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CYCLOHEXIMIDE TREATMENT OF MOUSE MAST CELLS INHIBITS SEROTONIN RELEASE

EVIDENCE OF A REQUIREMENT FOR NEWLY SYNTHESIZED PROTEIN IN THE EXOCYTOTIC RESPONSE

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Abstract—Treatment of mouse peritoneal mast cells and mouse bone marrow-derived cloned mast cells with the protein synthesis inhibitor cycloheximide led to a marked abrogation of serotonin (5-hydroxytryptamine; 5-HT) release induced by a range of activators including antigen, anti-immunoglobulin E (anti-IgE) antibody, calcium ionophore A23187, and the polycation polylysine. Significant inhibition (28%) of IgE-mediated secretion was attained after incubation of peritoneal cells for only 2 hr, and inhibition progressed over a 24 hr period, reaching >80% after 15 hr. When peritoneal mast cells were exposed to cycloheximide and then washed and returned to culture conditions, a substantial recovery of responsiveness to anti-IgE was seen after 5 hr. Under the same conditions to those used in functional studies, cycloheximide inhibited protein synthesis, measured as incorporation of [^{35}S]-methionine, by purified peritoneal mast cells and cloned mast cells to 18.5% and 7.9% of control levels, respectively. These results show that synthesis of new protein over a period of a few hours is required to render mast cells fully responsive to stimuli that act via the IgE receptor, and to certain other stimuli that are receptor-independent.

Mast cells are secretory cells located in connective tissues, beneath and within epithelia of the lungs, gut and skin, and in the serosal cavities of rodents. Their function is to promote tissue defence and repair by releasing multiple chemical mediators of inflammation; however, excessive activation of these cells can lead to allergic disease. The immunological activation of mast cells is initiated by crosslinking of cell surface receptor-bound immunoglobulin E (IgE†) antibody by antigen, as a consequence of which a number of complex biochemical changes occur that have been implicated in signal transduction. These events include phosphorylation of the IgE receptor [1], hydrolysis of phosphoinositides [2] and influx/mobilization of calcium [3]. We have focused in previous studies on the earliest event in the sequence, namely the process by which specific IgE antibody binds (via its Fc region) to high affinity receptors to render the cells responsive to antigen—a process referred to as cell sensitization. Studies of the passive sensitization of mouse peritoneal mast cells *in vitro* by monoclonal IgE antibody [4] and of active sensitization of rat peritoneal mast cells *in vivo* by immunization [5] have demonstrated a lag phase of several hours between addition of IgE (*in vitro*) or the appearance of circulating IgE (*in vivo*) and the acquisition of cellular responsiveness to antigen. This delay in sensitization may be due to a requirement for long term exposure to IgE before sufficient receptors become occupied (binding may be prolonged if it is limited by the dissociation rate of IgE that may already occupy the receptors). Alternatively, as has been postulated before [4, 5], there may be a requirement for a second cellular event following occupation of the receptors, that is required before the cells become functionally sensitized. We consider the possibility that such an event may involve the *de novo* synthesis of protein, and we now report experiments designed to investigate whether inhibition of protein synthesis might selectively inhibit the sensitization process. Although our results failed to demonstrate selective inhibition of sensitization, we uncovered a requirement for protein synthesis for full responsiveness of tissue and cloned mast cells to a number of stimuli. We characterize further the time course of inhibition and recovery of tissue mast cell function after inhibition of protein synthesis.

