Horse Eosinophil Degranulation Induced by the Ionophore A23187

Ultrastructure and Role of Phospholipase A_2

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Horse eosinophils stimulated with the calcium ionophore A23187 were examined by transmission and scanning electron microscopy. Secretion was characterized by granule movement to the cell periphery and fusion of adjacent granules. The granules became swollen and less electron-dense as their contents were released into large intracellular vacuoles, which opened to the outside of the cell through surface pores. A23187-induced eosinophil peroxidase (EPO) release, as measured by guaiacol oxidation, was blocked by eicosa-5,8,11,14-tetraynoic acid (ETYA) (which inhibits both the cyclooxygenase and lipoxygense pathways of arachidonic acid metabolism) but not by indomethacin (which inhibits only the cyclooxygenase pathway). Highly purified porcine phospholipase A_2 induced noncytotoxic eosinophil degranulation (as measured by the release of EPO without the concomitant release of the cytoplasmic marker lactate dehydrogenase), which was blocked by pretreatment of the enzyme with the phospholipase A_2 inhibitor 4-bromophenacyl bromide. These results suggest that calcium-dependent activation of phospholipase A_2 and generation of lipoxygenase products of arachidonic acid metabolism are important in the initiation of eosinophil degranulation (Am J Pathol 1983, 111:341–349)

EOSINOPHILS, which are a prominent constituent of immediate hypersensitivity reactions, may play an important role in the host defense against certain parasites and tumor cells. Eosinophils are characterized by their large cytoplasmic granules, which contain high concentrations of peroxidase (eosinophil peroxidase, EPO)[1,2] and of basic proteins.[3] Eosinophils attracted to the site of inflammation may be stimulated by reaction with an appropriately opsonized particle or other stimulus to release extracellularly their granule components[4] as well as nongranule low-molecular-weight compounds such as hydrogen peroxide (H_2O_2)[5,7] and leukotrienes.[8] EPO, when combined with H_2O_2 and a halide, has potent cytotoxic[9,10] and microbicidal[11–14] activity, and can inactivate soluble mediators[7] and induce mast-cell secretion.[15]

There have been few ultrastructural studies of eosinophil degranulation in comparison with the extensive literature on degranulation by other pathocytes. We report here the ultrastructural changes in horse eosinophils induced to degranulate by the calcium ionophore A23187. Characteristic features of this secretory process are the fusion of intracellular perigranular membranes with formation of large intracellular vacuoles, which open to the outside of the cell through surface pores. EPO is released into these channels and discharged extracellularly. Highly purified porcine phospholipase A_2 can initiate noncy-

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totoxic eosinophil degranulation, and evidence suggesting that lipoxygenase products of arachidonic acid metabolism may be important in the secretory process is provided.

Materials and Methods

Special Reagents

Phospholipase A_2 was kindly provided by Dr. G. H. deHaas, Rijksuniversiteit, Utrecht, the Netherlands. It was isolated in pure form from porcine pancreas[16] and had a specific activity of 1400 units/mg protein, determined by the use of titrimetric assay with phosphatidylcholine as substrate.[17] One enzyme unit of phospholipase A_2 generates 1 μEq of free fatty acid per minute. Eicosa-5,8,11,14-tetraynoic acid (ETYA) was kindly provided by Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, New Jersey. 4-Bromophenacyl bromide (BPB) was obtained from Sigma Chemical Co., St. Louis, Missouri, 3,3-diaminobenzidine tetrahydrochloride (DAB) from Scientific Chemical Co., Huntington Beach, California, and the calcium ionophore A23187 from CalBiochem, La Jolla, California. All other commercial reagents were of the highest grade available.

Isolation of Eosinophils

Eosinophils were isolated from horse blood (kindly supplied by the Florence Packing Co., Florence, Wash) by polyvinylpyrrolidone sedimentation as previously described.[18] The cell preparations contained 98–100% eosinophils, which were greater than 98% viable as seen by trypan blue exclusion. The eosinophils were suspended in a standard salt solution, pH 7.2, consisting of 2.7 mM KCl, 154 mM NaCl, 4 mM NaH_2PO_4, 2.7 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.9 mM $CaCl_2$, and 6 mM glucose.

