTP) and pharmacologic response to fluoride. Structural analogues of adenosine with an intact ribose act through a membrane R site, by a GTP-G/F protein-dependent mechanism, to activate the catalytic unit of adenylate cyclase and to augment simultaneously IgE-Fc receptor-initiated mediator release (5). Conversely, structural analogues of adenosine with an intact purine moiety occupy an intracellular P site, possibly at the guanosine diphosphate (GDP)-G/F protein complex, to maintain this form of the complex, thereby suppressing activation of the catalytic unit, and simultaneously inhibiting IgE-Fc receptor-initiated granule secretion (5). The linear relationship between attenuation of the IgE-Fc receptor-initiated rise in cyclic AMP and inhibition of mediator release by a P site agonist indicates a direct relationship between IgE-Fc receptor perturbation, transmembrane activation of adenylate cyclase, and granule secretion (5). The additional finding that dose-dependent immunologic activation of mast cells for histamine release was associated with progressive activation of cyclic AMP-dependent protein kinase isoenzymes (6) suggested that cyclic AMP represented a messenger between the membrane and cytoplasmic stages of coupled activation-secretion. Indeed, there was a linear relationship between the activation of cyclic AMP-dependent protein kinase isoenzymes and mediator release when incremental amounts of a P site agonist were used to suppress adenylate cyclase. Furthermore, differential analysis reveals activation of both the type I and type II cyclic AMP-dependent protein kinase isoenzymes with and without a P site agonist in the secretory response to IgE-Fc receptor perturbation.

Materials and Methods

Materials. Porcine heparin, *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside, DL-dithiothreitol (DTT),[1] ethylene glycol-*bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), ω-

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[1] *Abbreviations used in this paper:* DDA, 2′,5′-dideoxyadenosine; DEAE, diethylaminoethyl; DTT, DL-dithiothreitol; EGTA, ethylene glycol-*bis*-(β-aminoethylether)*N,N'*-tetraacetic acid; ω-NH_2-C_6, ω-aminohexyl-agarose; GDP, guanosine diphosphate; TGD^-, calcium-free Tyrode's buffer containing 1 mg/ml gelatin and 1 mg/100 ml DNase; TGD, TGD^- containing 1.36 mM $CaCl_2 \cdot 2H_2O$.

aminohexyl-agarose (ω-NH_2-C_6), β-glycerolphosphate (Sigma Chemical Co., St. Louis, Mo.); deoxyribonuclease I (DNase) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.); metrizamide (Accurate Chemical & Scientific Corp., Hicksville, N. Y.); gelatin (Difco Laboratories, Detroit, Mich.); P-81 phosphocellulose chromatography paper (Whatman, Inc., Clifton, N. J.); adenosine 5′-triphosphate tetra (triethyl ammonium) salt, [γ^{32}-P] (1,000–3,000 Ci/mmol) (New England Nuclear, Boston, Mass.); and 2′,5′-dideoxyadenosine (DDA) (P. L. Biochemicals, Inc., Milwaukee, Wis.) were obtained from the manufacturers. Rabbit IgG anti-rat IgE was prepared by ammonium sulfate fractionation and DEAE ion-exchange chromatography (7).

Mast Cell Purification and Activation. Rat serosal cells were obtained by pleural and peritoneal lavage of 50–100 adult Sprague-Dawley rats with calcium-free Tyrode's buffer containing 1 mg/ml gelatin, 1 mg/100 ml DNase (TGD^-), and 5 mg/100 ml heparin. Mast cells were isolated by isopyknic sedimentation at 400 *g* for 15 min with 22.5% metrizamide in TGD^- at 23°C, followed by velocity sedimentation through a continuous 3–9% metrizamide gradient in TGD^- at 35–55 *g* for 12 min at 23°C. The mast cells were 97–99% pure when assessed by light microscopy of suspensions stained with 0.1% toluidine blue in ethyl alcohol. Contaminating erythrocytes, if present, were removed by a second centrifugation through 3–9% metrizamide.