MATERIALS AND METHODS

Cells. Tissue mast cells were obtained by peritoneal lavage of CD1 Swiss White mice (departmental outbred stock) with RPMI-1640 medium (Gibco, Uxbridge, U.K.). The cells were pelleted by centrifugation (150 *g* for 5 min), washed once in RPMI-1640, and resuspended in RPMI-1640 medium supplemented with 5% foetal calf serum (FCS; Seralab, Sussex, U.K.), 2 mM L-glutamine and 40 μg/mL gentamicin (complete medium). These peritoneal cell preparations, which contained 1–2% mast cells as determined by staining in 0.01% toluidine blue, will be referred to as unfractionated

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† Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); C1.MC/C57.1, a mouse bone marrow-derived mast cell line; DMEM, Dulbecco's Modified Eagles Medium; DNP, dinitrophenyl; HSA; human serum albumin; FCS, foetal calf serum; IgE, immunoglobulin E; TCA, trichloroacetic acid.

peritoneal or tissue mast cells. In experiments requiring purified mast cells, pelleted peritoneal cells were suspended in 72.5% isotonic Percoll (Pharmacia, Uppsala, Sweden) in RPMI-1640 medium containing 1% FCS, and centrifuged at 350 *g* for 10 min. The pelleted cells, which comprised approximately 90% mast cells (henceforth referred to as purified peritoneal mast cells) were washed twice and suspended in complete RPMI-1640 medium. Unfractionated and purified peritoneal mast cells were maintained in complete RPMI-1640 medium throughout all experimental procedures.

Cells of a growth factor-independent mast cell line derived from the bone marrow cells of C57BL/6J mice and designated clone C1.MC/C57.1 [6] were kindly provided by Dr S. J. Galli, Beth Israel Hospital, Boston, MA, U.S.A. They were grown in complete DMEM (containing 10% FCS, 4 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) at a cell density of approximately 10^6/mL in an atmosphere of 5% CO_2 at 37°. These cells, which will be referred to as cloned mast cells, were maintained in complete DMEM throughout all experimental procedures. Their viability, assessed by exclusion of 0.02% trypan blue, was >95% in all experimental preparations.

Experiments to investigate the effect of cycloheximide on the secretory response of mast cells. Peritoneal and cloned mast cells were treated with cycloheximide before challenge with a range of cell-activating stimuli. In experiments that involved challenge of tissue mast cells with different stimuli including antigen, unfractionated rather than purified peritoneal mast cells were used, since, in line with previous reports [7], we found that mast cells that had been sedimented through density gradients were relatively refractory to sensitization by IgE antibody (the process by which mast cells are rendered responsive to antigen) but retained a reasonable level of responsiveness to anti-IgE antibody, which crosslinks total cell surface IgE regardless of its antigen specificity. Hence, in experiments that utilized purified tissue mast cells, the cells were not passively sensitized with IgE antibody and were challenged with anti-IgE. Unfractionated and purified peritoneal mast cells (2×10^4 mast cells/mL/well) were incubated in 24-well tissue culture plates (Costar, U.K.) and cloned mast cells (10^6 cells/mL in a total volume of 5 mL) were incubated in 25 cm^2 tissue culture flasks. Mouse monoclonal IgE anti-dinitrophenyl (DNP) antibody (ICN-Flow, High Wycombe, U.K.) was added as required to the cells for 24 hr at concentrations of 0.25–0.5 μg/mL for peritoneal mast cells, and 0.1 μg/mL for cloned mast cells—concentrations that had been shown in preliminary experiments to be optimal for sensitization of the respective cell types. Cycloheximide (Sigma Chemical Co., Poole, U.K.) was added to duplicate cell cultures at the start of the 24 hr incubations, or at various time points in kinetic studies, at concentrations that had been selected from preliminary experiments to give comparable effects on the two cell types at the cell densities used (2 μg/mL for peritoneal mast cells and 5 μg/mL for cloned mast cells).

In experiments to look at the recovery of tissue mast cells from cycloheximide, peritoneal cells were cultured in 1 mL aliquots (10^5 mast cells) in conical tubes containing 2 μg/mL cycloheximide (or no cycloheximide as control) for sufficient time (16 hr) to give appreciable inhibition of 5 hydroxytryptamine (serotonin; 5-HT) release. After exposure to the drug, duplicate cell cultures were washed twice and restored to culture in the same volume of complete medium for various time periods. In these experiments the cells were not exposed to IgE because of the difficulty of ensuring a constant time of exposure to the antibody. Hence it was necessary to challenge the cells with anti-IgE antibody rather than antigen.