EPO and Lactate Dehydrogenase (LDH) Release From Eosinophils

Eosinophil preparations in duplicate were preincubated for 5 minutes at 37 C in a shaking water bath in the presence or absence of inhibitors (see legend to Table 1). Either the calcium ionophore A23187 or pancreatic phospholipase A_2 was added to make a final volume of 1.0 ml, and the incubation was continued for an additional 15 minutes. The samples were then centrifuged at 400*g* for 6 minutes at 4 C, and the supernatants and pellets were collected and assayed for peroxidase and lactate dehydrogenase (LDH) release as previously described.[7,15] One peroxidase enzyme unit is defined as the amount that oxidizes 1 μmole of guaiacol per minute at 25 C. A molar absorbancy for tetraguaiacol of 2.66×10^4 M^{-1} cm^{-1} at 470 nm was employed.[19] The percentage of peroxidase released was calculated as follows: (supernatant EPO/pellet + supernatant EPO) × 100 and the percentage of LDH release as follows: (control − experimental pellet LDH/control pellet LDH) × 100.

In some experiments, the pancreatic phospholipase A_2 was inactivated by preincubation with BPB.[20] We added 0.025 ml of BPB (2×10^{-3} M in acetone) to 0.475 ml of 0.1 M phosphate buffer, pH 7.0, containing 250 μg of phospholipase A_2 to make a final BPB concentration of 1×10^{-4} M. Following incubation for 6 hours at 37 C, the mixture was dialyzed against water with the use of Spectra/Por hollow fiber membranes (Spectrum Medical Industries, Queens, NY) for removal of BPB and acetone. This BPB-treated phospholipase A_2 had less than 10% residual enzymatic activity.

Electron-Microscopic Studies

Pellets of eosinophils incubated with the various components of the reaction mixture (see legends to figures) were collected by centrifugation at 400*g* and prepared for transmission[15] or scanning[21] electron-microscopic (EM) examination as previously described. All preparations for transmission EM examination were stained in the block with uranyl acetate and in the section with lead citrate unless otherwise indicated. For peroxidase detection, some pellets were treated with a saturated solution of DAB and 0.015% H_2O_2[22]. The transmission EM studies were performed with a JEOL 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan) at 60 kv, and the scanning EM studies with a JEOL JSM 35C electron microscope at 19 kv.

Statistical Analysis

The data are reported as the mean ± SE of the combined experiments, and differences were analyzed for significance with the use of the Student two-tailed *t* test for independent means (not significant, $P > 0.05$).

Results

Transmission electron micrographs of untreated and A23187-stimulated eosinophils are shown in Figure 1. Unstimulated horse eosinophils are characterized by their large, uniformly electron-dense, mem-

brane-bound cytoplasmic granules (in contrast to human eosinophils, whose granules contain an electron-dense crystalloid core and a less dense matrix) (Figure 1a). The nucleus is typically segmented, other cytoplasmic organelles (endoplasmic reticulum, Golgi vesicles, mitochondria) are present, and the plasma membrane contains numerous microridges. Fewer than 2% of the control cells showed ultrastructural evidence of degranulation.

In contrast, greater than 70% of the eosinophils showed evidence of degranulation when incubated with 10 μg/ml A23187 for 5–15 minutes at 37 C (Figure 1b–e). At 5 minutes, cytoplasmic granules lost some of their electron-density, and pentalaminar membrane structures formed between adjacent granules (Figure 1b and c). Surface bulges due to submembranous granules were seen (Figure 1b), and there was a loss of plasma membrane microridges. At 15 minutes, large intracellular vacuoles and channels were seen (Figure 1d and e), which opened to the outside of the cell through surface pores (arrows). The channels contained some electron-dense material largely subjacent to the membrane; however, no intact granular matrices were seen either within the channels or extracellularly, and many channels appeared largely empty. There were no structural changes suggestive of cytotoxic damage to the cells. Degranulation of eosinophils by A23187 was not observed when calcium was excluded from the reaction mixture (data not shown).

