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Rapid Communication

Localization of Cyclo-oxygenase and Prostaglandin E₂ in the Secretory Granule of the Mast Cell

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The application of anti-cyclo-oxygenase and anti-prostaglandin E₂ immunoglobulins to A23187-stimulated rat connective tissue mast cells has permitted the localization of cyclo-oxygenase activity (prostaglandin H₂ synthetase) and the site of prostaglandin E₂ (PGE₂) formation in the secretory granules. Because binding was carried out after stimulation but before dehydration and embedding, we have limited the loss of these antigens due to normal degradation and to aqueous and solvent washes. As this method permits labeling of exposed cell surfaces, only granules that have been exteriorized can be labeled. Contrary to what might have been expected, no labeling was associated with plasma membranes or with any portion of damaged cells. Antibodies to PGE₂

were bound evenly over the surface of the granule matrix, whereas antibodies to cyclo-oxygenase appeared to be bound to strands of proteo-heparin projecting from the surface of the granule matrix. Where granule matrix had become unraveled and dispersed, label appeared to adhere throughout the ribbon-like proteo-heparin strands. These results support our previous conclusion that the secretory granule is the site of the arachidonic acid cascade during exocytosis. (*J Histochem Cytochem* 37:1319-1328, 1989)

KEY WORDS: Cyclo-oxygenase; Prostaglandin E₂; Secretory granules; Mast cell; Exocytosis; Lipid mediators; Inflammation; Arachidonic acid; Eicosanoids; Immunocytochemistry.

Introduction

It has long been assumed that the cell membrane is the source of phospholipid which provides the arachidonic acid required for synthesis of prostaglandins and other eicosanoids (1-5). Eicosanoid release has also been assumed to occur as a result of cell and membrane damage (3,4). However, membrane damage cannot be the source for the large pool of free arachidonic acid required for eicosanoid synthesis, as calcium ionophore-stimulated release of eicosanoids from neutrophils can occur without damage to membrane or loss of cell function (6). In addition, macrophages that produce large amounts of eicosanoids are known to contain no stored free arachidonic acid (7). These contradictions are compounded when one considers that phospholipase A₂, the enzyme necessary for arachidonic acid release from phospholipid, requires millimolar calcium concentrations for its activity (4,8). This calcium requirement cannot be met by a membrane bilayer source.

Recently, we found that the secretory granules of the mast cell contain a large non-bilayer phospholipid store (9). During granule activation, a portion of this phospholipid spontaneously assembles

into vesicles as a result of a water influx from the cytoplasm into the granule (9-11). Fusion of these newly assembled membrane vesicles with the perigranular membrane enables the activated granule to enlarge and its perigranular membrane to lift from the surface of the granule matrix. The contact and fusion of the expanding perigranular membrane with the plasma membrane culminate in exocytosis (10-13). Like many other secretory systems, the mast cell also produces a variety of lipid-derived mediators, such as prostaglandins and leukotrienes, during histamine release (14). Because mast cell phospholipid contains a high concentration of arachidonic acid (15), the remaining matrix-bound phospholipid would be a convenient arachidonic acid source for eicosanoid synthesis if the enzymes of the arachidonic acid cascade are also found in the granule. To this end, we have demonstrated that the mast cell granule not only contains the substrate for eicosanoid synthesis but also contains the machinery for rapid production of eicosanoids during granule activation (16). In this communication, we further establish that the granule is the site as well as the source of eicosanoid production, by localizing the presence of cyclo-oxygenase and its product, prostaglandin E₂ (PGE₂) to the granule matrix using immunocytochemical techniques.