Cell challenge and measurement of serotonin release. Tritiated serotonin (5-[1,2-^{3}H(N)]-hydroxytryptamine creatinine sulphate, supplied by NEN, Dreiech, Germany; sp. act. 27 Ci/mmol) was added (1 μCi/mL) to cell preparations 90 min before challenge. The cells were then washed three times in culture medium containing 1% FCS (challenge medium) to remove unincorporated [^{3}H]5-HT and cycloheximide and unbound IgE, and resuspended in an appropriate volume (1–2 mL) of culture medium containing 1% FCS. Aliquots of cell suspension (150 μL) were then added to an equal volume of the cell-activating compound in challenge medium, challenge medium alone, or 0.05% Triton X-100, all of which had been pre-warmed to 37°. Challenge was performed in duplicate tubes, and proceeded for 10 min in a water bath at 37°. The cell activators employed were compound 48/80, polylysine (M_r 59,000), calcium ionophore A23187 (all from Sigma), rat monoclonal anti-mouse IgE (kindly provided by Dr F. D. Finkelman, Uniformed services University, Bethesda, MD, U.S.A.) and DNP_{13}–HSA antigen [4]. The cells were then sedimented (300 *g*, 3 min) and 150 μL of supernatant fraction was removed for scintillation counting. Release of 5-HT, which is a specific marker of exocytotic secretion from rodent mast cells, was calculated according to the equation: net % release = $[(a - b) \div c] \times 100$, where a is the radioactivity released by stimulated cells, b is the radioactivity released from unstimulated cells, and c is the radioactivity in the Triton X-100 detergent-lysed samples (total incorporated cellular radioactivity).

Protein synthesis assay. Purified peritoneal mast cells ($2–6.5 \times 10^5$/mL) or cloned mast cells (1×10^6/mL) were incubated for 24 hr at 37° with cycloheximide in complete medium (100 μL) in sterile 15 mL conical tubes. [^{35}S]L-Methionine (ICN, Irvine, CA, U.S.A.; sp. act. 1168 Ci/mmol), which is incorporated into newly synthesized proteins, was added to the cultures at 100 μCi/mL for the last 3 hr of culture, after which the unabsorbed radiolabel was removed by washing the cells twice in 0.15 M phosphate buffered saline. The cells were then resuspended in 30% trichloroacetic acid (TCA) on ice for 5 min to precipitate the protein, and the precipitates were collected on Whatman GF/B glass microfiber filters (Whatman, Maidstone, U.K.) using a vacuum manifold apparatus (Millipore Corporation, Bedford, MA, U.S.A.). The TCA-precipitated protein deposited on the filters was washed with 3 mL of 30%, 5%, then 1% TCA