The degranulation of A23187-stimulated eosinophils was confirmed by scanning EM examination (Figure 2). Control eosinophils were spherical, with numerous surface ridges (Figure 2a). Following stimulation by A23187, prominent bulging of the surface over underlying granules was seen, and pores were often noted in the cell surface (Figure 2b). Corresponding transmission electron micrographs confirmed the presence of marked bulging and the absence of extracellular granules (Figure 2c).

The localization of peroxidase in control and A23187-stimulated eosinophils is shown in Figure 3. In normal cells, peroxidase activity was present predominantly in the large cytoplasmic granules (Figure 3a). Following stimulation of degranulation by A23187, peroxidase-positive material was seen lining cytoplasmic vacuoles and bound to the surface membrane (Figure 3b). Omission of H_2O_2 from the DAB mixture abolished this reaction.

In agreement with our prior studies,[7] 10.2% of the granule EPO was detected in the extracellular fluid following treatment of eosinophils with the ionophore A23187 (10 μg/ml) under conditions in which LDH release was less than 5% (Table 1). A23187-induced EPO release was unaffected by indomethacin (10^{-5}–10^{-6} M) but was significantly decreased by ETYA (10^{-5} M) (Table 1). The addition of phospholipase A_2 (2 IU/ml) to eosinophils induced peroxidase release comparable to that seen with A23187. Pretreatment of the enzyme with BPB significantly decreased EPO release, whereas phospholipase A_2, a heat-stable enzyme,[23] retained its eosinophil-degranulating activity when heated at 100 C for 1 hour (Table 1). There was no significant release of EPO when eosinophils and phospholipase A_2 were incubated in buffer without calcium. The ultrastructural features of phospholipase A_2-induced eosinophil degranulation were comparable to those seen when the calcium ionophore A23187 was employed (data not shown).

Discussion

Prior morphologic studies have indicated that degranulation follows phagocytosis by eosinophils, with the discharge of granule contents, including peroxidase, into the phagosome.[24] Granule rupture can occur prior to the completion of the act of engulfment with the extracellular release of granule components.[1] Exocytosis is particularly apparent when eosinophils encounter particles too large to be ingested. Thus, eosinophils adherent to the surface of antibody-coated schistosomula release their granule components extracellularly, and peroxidase can be detected on the surface of the organism.[4] Our studies using transmission and scanning electron microscopy indicate that eosinophil exocytosis also is induced by the ionophore A23187.

Degranulation by the ionophore was characterized initially by a loss of granule density, the presence of pentalaminar structures between adjacent granules, and the formation of surface bulges overlying submembranous granules. Large intracellular vacuoles communicated with the cell exterior through surface pores. Intact granule matrices were seen neither within the cytoplasmic vacuoles nor extracellularly, suggesting that the granule material dissolves completely and is secreted. The disparity between the extensive release of granule contents observed ultrastructurally and the recovery of relatively small proportion of the total peroxidase in the supernatant fluid of stimulated cells (10.2%) may be due in part to extensive binding of the released EPO to the vacuolar and plasma membranes, as detected by diaminobenzidine cytochemical study (Figure 3b). A23187 at the concentration employed (10 μg/ml) caused less than 5% release of the cytoplasmic enzyme LDH and had no discernible effect on the morphologic characteristics