Materials and Methods

Rat serosal mast cells were obtained by peritoneal lavage according to a pub-

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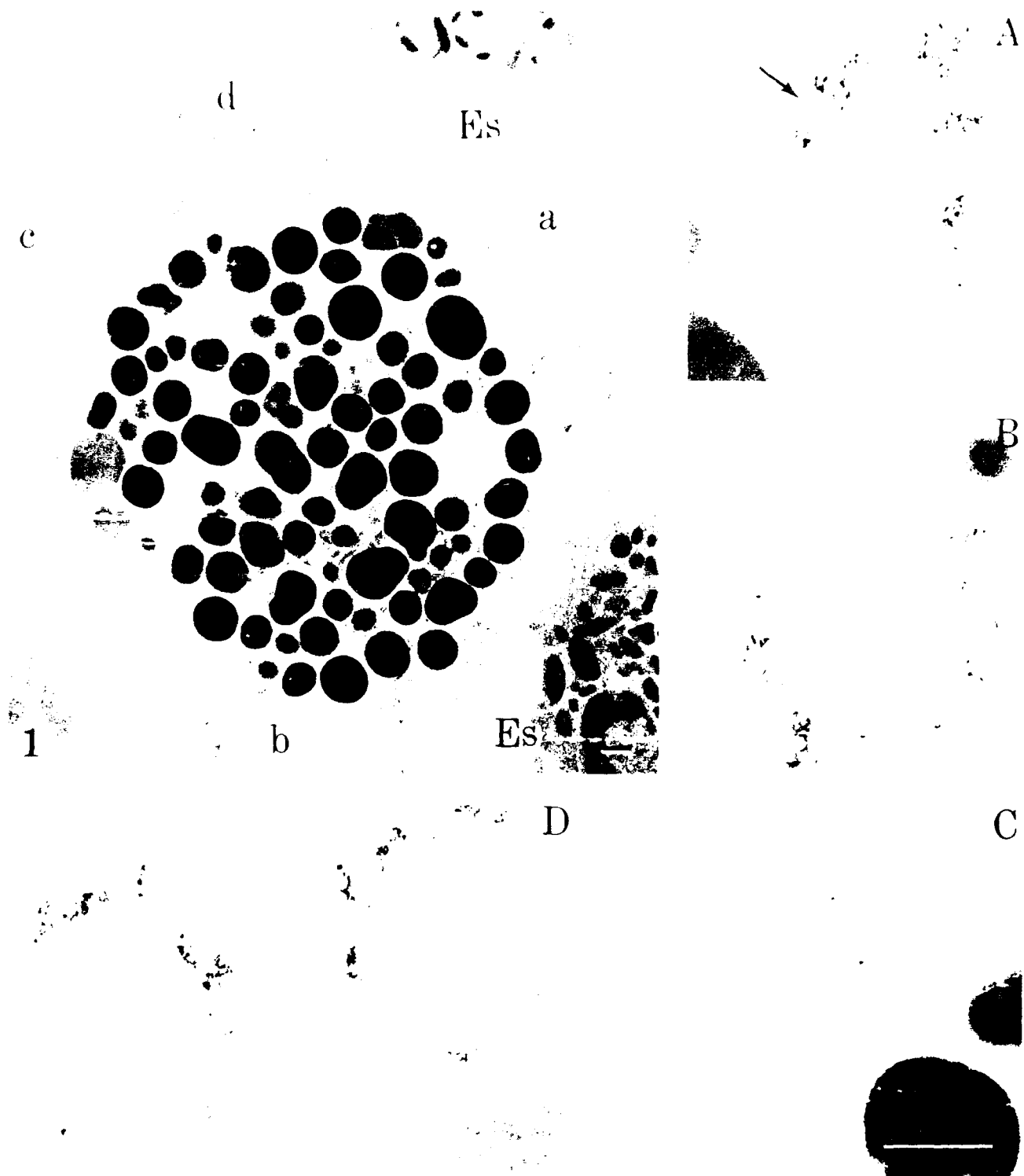
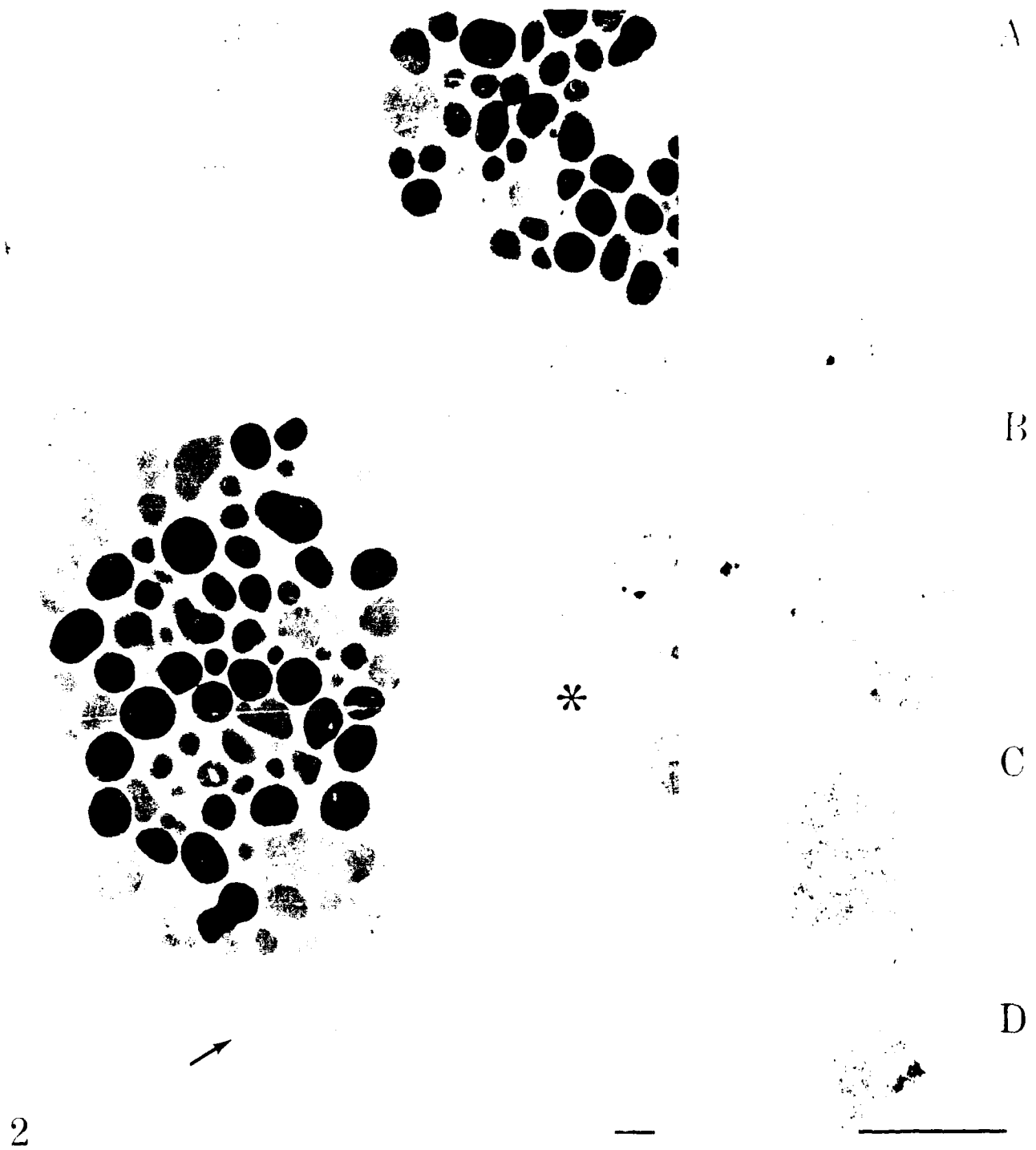