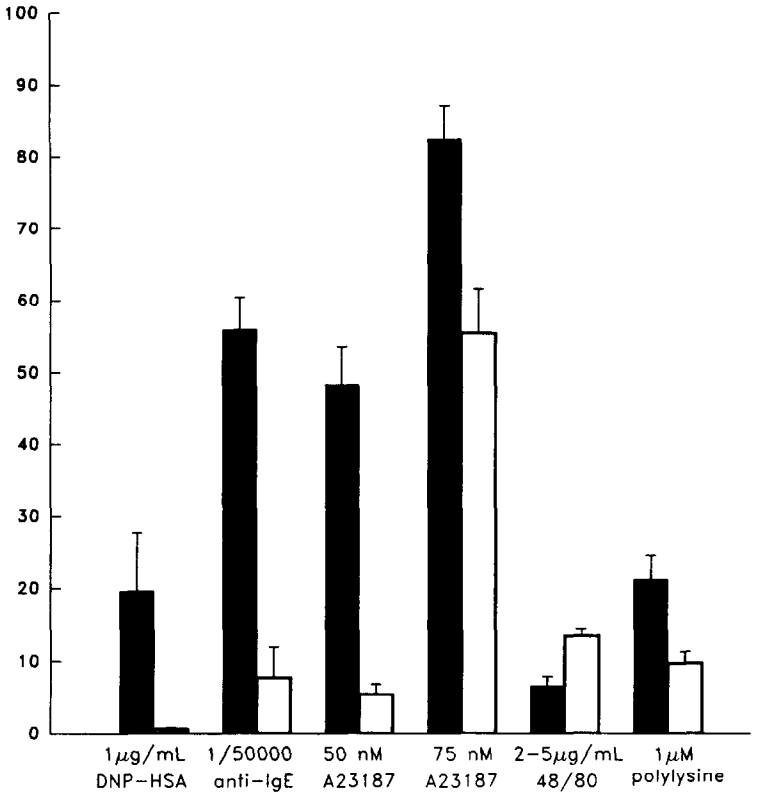

Fig. 1. Release of 5-HT from unfractionated mouse peritoneal mast cells incubated with (open bars) or without (filled bars) 2 μg/mL cycloheximide for 24 hr prior to challenge for 10 min with a range of cell activators. Results are means ± SEM for at least four experiments. The differences between treated and untreated cells were statistically significant ($P < 0.05$ by paired Student's *t*-test, 2-tailed) for each cell activator except compound 48/80.

followed by 5 mL of ethanol. The filters were then left to dry before scintillation counting to assess ^{35}S content.

RESULTS

Effect of treatment of mouse mast cells with cycloheximide on secretion induced by various cell activators

Treatment of unfractionated mouse peritoneal mast cells with cycloheximide (2 μg/mL) for 24 hr led to a significant inhibition of 5-HT release induced by DNP–human serum albumin (HSA) antigen, anti-IgE, calcium ionophore A23187 and polylysine (Fig. 1). Release of 5-HT induced by the higher concentration of A23187 tested (75 nM) was not inhibited so strongly as that induced by the lower concentration (50 nM), indicating that inhibition by cycloheximide is more pronounced at a lower intensity of cell stimulation. In contrast to the other cell activators, secretion induced by compound 48/80 was not inhibited by cycloheximide, despite this agonist being the least efficacious of those we tested (Fig. 1). Antigen-induced release of 5-HT was inhibited by an average of 96%, and anti-IgE-induced release by an average of 87%. Release induced by polylysine was not so markedly inhibited by cycloheximide (to 46% of control values) as was seen for the IgE-directed ligands, or calcium ionophore, even though the intensity of cell stimulation provided by polylysine was equivalent to that given by antigen (Fig. 1). Cycloheximide produced a similar degree of inhibition of 5-HT release induced by anti-IgE from both unfractionated and purified mast cells (Table 1).

Table 1. Effect of treatment of unfractionated and purified mast cells with cycloheximide (2 μg/mL) on net percentage 5-HT release in response to anti-IgE (1/50,000 for mixed peritoneal cells and 1/10,000 for purified mast cells)

	Unfractionated peritoneal cells	Purified peritoneal mast cells
Control	55.9 ± 4.4	20.2 ± 5.5
Cycloheximide	7.7 ± 4.3	5.5 ± 2.2

Unfractionated peritoneal cells contained 1–2% mast cells and purified preparations 86.1 ± 4.4% mast cells. Cycloheximide significantly reduced anti-IgE-induced 5-HT release from both unfractionated and purified mouse mast cells ($P < 0.005$ by paired Student's *t*-test, 2-tailed). Results are means ± SEM for four or five experiments.