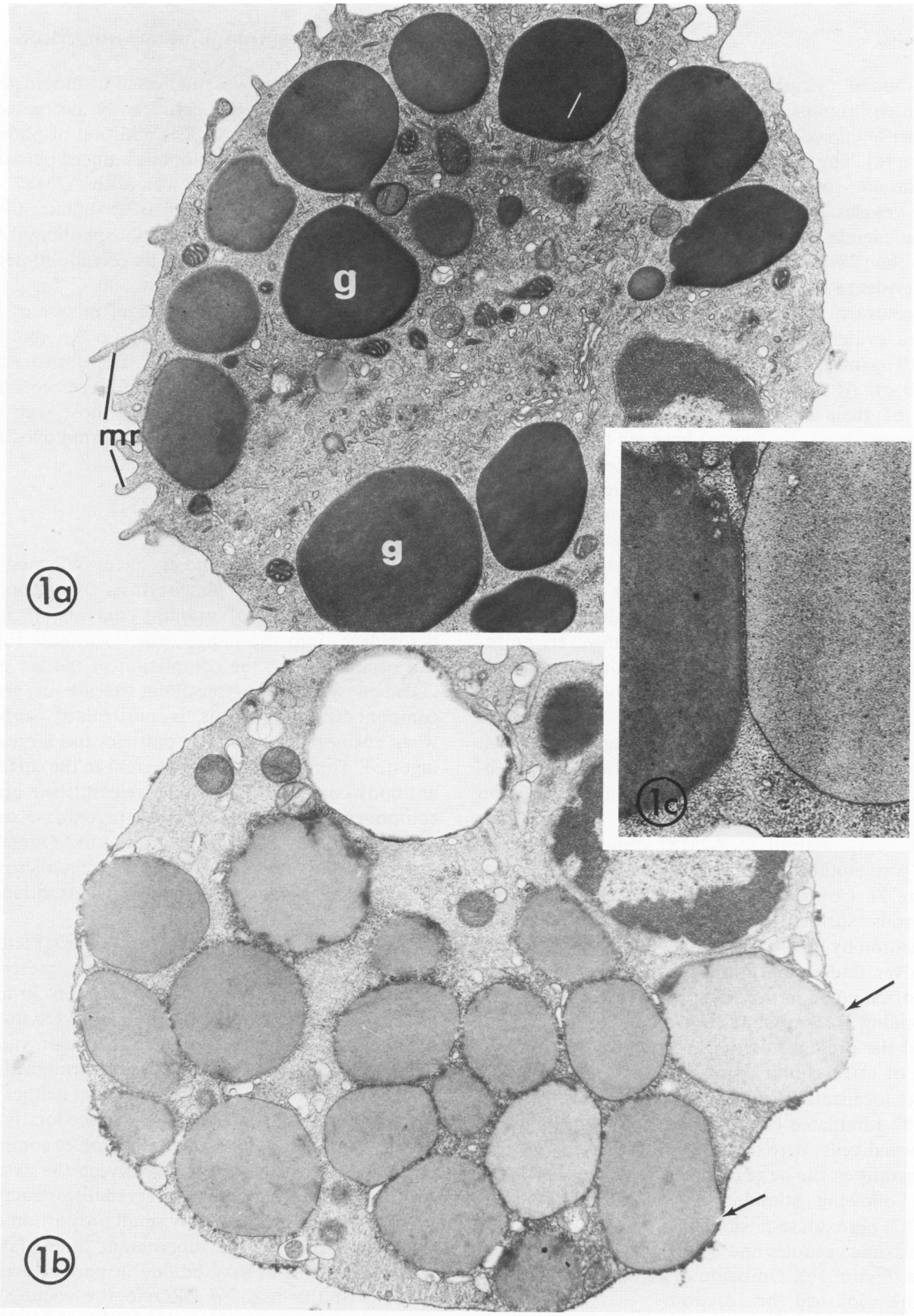
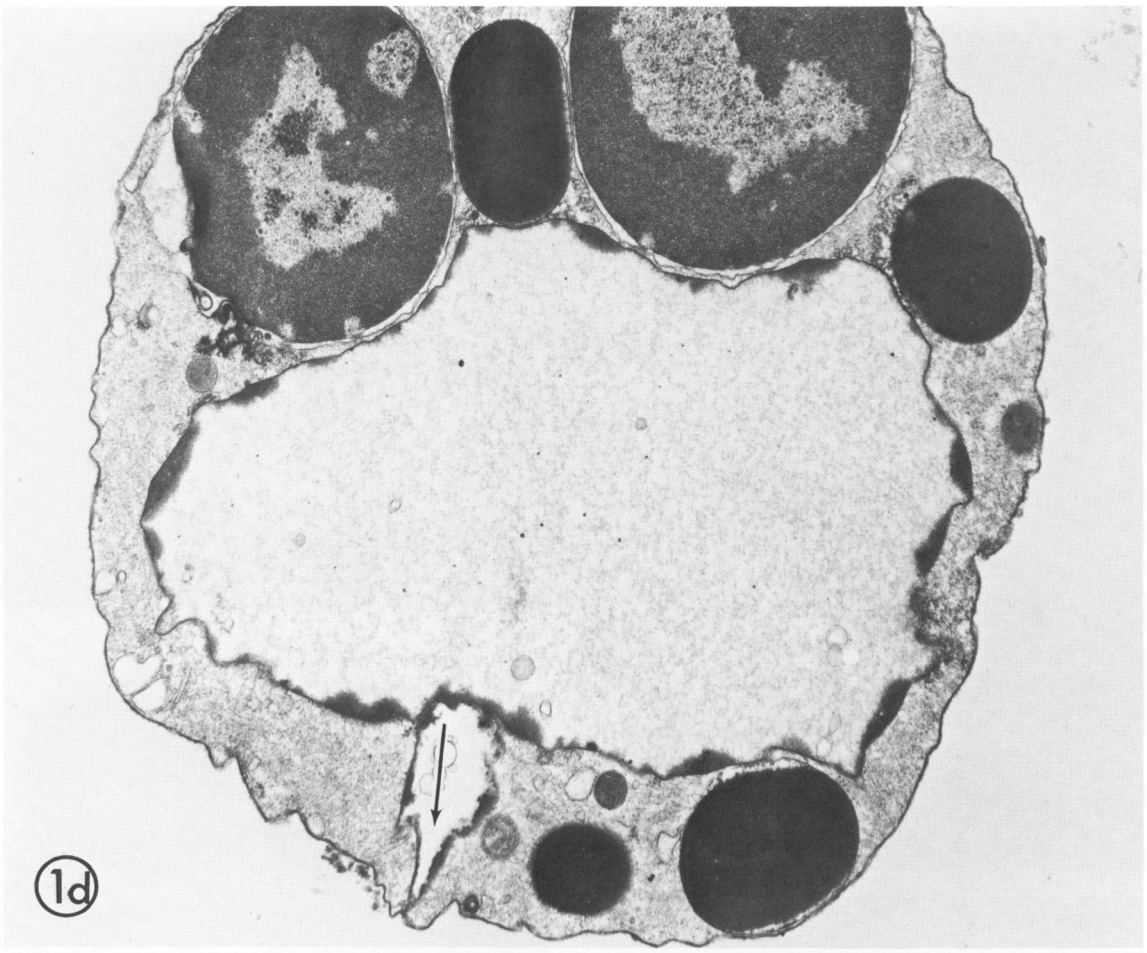