To assess the kinetics of activation of protein kinase-mixed isoenzymes and mediator release, five duplicate suspensions of 10^6 mast cells in 100 μl of TGD^- containing 1.36 mM $CaCl_2 \cdot 2H_2O$ (TGD) were preincubated at 37°C for 5 min, and challenged with rabbit IgG anti-rat IgE at a final dilution of 1:25 (vol:vol) at 37°C. The reactions were terminated at 0, 15, 30, 60, or 120 s by the addition of 150 μl iced TGD^- and centrifugation at 100 *g* for 5 min at 4°C. The supernates were removed, and the cell pellets were resuspended in 250 μl TGD^- and disrupted by sonication at 4°C (model 350 Sonifier; Branson Sonic Power Co., Danbury, Conn. used at power 3, 50% pulse cycle, 5 pulses). β-Hexosaminidase was assayed in mast cell supernates and pellets by cleavage of *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside. The net percent release of β-hexosaminidase was calculated as follows: net percent release = [stimulated release minus unstimulated release/unstimulated (release plus residual)] × 100. This measurement has been shown to be highly reproducible, parallels the release of histamine, and is unaffected by DDA in this concentration range (5, 8). Protein kinase activity was assessed in the extracted pellets in the absence and presence of 2 μM cyclic AMP and expressed as incorporation of ^{32}P counts per minute into protamine (6). Net percent activation of cyclic AMP dependent protein kinase was calculated as follows: net percent activation of cyclic AMP-dependent protein kinase = [activity in unstimulated cell pellet minus activity in stimulated cell pellet/activity in unstimulated cell pellet] × 100.

To determine the effect of the P site agonist, DDA, on the activation of protein kinase isoenzymes, six duplicate suspensions of 10^6 mast cells in 100 μl of TGD were preincubated with 0, 50, 100, 250, 500, or 1,000 μM concentrations of DDA for 5 min before challenge with anti-rat IgE at a final dilution of 1:25 (vol:vol). The reactions were terminated at 1 min by the addition of 100 μl ice-cold TGD^- and the net percent β-hexosaminidase release and net percent activation of cyclic AMP-dependent protein kinase were assessed. Cyclic AMP-dependent protein kinase activity in cells exposed to DDA alone was unaffected as compared with cells treated with buffer.

The net percent activation of the isoenzymes of mast cell cyclic AMP-dependent protein kinases was determined after their separation on ω-NH_2-C_6. Suspensions of 10^7 mast cells in 1 ml TGD were preincubated at 37°C for 5 min with or without added DDA. The cells were challenged with IgG anti-rat IgE at a final dilution of 1:25, and the reactions were terminated at 1 min with 1 ml ice-cold TGD^-. The mixtures were centrifuged, and the supernates were removed and assessed for β-hexosaminidase. The pellets were resuspended in 500 μl 50 mM glycerol phosphate, pH 7.0, containing 1 mM DTT (buffer A), and disrupted by sonication as described above. 25-μl samples of each replicate were further sonicated in 500 μl 1 M NaCl, and 50 μl of this mixture was analyzed for β-hexosaminidase. The remaining original 475 μl of cell pellets sonicated in buffer A were each sedimented at 2,000 *g* at 4°C for 15 min, and the supernate was retained; the pellets were re-extracted with 100 μl buffer A. The combined buffer A cell extracts from each replicate were applied to individual 500-μl ω-NH_2-C_6 columns previously equilibrated with buffer A. The columns were washed with buffer A for 15 fractions

(500 μl) to elute type I cyclic AMP-dependent protein kinase, and then with buffer A containing 0.5 M NaCl to elute type II. Samples of each fraction were assayed for protein kinase activity in the absence and in the presence of 2 μM cyclic AMP. Recovery from the columns was 102 ± 8.6% (mean ± SEM, $n = 8$).

Results

Activation of Types I and II Cyclic AMP-dependent Protein Kinase. Immunologic activation of cyclic AMP-dependent protein kinase isoenzymes was maximal and plateaued at 30 s; release of β-hexosaminidase was maximal at 1 min. Thus, in all experiments activation of protein kinase was assessed at 1 min after the addition of anti-IgE.

Chromatography of cell sonicates of immunologically challenged rat mast cells on ω-NH_2-C_6-agarose completely separated type I from type II. In the experiment depicted in Fig. 1, the net percent activation (mean ± SEM) of types I and II isoenzymes was 25.1 ± 2.4 and 36.0 ± 8.2%, respectively, and the net percent release of β-hexosaminidase was 39.8 ± 2.4% ($P < 0.001$). In eight preparations of rat serosal

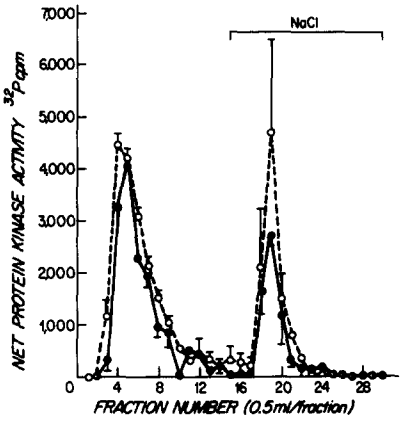

FIG. 1. ω-Aminohexyl-agarose chromatography of rat mast cell cyclic AMP-dependent protein kinases with (●) and without (○) prior activation with IgG anti-rat IgE. The results depict a single representative experiment in which duplicate samples of cells were challenged or exposed to buffer alone. Assays depict the increment in the presence of cyclic AMP, as there was no cyclic AMP-independent protein kinase activity in the fractions.