Figure 1. Cyclo-oxygenase localization in mast cells stimulated with A23187. The cyclo-oxygenase activity was localized by using monoclonal anti-cyclo-oxygenase antibodies and the resulted antigen-antibody complex was visualized ultrastructurally by binding of ferritin-conjugated antibodies. The ferritin label was confined to the strands of proteo-heparin projecting from the surface of the secreted granules. Eosinophils (Es) showed no label. Membrane vesicles were often seen in association with secreted granules (arrow). (A-D) High-magnification images of areas marked a through d. Original magnification $\times 10,120$; A-D $\times 36,550$. Bars = $0.5 \mu\text{m}$.



2

Figure 2. Association of cyclo-oxygenase with vesicles assembled from granule matrix phospholipid. The presence of cyclo-oxygenase activity, as visualized by the ferritin label, is seen to decorate the secreted membrane vesicles (arrow). (A-D) High-magnification images of serial sections of the cluster of vesicles. Note that the damaged mast cell (*) shows no ferritin label. Original magnification $\times 1,362$; A-D $\times 48,375$. Bars = 0.5 μm .

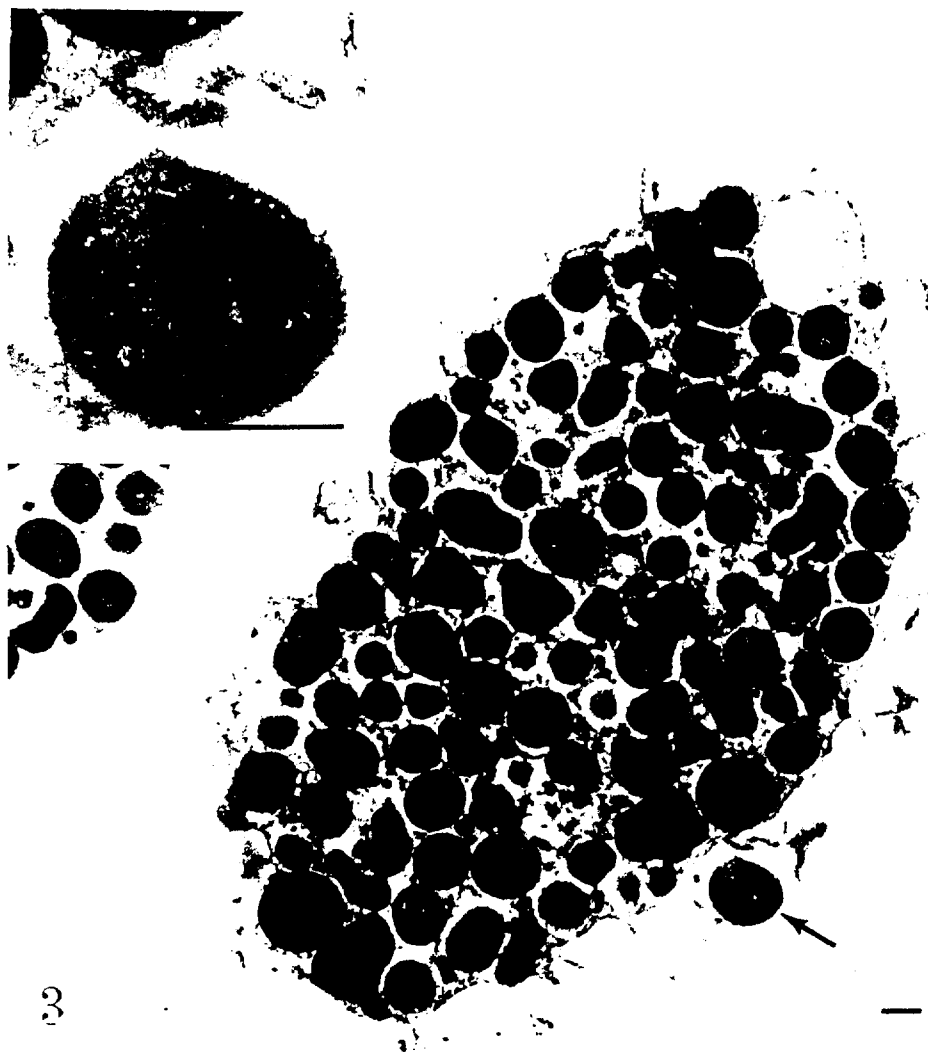


Figure 3. Control experiment for cyclo-oxygenase localization. Secreting mast cells incubated with pre-immune serum in place of the anti-cyclo-oxygenase antibodies show essentially no ferritin label. (Inset) High-magnification image of the granule (arrow). Original magnification $\times 10,350$; Inset $\times 40,850$. Bars = $0.5 \mu\text{m}$.