Figure 1 — A23187-induced eosinophil degranulation (transmission electron microscopy). Eosinophils (5×10^6) were incubated in 1.0 ml of standard salt solution either alone (**a**, ×12,500) or supplemented with 10 μg ionophore A23187 for 5 (**b**, ×9000; **c**, ×30,000) or 15 minutes (**d**, ×13,500; **e**, ×12,000) at 37 C. Control cells (**a**) contained homogeneous, electron-dense granules (*g*) with no evidence of degranulation. Many microridges (*mr*) extended from the cell surface. Golgi vesicles, mitochondria, and other cytoplasmic organelles appeared normal, as did the characteristically segmented nucleus. Five minutes after A23187 stimulation, the cytoplasmic granules were less electron-dense and were in contact with each other and with the cell surface, with the most peripheral granules causing surface bulges (*arrows*) (**b**). Pentalaminar perigranular membrane fusion occurred between adjacent granules (**b** and **c**), and surface microridges were less evident (**b**). Fifteen minutes after stimulation with the ionophore (**d** and **e**), large intracellular vacuoles were found that communicated with the extracellular environment through surface openings (*arrows*). No intact granule matrices were noted either within the channels or outside the cell. While some channels contained amorphous material (**d**), most channels appeared devoid of electron-dense material (**e**). The nucleus, mitochondria, Golgi vesicles, and other intracellular organelles appeared unaffected.