TABLE I

Immunologic Activation by Anti-IgE of Rat Mast Cell Protein Kinase Isoenzymes

	Mean ± SEM (net %)	*P* value*
β-Hexosaminidase release	32.0 ± 4.3	<0.001
Type I activation vs. total protein kinase in TGD control	18.4 ± 2.6	<0.001
Type II activation vs. total protein kinase in TGD control	8.6 ± 1.5	<0.001
Type I activation vs. type I in TGD control	27.1 ± 3.5	<0.001
Type II activation vs. type II in TGD control	26.6 ± 4.5	<0.001

* Results are compared in a two-tailed statistical analysis for $n = 8$ determinations.

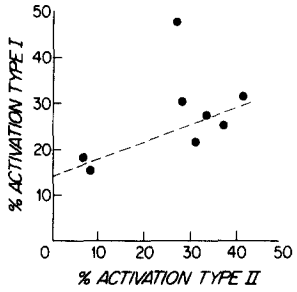

FIG. 2. Comparison of net percent activation of type I cyclic AMP-dependent protein kinase vs. type II isoenzyme in rat mast cells challenged with anti-IgE. The results are from seven of eight experiments, and data were averaged when duplicate determinations were carried out with the same mast cell preparation.

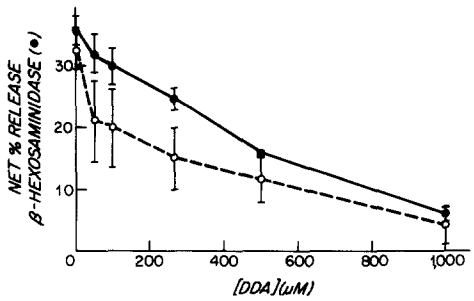

FIG. 3. Dose-response effect of DDA on rat mast cell net percent β-hexosaminidase release (●) and net percent activation of cyclic AMP-dependent protein kinase (○). The results are expressed as mean ± SEM for four determinations.

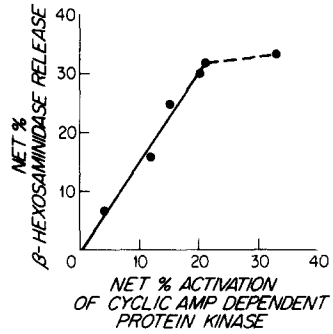

FIG. 4. Net percent release of β-hexosaminidase as a function of cyclic AMP-dependent protein kinase activation. Data are from Fig. 3 (correlation coefficient = 0.992, $n = 5$, $P < 0.001$).

mast cells there was no difference in the net percent activation of type I (27.1 ± 3.5%, $P < 0.001$) and type II (26.6 ± 4.5%, $P < 0.001$) isoenzymes (Table I). However, activation of type I represented 18.4 ± 2.6% ($P < 0.001$) of the total amount of cyclic AMP-dependent protein kinase and activation of type II was 8.6 ± 1.5% ($P < 0.001$) because there is twice as much type I as type II available in the unstimulated cell. A plot of the net percent of type I activated vs. the net percent of type II activated using seven of eight separate experiments, summarized in Table I, gives a straight line (correlation coefficient = 0.829, $P < 0.01$), which passes through 14.4% on the type I

axis (Fig. 2), suggesting that at least 14.4% of the type I protein kinase may be activated independently of type II.

Effect of P Site Adenylate Cyclase Inhibitors on Activation of Mast Cell Cyclic AMP-dependent Protein Kinases. Type I or type II cyclic AMP-dependent protein kinase activation during release of mediators from the mast cell was further investigated using a P site inhibitor of adenylate cyclase to regulate the levels of cyclic AMP and corresponding activation of protein kinases. In four experiments with concentrations of DDA ranging from 50 to 1,000 μM, there was a dose-related inhibition of β-hexosaminidase release and suppression of activation of cyclic AMP-dependent protein kinase isoenzymes (Fig. 3). Net percent β-hexosaminidase release directly correlates with net percent activation of cyclic AMP-dependent protein kinase isoenzymes, giving a straight line (correlation coefficient = 0.992, $P < 0.001$), which passes through the origin (Fig. 4). The single point of >30% activation of cyclic AMP-dependent protein kinase obtained without any DDA did not fall on the line, suggesting a possible threshold function between inhibition of activation of the isoenzymes and a decrease in the secretory response.