lished procedure (9), except that plain normal Hank's balanced salt solution (HBSS) was used as a lavage buffer. After one wash with HBSS and centrifugation at $30 \times g$ for 10 min, mast cells were activated by suspension in HBSS containing $1 \mu\text{g/ml}$ A23187 for 5 min at 20°C . The reaction was stopped and cells were fixed by the addition of an equal volume of fixative containing 5% glutaraldehyde, 100 mM cacodylate, and 4 mM MgCl_2 . After a 30-min incubation at 20°C , the cells were washed three times with HEPES-buffered saline (0.15 M NaCl and 20 mM HEPES, pH 6.8). Each wash was for 15 min with gentle agitation and was followed by a 2-min 1500-rpm centrifugation in a Beckman microfuge 12. During the third wash, the cell suspension was divided into aliquots for the various intended experiments. After centrifugation, the cell pellets were re-suspended in the respective antibodies or in pre-immune serum for the corresponding control experiments.

For localization of cyclo-oxygenase, the cells were re-suspended and incubated for 75 min with monoclonal mouse anti-cyclo-oxygenase immunoglobulin G₁ (Cayman Chemical; Ann Arbor, MI) diluted to an approximate concentration of $50 \mu\text{g/ml}$ in HEPES-buffered saline containing 20 μM digitonin (Fluka; Buchs, Switzerland) and 10 mM EDTA. A cell sample for the corresponding control experiment was incubated in pre-immune mouse serum diluted to $50 \mu\text{g/ml}$ in the same buffer.

For localization of PGE_2 , the cells were incubated for 75 min with polyclonal rabbit anti- PGE_2 immunoglobulin G (Cayman Chemical) diluted to about $50 \mu\text{g/ml}$ as for the anti-cyclo-oxygenase antibody. The corresponding control was incubated with pre-immune rabbit serum at about $50 \mu\text{g/ml}$.

After incubation, the cells were treated with three 5-min washes in HEPES-buffered saline to remove unbound antibodies. To visualize the immunogenic activity of the cyclo-oxygenase, both the control and the experimental samples were incubated with about 1 mg/ml ferritin-conjugated goat anti-mouse IgG antiserum (Cappel; West Chester, PA) for 60 min, followed by three 5-min washes with HEPES-buffered saline to remove unbound ferritinated antiserum. To visualize the PGE_2 , the control and the experimental samples were incubated with diluted ferritin-conjugated goat anti-rabbit IgG antiserum (Sigma; St. Louis, MO) for 60 min, followed by washes to rid of excess unbound antibodies. Similarly, gold-conjugated antiserum (Sigma) was also used in place of the ferritin-conjugated antisera for ultrastructural localization of PGE_2 .

After a last wash to rid the cells of the unbound heavy metal-conjugated antisera, the specimens were osmicated for 20 min in 1% osmium tetroxide, followed by routine dehydration and embedding in Epon 812 according to established procedures (12). Unstained thin sections were examined with a Phillips 400 transmission electron microscope.

Results

Cyclo-oxygenase Localization

The application of monoclonal anti-cyclo-oxygenase antibodies to A23187-stimulated peritoneal lavage cells permitted the ultrastruc-