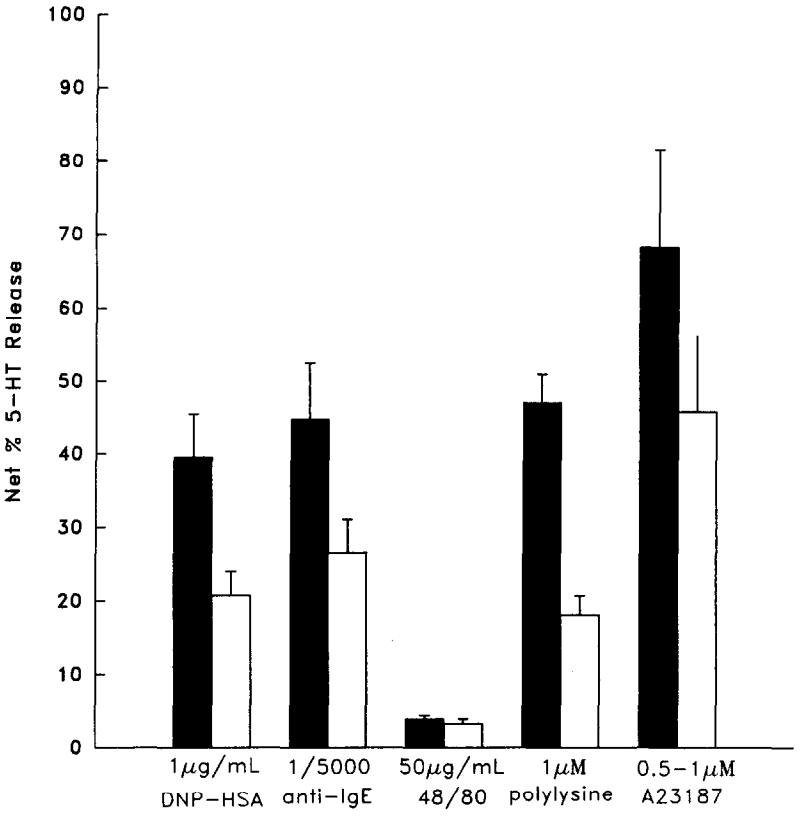

Fig. 2. Release of 5-HT from mouse cloned mast cells incubated with (open bars) or without (filled bars) 5 μg/mL cycloheximide for 24 hr prior to challenge for 10 min with a range of cell activators. Results are means ± SEM for at least four experiments. The differences between treated and untreated cells were statistically significant ($P < 0.05$ by paired Student's *t*-test, 2-tailed) for each cell activator except compound 48/80.

Treatment of mast cells of clone C1.MC/C57.1 with cycloheximide significantly inhibited secretory responses to DNP–HSA antigen, anti-IgE, calcium ionophore and polylysine (Fig. 2). These cells proved to be refractory to compound 48/80, even at a concentration of 50 μg/mL (Fig. 2).

Treatment of tissue and cloned mast cells with cycloheximide did not alter cell viability measured as trypan blue uptake, or cellular uptake of [^{3}H]5-HT.

Time course of cycloheximide-mediated inhibition of secretion by tissue mast cells and cell recovery

Over a 24-hr incubation period the inhibitory effect of cycloheximide on DNP–HSA antigen-induced 5-HT release from unfractionated peritoneal mast cells increased progressively with time (Fig. 3). Significant inhibition was obtained when the drug was added to the cells only 2 hr before challenge (28% inhibition) and the degree of inhibition increased to greater than 80% after a 15 hr incubation period (Fig. 3).

When the cells were exposed to cycloheximide and subsequently washed and recultured in cycloheximide-free medium for various times, release of

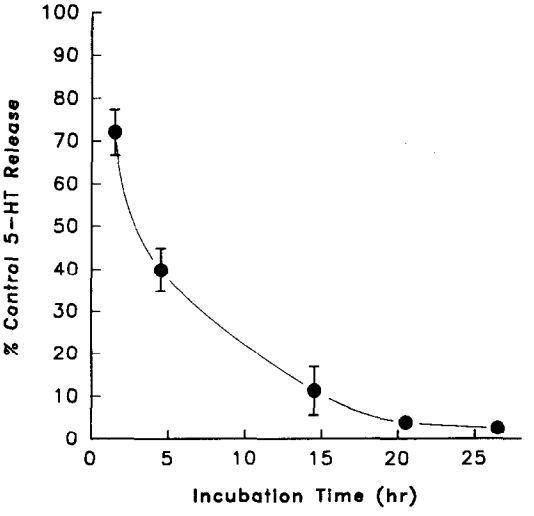

Fig. 3. Effect of time of incubation of peritoneal mast cells with 2 μg/mL cycloheximide on antigen-induced release of 5-HT. Results are means ± SEM for four experiments.