To examine differential activation of the isoenzymes, concentrations were selected that have a threshold (100 μM), a moderate (250 and 500 μM), or a maximal (1,000 μM) inhibitory effect on the activation of cyclic AMP-dependent protein kinase isoenzymes and release of β-hexosaminidase. Six separate experiments were conducted in order to have four experimental points for each concentration of DDA. 1 mM DDA diminished the net percent β-hexosaminidase release from 35.0 ± 3.6 to 7.1 ± 2.1%. Type I protein kinase net percent activation was reduced from 26.8 ± 4.2 to 11.0 ± 5.9% and type II from 26.1 ± 5.0 to 16.7 ± 7.3%. At a threshold dose of DDA (100 μM), β-hexosaminidase release was unaffected; there was a decrease in net percent activation of type I protein kinase to 23.0 ± 6.8% and an increase in the amount of type II isoenzyme activated to 27.5 ± 6.0%. The net percent release of β-hexosaminidase decreased to 21.0 ± 3.2 and 14.82 ± 1.7% with 250 and 500 μM DDA, respectively. The net percent activation of type I cyclic AMP-dependent protein kinase was reduced to 19.1 ± 5.3 and 10.1 ± 4.0%, respectively. For the two doses of DDA, type II cyclic AMP-dependent protein kinase isoenzyme net percent activation fell to 22.6 ± 6.7% at 250 μM and 17.8 ± 6.8% at 500 μM.

The net percent release of β-hexosaminidase and the specific activation of the protein kinase isoenzymes for each concentration of DDA were plotted as a percent relative to each function in cells exposed to immunologic challenge in the absence of

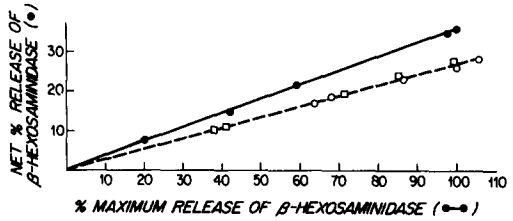

FIG. 5. Net percent activation of rat mast cell cyclic AMP-dependent protein kinase isoenzymes (- - -) type I (□) and type II (○) and β-hexosaminidase release (●), as a function of percent maximum β-hexosaminidase release (35.0%), and corresponding protein kinase activation (type I, 26.8%, and type II, 26.1%) with immunologic challenge in the absence of DDA. Each point is the mean of four experiments.

DDA. This was done to normalize the data relative to a maximal percent in order to allow examination of the relationship of the three parameters of cell response in six different cell preparations (Fig. 5). β-Hexosaminidase release corresponded to a slope of 0.350, whereas the slope for activation of types I and II was 0.264. These results suggest that activation of both types I and II cyclic AMP-dependent protein kinase isoenzymes correlates with release of β-hexosaminidase from immunologically challenged rat serosal mast cells.

Discussion

The IgE receptor-initiated transmembrane activation of adenylate cyclase was suspected by the time relationship between the early transient rise in cyclic AMP and the immediately subsequent mediator release (1, 2). The quantitative nature of this relationship was not established until mast cells were activated by anti-IgE in the presence of increasing concentrations of the P site agonist, DDA, so as to achieve progressive inhibition of adenylate cyclase, and corresponding attenuation of the rise of cyclic AMP and of the release of secretory granule constituents (5). The earlier finding that incremental activation of rat mast cells by anti-light chain antiserum and histamine release was associated with a progressive decline in cellular cyclic AMP-dependent protein kinase holoenzyme did not reveal whether the activation of the cyclic AMP-dependent protein kinase was essential for the secretory reaction to proceed or represented a negative feedback regulatory event (6). The linear relationship between the net percent β-hexosaminidase release and the net percent activation of cyclic AMP-dependent protein kinase isoenzymes, when the IgE-dependent activation of adenylate cyclase is suppressed by incremental concentrations of DDA, indicated a direct relationship between the activation of cyclic AMP-dependent protein kinase and the secretory response (Figs. 3 and 4). Assessment of residual cyclic AMP-dependent protein kinase activity in cell extracts was used to define percent activation of the combined isoenzymes after immunologic challenge because recovery of free catalytic subunit is incomplete in physiologic buffer. Attempts to recover free catalytic subunit activity using available techniques, such as high salt extraction, result in both partial activation of the holoenzyme and inactivation of the free catalytic subunit (9–11). Measurement of holoenzyme depletion to assess activation of combined or chromatographically separated cyclic AMP-dependent protein kinase isoenzymes has been reliably employed in other cell systems (12–14).