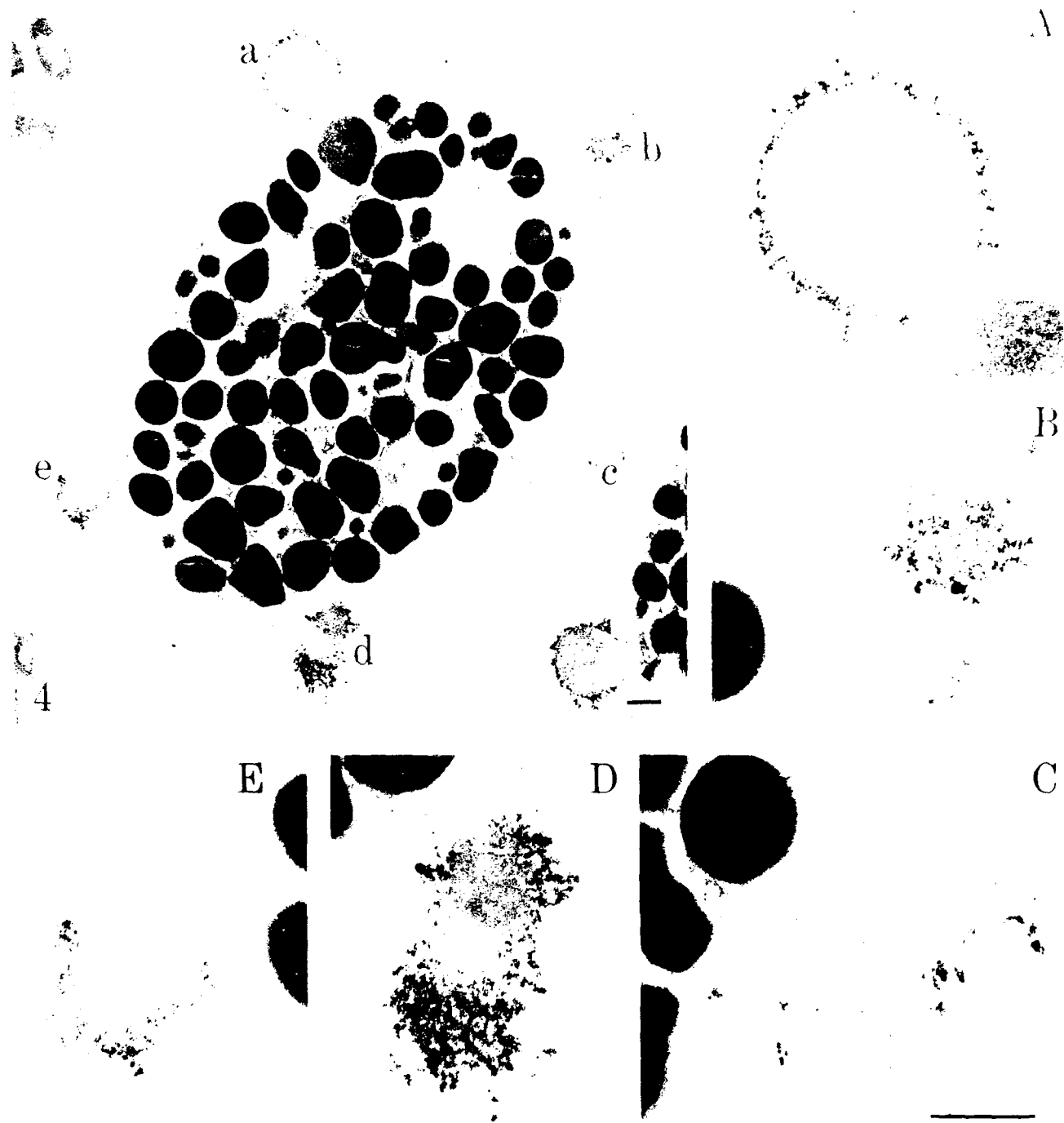


Figure 4 Localization of the secretory granule as the site of PGE₂ production. Mast cells were stimulated with A23187 and PGE₂ was localized by using a polyclonal anti-PGE₂ antibody. The resulting antigen-antibody complex was visualized ultrastructurally by binding of ferritin-conjugated IgG. PGE₂ is confined to the exposed surface of the secreted granules (A-E) High-magnification images of areas labeled a through e. Original magnification $\times 11,730$; A-E $\times 34,400$. Bars = 0.5 μ m

tural localization of cyclo-oxygenase to the secretory granule of the mast cell. The association of cyclo-oxygenase with secreted mast cell granule matrix was readily visualized (Figure 1). Antibody binding was prominently localized to the proteo-heparin strands project

ing from the surfaces of the secreted granules. All secreted mast cell granules were labeled. Binding was not seen in the interior of the granules. This may indicate the inaccessibility of the condensed granule matrix to the antibodies. Dispersed granules which re-