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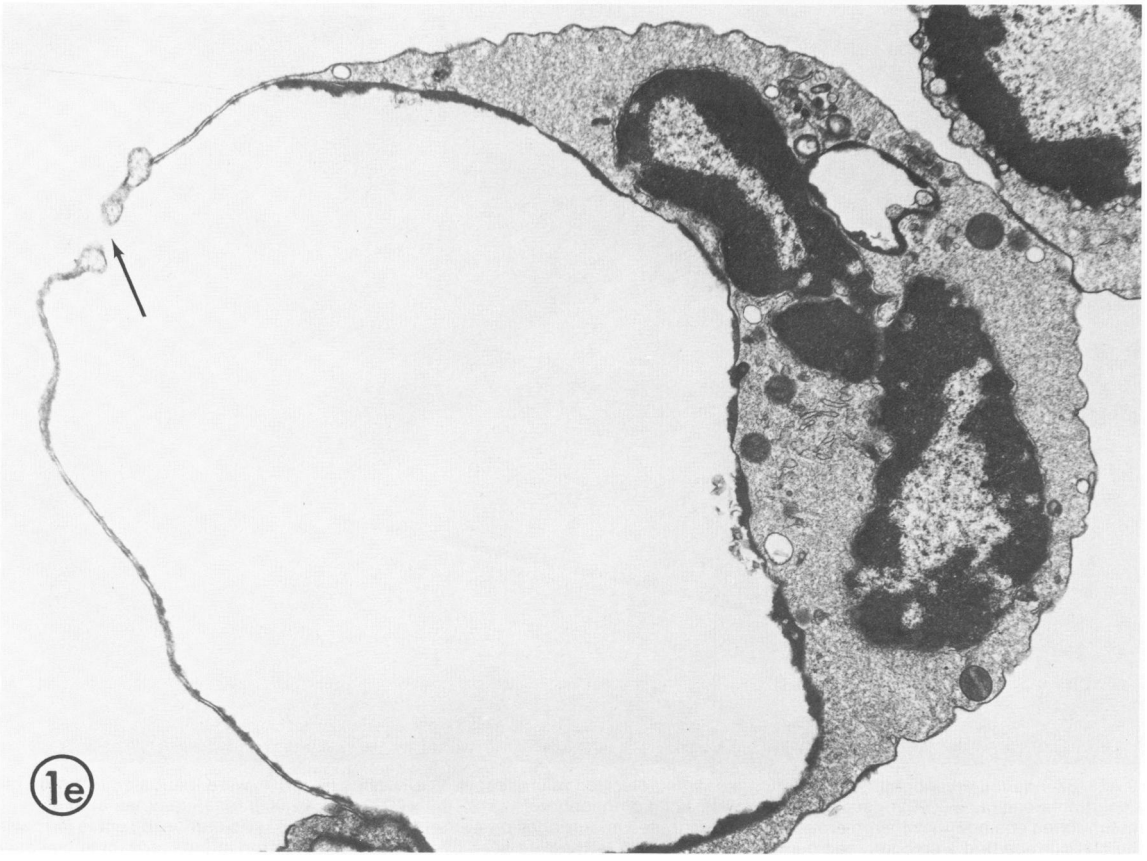