The rat mast cell contains cyclic AMP-dependent protein kinase isoenzymes types I and II that can be separated without overlap (Fig. 1) by ω-NH_2-C_6 chromatography (6, 14–16). Activation of the individual isoenzymes was measured by residual cyclic AMP-dependent types I and II activity after chromatographic separation, wherein the recovery of the isoenzymes is 100% of the cyclic AMP-dependent protein kinase holoenzyme activity in the cell extracts applied for separation. In eight consecutive experiments in which purified rat mast cells were activated with anti-IgE, the mean net β-hexosaminidase release of 32% was accompanied by a net percent activation of cyclic AMP-dependent protein kinase that was 27% for both isoenzymes (Table I). As the type I protein kinase was twice as abundant as the type II, the percent activation relative to the total holoenzyme pool was twice as great for type I. Although the activation of the cyclic AMP-dependent isoenzymes during coupled activation and secretion was related in a linear fashion, the extrapolated intercept implied that

almost 15% of the type I could be activated without type II (Fig. 2). The differential activation of the isoenzymes apparent in Fig. 2 raises the question of different functions. Opposing biological effects have been implied in the literature for selective activation of cytoplasmic isoenzymes (14), and there is cell cycle-specific expression of types I and II cyclic AMP-dependent protein kinases in metabolically synchronized Chinese hamster ovary cells (17). There is also evidence that particulate type II, but not cytosolic type I, cyclic AMP-dependent protein kinase in rat myocardium responds to the appropriate agonist, with activation of phosphorylase and an increase in myocardial contractibility (18).

The IgE receptor-initiated activation of adenylate cyclase was suppressed with the P site agonist DDA to determine whether the corresponding inhibition of mediator release could be related to a preferential failure to activate one of the isoenzymes. There was parallel suppression of activation of both isoenzymes (Fig. 5). Maximal activation of either isoenzyme was 25–30%, and release of preformed mediators was directly related to activation of the two isoenzymes. This finding contrasts with the dose-related effects of theophylline in raising cyclic AMP and yielding a percent inhibition of mediator release and percent activation of cyclic AMP-dependent protein kinase type I with superimposable slopes progressing to 100%. The percent activation of type II cyclic AMP-dependent protein kinase was approximately half that of type I at each theophylline concentration and plateaued at ~50% when type I activation was at 100% and mediator release was completely suppressed (19, 20). The down-regulating effect of theophylline may result either from the depletion of cyclic AMP-dependent protein kinase essential for the integrated release response or from the phosphorylation by the protein kinase of an inhibitory protein. The dose-related effects of an adenylate cyclase P site inhibitor on activation of cyclic AMP-dependent protein kinases and mediator release establish the second messenger function of cyclic AMP in mast cell-coupled activation and secretion, linking extracellular IgE-Fc receptor perturbation with membrane-associated adenylate cyclase, and the intracellular events that lead to the response to stimulus, i.e., degranulation.

Summary

Stereo-specific perturbation of the IgE-receptor (shown in previous studies) produces a monophasic rise in cyclic AMP that peaks at 15 s and a depletion of cyclic AMP-dependent protein kinase that plateaus at 30–60 s. The previously observed linear relationship between the attenuation in the monophasic rise in cyclic AMP and the quantity of mediator release in the presence of incremental concentrations of the adenosine analogue 2′,5′-dideoxyadenosine, DDA, which is known to inhibit adenylate cyclase, indicated a direct relationship between receptor perturbation, transmembrane activation of adenylate cyclase, and granule secretion. The role of cyclic AMP as a second messenger in this sequence is now apparent from the linear relationship between net percent mediator release and net percent activation of cyclic AMP-dependent protein kinase isoenzyme when IgE-dependent activation of adenylate cyclase is suppressed by incremental quantities of DDA. There was a comparable percent activation of both types I and II mast cell cyclic AMP-dependent protein kinase isoenzymes with anti-IgE-induced activation and secretion, and there was a parallel suppression of the activation of both isoenzymes in the presence of DDA. Although these studies firmly link the activation of cytoplasmic cyclic AMP-dependent

protein kinase to the IgE receptor-initiated transmembrane activation of adenylate cyclase, they do not discriminate among the functions of the two isoenzymes.

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