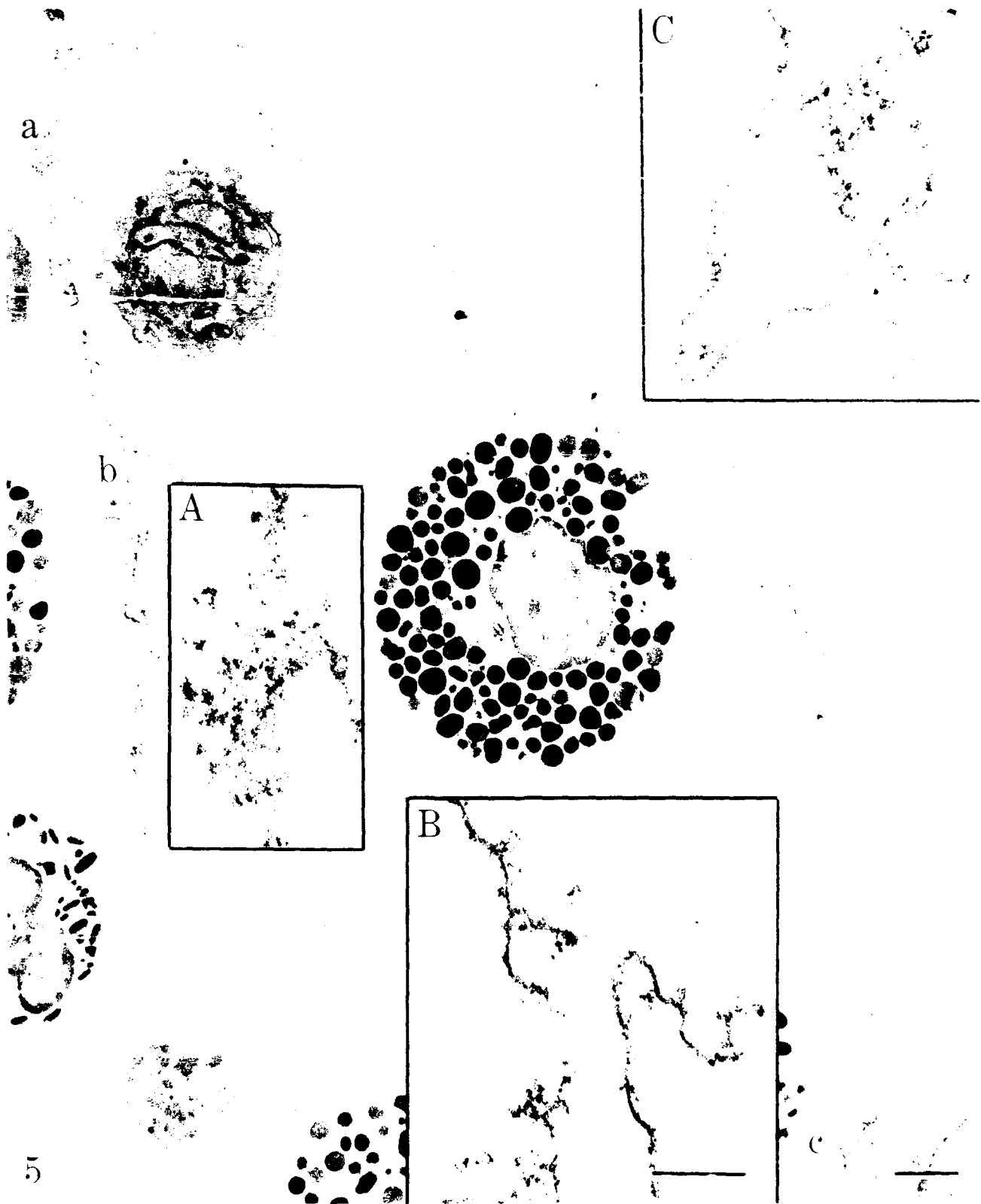
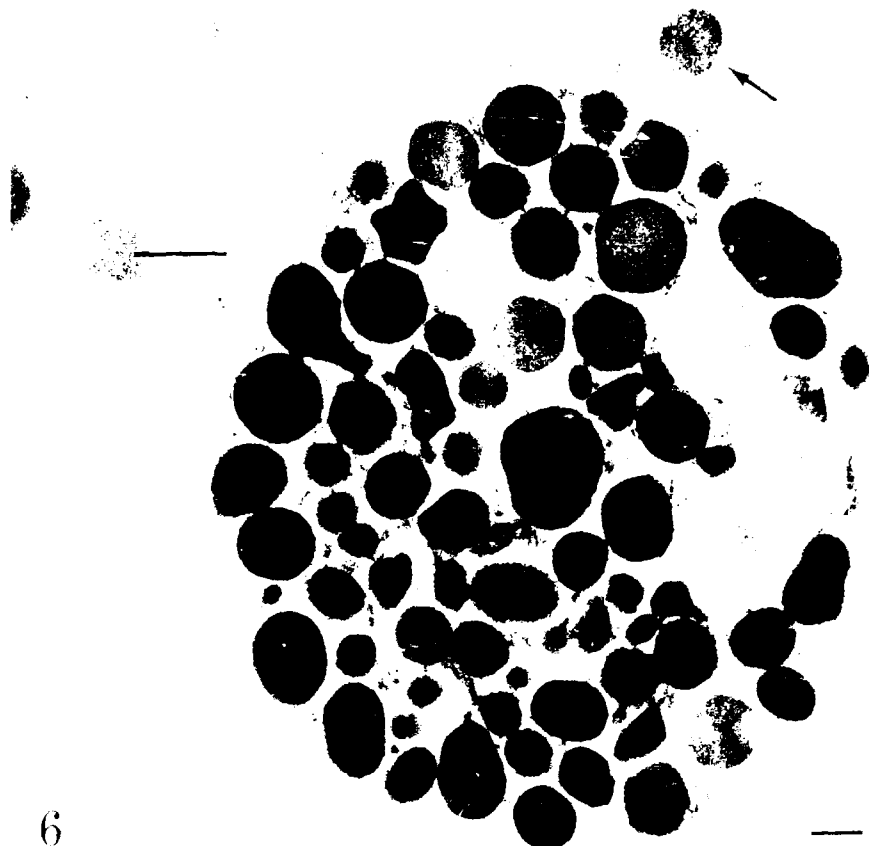


Figure 5. PGE₂ localization on unraveled secreted granule. Complete unraveling of a secreted granule has permitted visualization of PGE₂ binding on the ribbon-like proteo-heparin strand. Bar at the lower right-hand corner = 2 μ m. Insets A, B, and C correspond to areas a, b, and c on the strand. Even distribution of ferritin label on the matrix proteo-heparin strands can be observed. Original magnification \times 5698, A-C \times 33,325. Inset bar = 0.5 μ m.