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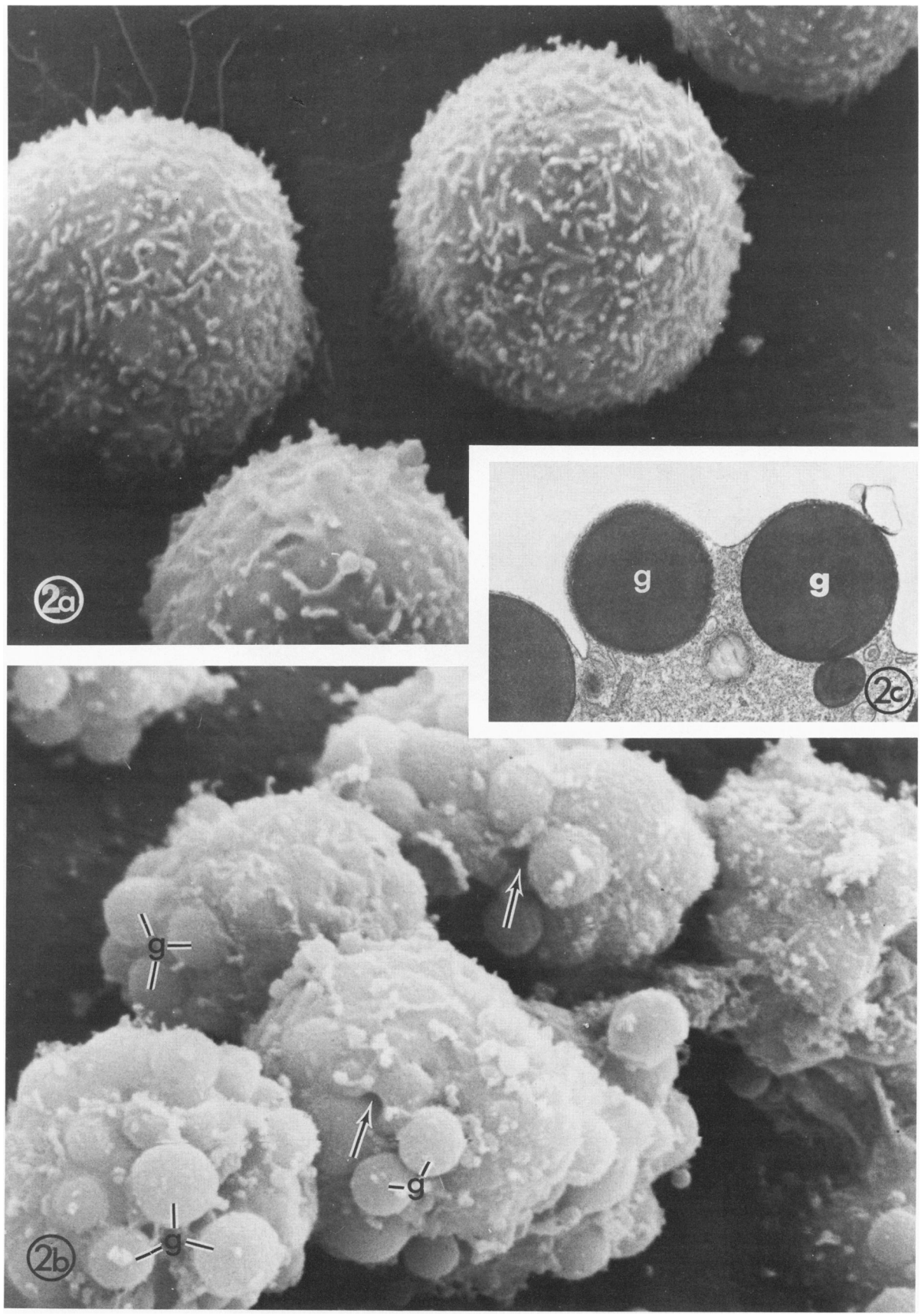

Figure 2 — A23187-induced eosinophil degranulation (scanning electron microscopy). Eosinophils (5×10^6) were incubated in 1.0 ml of standard salt solution alone (**a**, ×6000) or supplemented with 10 μg of ionophore A23187 (**b**, ×6000; **c**, ×22,000) for 15 minutes at 37 C. **a** — A typical unstimulated eosinophil with numerous, evenly distributed microridges on its surface. After A23187 stimulation (**b**), pores (*arrows*) were seen in the cell surface that also contained numerous bulges. The latter were seen by transmission electron microscopy (**c**) to be caused by underlying cytoplasmic granules (*g*).