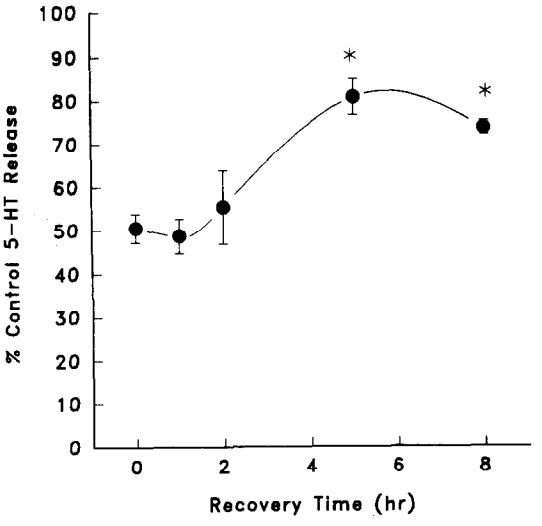

Fig. 4. Recovery of responses of mouse peritoneal mast cells to anti-IgE after treatment with 2 μg/mL cycloheximide. The cells were treated with the drug for at least 16 hr, then washed and incubated in culture medium for various times before challenge. Results are means ± SEM for four experiments.

5-HT in response to anti-IgE recovered significantly by 5 hr (Fig. 4).

Inhibition of protein synthesis in purified peritoneal mast cells and C1.MC/C57.1 cells by cycloheximide

Treatment of mast cells with cycloheximide under the same conditions to those used in functional studies (2 μg/mL for tissue mast cells and 5 μg/mL for cloned mast cells, for 24 hr) reduced the rate of protein translation (measured as incorporation of [^{35}S]methionine) to 18.5% and 7.9% of control levels for the two respective cell types. Increasing the cycloheximide concentration to 100 μg/mL led to inhibition to 7.7% and 2.7% of control values for purified tissue mast cells and cloned mast cells, respectively (Table 2). Thus, the conditions used in the functional experiments were sufficient to give a high degree but not maximal inhibition of protein synthesis.

Table 2. Effect of cycloheximide on protein synthesis by purified peritoneal mast cells and cloned mast cells

	Cycloheximide (μg/mL)	Protein synthesis (% control)
Peritoneal mast cells		
	2	18.5 ± 1.2
	100	7.7 ± 2.5
Cloned mast cells		
	5	7.9 ± 0.4
	100	2.7 ± 0.5

Peritoneal mast cells were enriched to 90.28 ± 1.01% purity. The cells were cultured for 24 hr, and [^{35}S]-methionine was added 3 hr before precipitation of the proteins with TCA. The control level of [^{35}S]methionine incorporation for peritoneal mast cells was 4393 ± 1660 cpm and for cloned mast cells was 43,124 ± 6696 cpm. In both cell types, cycloheximide significantly reduced protein synthesis at each of the concentrations used ($P < 0.001$).

Results are shown as means ± SEM for four experiments.