Figure 6. Control experiment for PGE₂ localization. Parallel experiment in which pre-immune serum is used in place of the polyclonal anti-PGE₂ antiserum. (Inset) High-magnification of the secreted granule (arrow). Original magnification $\times 13,570$; inset $\times 24,725$. Bars = 0.5 μm .



mained within the confines of the cell but which were in communication with the outside via a pore often had little or no label. No cyclo-oxygenase was localized to the plasma membranes of any cell type present in the lavage.

The lipid nature of the granule contents could be seen, to a limited extent, with the appearance of a single vesicle adjacent to a secreted granule (Figure 1A). It became more apparent in Figure 2, where a large cluster of vesicles was associated with a partially secreted mast cell. This mass of vesicles was prominently labeled for cyclo-oxygenase, especially in the intervesicular regions of the mass.

A control cell which received pre-immune serum rather than anti-cyclo-oxygenase antibodies is shown in Figure 3. The absence of label on the secreted granules of the control cells illustrated the specificity of the antigen-antibody reaction.

Prostaglandin E₂ Localization

The application of polyclonal anti-PGE₂ antibodies to A23187-stimulated peritoneal lavage cells has established the mast cell granule as the site of PGE₂ synthesis during histamine release (Figure 4). All secreted mast cell granules were labeled. Although other granule-containing cell types were present at the lavage, including

eosinophils and neutrophils, these cells did not appear to have undergone secretion and were generally not associated with PGE₂ label. Antibody binding was predominantly localized to the periphery of the secreted granules. Unlike the cyclo-oxygenase label which was apparent on the proteo-heparin projections from the granule matrix, the PGE₂ label was distributed evenly and in a more continuous fashion on the surface of the matrix. This suggests a strong hydrophobic interaction between the PGE₂ and the hydrophobic components of the matrix.

It is not uncommon to find extruded granules that have become so dispersed and unraveled that they reveal their proteo-heparin cores as being made up of long ribbon-like strands (17). A portion of an unraveled granule can be seen in Figures 5A-5C. The adherence of the label throughout the strand indicates that much of the newly synthesized prostaglandin remains bound to the granule matrix long after the extrusion of the granule. We never observed any gradient of label in association with the granule matrix. The label was always tightly associated with the matrix protein and did not appear to diffuse from this bound state.

Results of the control experiment in which cells received pre-immune serum rather than PGE₂ antibodies can be seen in Figure 6. The absence of label in the control sample lends credence to the results. The localization of both the cyclo-oxygenase and its



Figure 7. Ultrastructural visualization of PGE₂ antigen-antibody complex using a gold-conjugated antibody. Parallel experiment as in Figure 4, except that a gold-conjugated IgG antibody was used in place of the ferritin conjugated one for visualization of PGE₂. Like the ferritin label, the gold label is also confined to exposed surfaces of secreted granules, especially in areas where the granule is more dispersed and accessible to label penetration. Original magnification $\times 34,425$. Bar = 0.5 μm .

product, PGE₂, further supports the conclusion that the secretory granule is the site of the arachidonic acid cascade during exocytosis.

Analogous experiments which substituted the ferritin-conjugated antiserum with a gold-conjugated antiserum in the second antibody incubation were also carried out. The results were in agreement with those using ferritin-conjugated antibody for visualization of the antigens. Figure 7 shows a typical result using the immunogold method for localization of PGE₂.

Discussion

In agreement with our previous biochemical evidence, our current ultrastructural results also confirm the fact that the secretory granule of the mast cell is the site of cyclo-oxygenase activity and prostaglandin synthesis during exocytosis. Because we applied antibodies to secreting whole cells, the labeling was limited to exposed surfaces. The fact that only the matrices of the secreted granules were labeled (Figures 1, 4, 5, and 7) implies the presence of an eicosanoid-synthetic machinery in the secretory granule.

Contrary to what might have been expected, cell plasma membranes were never labeled with either cyclo-oxygenase or PGE₂ activity. Disrupted cells, broken or damaged as a result of handling,

also gave no evidence of cyclo-oxygenase activity or PGE₂ localization, even though the antibodies often had access to internal cytosolic locations (Figure 2).

Prostaglandin E₂ appeared to be bound to the granule matrix even when the granule was unraveled (Figure 5). Since PGE₂ is very hydrophobic, as evidenced by its low solubility in water, much of it may have remained bound to the hydrophobic matrix after secretion. Many cells of the immune system, such as neutrophils and macrophages, are rapidly attracted to secreted mast cell granules because their secretion is associated with the production of the so-called "slow-reacting substances of anaphylaxis" (SRSA). These SRSA, which are primarily leukotrienes, are potent leukocyte chemoattractants. In Figure 8, a granulocyte appears to be phagocytosing a labeled mast cell granule. Since the eicosanoids, as represented by PGE₂, remain bound to the granule matrix via hydrophobic interaction, it seems possible that these bound lipid mediators may confer a signal to the phagocytosing cells by activating their cell surface receptors at the points of contact. The phagocytosis of the granule matrix would result in formation of a lysosome which contains lipid mediators. However, it is also conceivable that mediator-containing vesicles, such as some of those shown in Figure 2, may fuse directly with a target cell on contact. Their fusion would result in the direct release of lipid mediators into the cytoplasm of the



Figure 8. Phagocytosis of ferritin-labeled secreted mast cell granule by granulocyte. A granulocyte is seen in the process of phagocytosing a mast cell granule (arrow) which has been labeled with ferritin for PGE₂ activity. Original magnification $\times 10,580$. Bar = 1 μm .

target cell. The internalization of non-membrane-delimited mediators opens up the possibility of some yet unknown intracellular functions for the lipid mediators. A role of arachidonic acid metabolites as intracellular modulators of K⁺ channel has been recently suggested (18).

Previously, eicosanoid synthesis was considered to originate from membrane phospholipid (1-5), with damage to these membranes as the event that made phospholipid available for eicosanoid synthesis. Our results indicate that prostaglandin formation may actually be the result of receptor-mediated granule exocytosis. The coupling of granule activation to triggering of the arachidonic acid cascade explains why, for the mast cell, the initial time course of histamine release closely parallels the time course for prostaglandin production (14,19). This explanation is also consistent with the coincident production of eicosanoids with the process of secretion in other secretory systems (20,21).

In the eicosanoid biosynthetic cascade, the first requirement for the synthesis of prostaglandins is availability of phospholipid. In the mast cell granule, this is easily met by the existence of a large non-bilayer phospholipid store bound to the granule matrix (9). The second requirement is a phospholipase to liberate the arachidonic acid from the phospholipid. Since arachidonic acid is usually esterified in the sn-2 position of phospholipid (7), the presence of a phospholipase A₂ is implied. The existence of a phospholipase A₂ in the mast cell granule has now been verified in our laboratory (submitted for publication). In fact, the formation of PGE₂ from endogenous granule phospholipid detected here by using a specific antibody to PGE₂ is in itself strong evidence for the presence of a phospholipase A₂ in the granule. This phospholipase A₂, like that found in the pancreas and in the macrophage, requires high calcium concentrations for its activity (4,8). This requirement can be easily met in the granule (22), but not by a membrane bilayer source. The phospholipase A₂ of macrophage granules was assumed to be bound to the granule membrane (4). In the mast cell, our studies suggest that it is bound to the granule matrix instead. The third requirement in the pathway is the presence of a cyclo-oxygenase, the topic of this study, to catalyze the conversion of arachidonic acid to PGE₂. Current theory had also placed this enzyme, along with other key enzymes of the cascade, in or on the plasma membrane. However, it should be pointed out here that fragmented granule matrix components can be easily sedimented with the microsomal fraction at 100,000 × g. Therefore, it is possible to confuse granule matrix components with membrane components. This confusion becomes more obvious in experiments involving the use of fragmented cells or tissues as starting materials. Our observations indicate that cyclo-oxygenase is tightly bound to the matrix of the secretory granule, where a large amount of phospholipid is stored. All these points, taken together, suggest that the secretory granule contains all the necessary ingredients to function as an eicosanoid-producing entity during granule exocytosis.

There are many inconsistencies surrounding the current dogma which places the prostaglandin-synthesizing machinery on the plasma membrane. Aside from the difficulty of coping with the consequence of placing many membrane-destructive enzymes on the plasma membrane, this concept also provides no credible explanation of how hydrophobic products such as prostaglandins can

be released from their hydrophobic membrane environment during exocytosis. More serious challenge to this dogma ensues after the identification of a key enzyme in the cascade, prostaglandin D-isomerase, being a cytosolic enzyme instead of an expected membrane enzyme (23). This difficulty of placing the cyclo-oxygenase complex on the plasma membrane while the D-isomerase/synthetase is in the cytosol, has been pointed out by Giles and Leff (24). Furthermore, the use of immunocytochemical technique also failed to demonstrate the presence of cyclo-oxygenase on the plasma membrane (25). Our present finding of localizing both the substrate and the necessary enzymes of the cascade to the secretory granules not only can overcome the difficulties facing the current dogma but also can provide the physical basis for linking of granule activation to the production of eicosanoids during exocytosis (16).

We now understand that stimulus in the form of trauma, infection, or radiation causes certain cells to secrete and thus produce various lipid mediators which can initiate an inflammatory response. Many of these mediators are also potent chemotactic factors. The involvement and the recruitment of the cells of the immune system into the site of inflammation usually results in tissue destruction. Under physiological conditions, the process of inflammation is self-contained in the sense that the release of a deleterious mediator eventually elicits the release of opposing factors via a feedback mechanism. This line of thinking is consistent with the concept of Chandler and Fulmer, who suggested that the inflammation-promoting eicosanoids are released to initiate an inflammatory response and that this inflammatory state is later terminated by the release of immunosuppressive eicosanoids (26). Many studies on the roles of eicosanoids in the mechanism of inflammation and how they interact with the various lymphokines, helper cells, and suppressor cells to elicit a specific immune response have been published (27-30). The localization of the site of eicosanoid production to the secretory granule and the linking of their production to the process of secretion will lead to a better understanding of the mechanism of inflammation and open the way to specific manipulation of the immune system.

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Animal usage was in compliance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

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