DISCUSSION

We investigated the effects of the protein synthesis inhibitor cycloheximide on the secretory response of mouse mast cells, measured as release of the granule-associated mediator 5-HT. Cycloheximide treatment of peritoneal mast cells and long-term cultured mast cells of the bone marrow-derived clone C1.MC/C57.1 led to a significant inhibition of 5-HT release induced by IgE-directed ligands (antigen and anti-IgE antibody), the calcium ionophore A23187 and the polycationic peptide polylysine. In further studies with peritoneal mast cells we found that substantial inhibition (28%) of IgE-mediated secretion was attained after only 2 hr of incubation of the cells with cycloheximide, and >80% inhibition was attained after 15 hr. The cells recovered their responsiveness within 5–8 hr of washing off the cycloheximide. The observation that the cells could recover from treatment, and the results of trypan blue exclusion tests, demonstrated that the drug was not cytotoxic at the concentrations used.

Our results provide evidence that the secretory responsiveness of mast cells to immunological, and some forms of chemical, stimulation is dependent on the active synthesis of new cellular protein(s). Indeed, we confirmed by measurement of incorporation of [^{35}S]methionine that under the experimental conditions used protein synthesis was substantially but not totally diminished. From the results of inhibition and recovery time-course experiments we estimate a half-life of 4–6 hr for the synthesis of these essential proteins. The mediator whose release was assayed in these experiments, namely tritiated serotonin, was incorporated into the cells before challenge, so that its release was not dependent on synthesis. In fact, it is a general rule that mast cell granule-associated mediators e.g. histamine, 5-HT, heparin and various proteases, are stored preformed, and do not require synthesis for release [8]. Therefore, the inhibitory effect of cycloheximide on the exocytotic response of mast cells must relate to inhibition of synthesis of proteins involved in secretion: either in energy production or in signal transduction.

The action of cycloheximide was not restricted to IgE-dependent cell activation since the drug inhibited mediator release induced by non-receptor directed stimuli such as calcium ionophore, provided that the intensity of stimulation was equivalent to that provided by antigen and anti-IgE. Inhibition by cycloheximide of release induced by calcium ionophore indicates that its inhibitory action is directed at least in part at target protein(s) required at a step in the secretory response subsequent to the influx or mobilization of calcium that is induced by this kind of stimulus [3]. Our data, particularly from tissue mast cells, also shows that cycloheximide was

less effective as an inhibitor of mediator release induced by polylysine and compound 48/80 than by IgE-directed ligands and ionophore—this is particularly apparent from Fig. 1 by comparison of polylysine with antigen, both of which induced similar control responses. Polycationic polyamines such as polylysine and compound 48/80 are thought [9, 10] to stimulate mast cells by activating directly a GTP-binding protein G_e that is involved at a late stage in the transduction sequence [11]. Hence, we conclude that essential newly synthesized protein(s) are probably required at a step prior to G_e activation.

Mast cells from the C1.MC/C57.1 line divide rapidly (with a doubling time of approximately 24 hr) and exhibit ultrastructural features characteristic of immature mast cells [6], and it is therefore perhaps not surprising that their secretory function is dependent on active protein synthesis. However, peritoneal mast cells, which are representative of mature connective tissue type mast cells [12], are generally thought to be metabolically rather inert until stimulated into exocytosis. Our results show that this is not the case, and that tissue mast cells require continuous protein synthesis to render them functionally primed to respond to immunological and other forms of stimuli.

We recently reported that the lymphocyte-derived cytokine interferon-γ inhibited serotonin release from mouse peritoneal mast cells triggered by a similar range of stimuli to those used in the present study, namely antigen, anti-IgE and calcium ionophore A23187 [13]. Interestingly however, the pattern of inhibition by interferon-γ does not parallel exactly that seen for cycloheximide in that the cytokine but not the drug abrogated responses to compound 48/80 [13]. Interferon-γ is known to act at cell surface receptors to regulate gene expression and the synthesis of selected cell proteins [14]. Although we may speculate that interferon-γ induces a selective down-regulation of proteins involved in the transduction of secretion by mast cells, we can infer from the current data that the proteins subject to interferon-γ regulation are not identical to those affected by the more general agent cycloheximide. The identity of the essential transduction proteins that are regulated by interferon-γ and those whose synthesis is inhibited by cycloheximide may merit further inquiry.

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