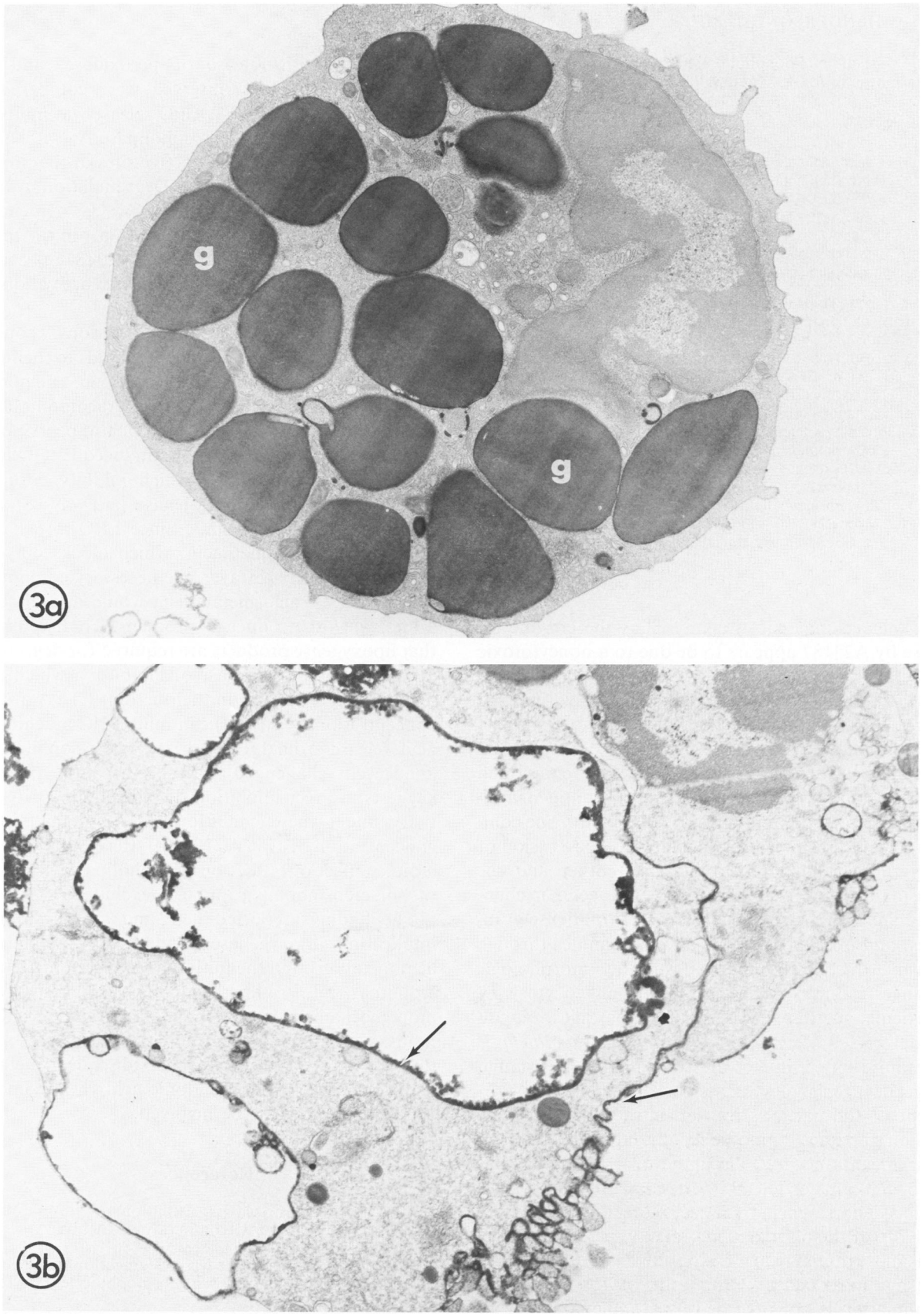

Figure 3 — EPO release from eosinophils. Eosinophils (5×10^6) were incubated in 1.0 ml of standard salt solution either alone (**a**, ×12,000) or supplemented with 10 μg of A23187 for 15 minutes at 37 C (**b**, ×16,000). The preparations were stained for peroxidase and were not counterstained with either uranyl acetate or lead. This caused the peroxidase-positive material to stand out from the remaining poorly stained cellular constituents. **a** — The large cytoplasmic granules (*g*) reacted homogeneously for peroxidase. **b** — After A23187 stimulation peroxidase-positive material (*arrows*) was noted lining the intracellular vacuoles and channels and on the cell surface.

Table 1—Role of Phospholipase A_2 and Arachidonic Acid Metabolites in A23187-Induced EPO Release*

Supplements	EPO release (%)	P
A23187	10.2 ± 1.9†	
A23187 + indomethacin (10^{-5} M)	8.9 ± 3.1	
A23187 + indomethacin (10^{-6} M)	9.8 ± 1.7	
A23187 + ETYA (10^{-5} M)	1.4 ± 0.7	< 0.01
A23187 + ETYA (10^{-6} M)	7.3 ± 2.1	
Phospholipase A_2	12.8 ± 2.5	
BPB-treated phospholipase A_2	1.5 ± 0.8	< 0.01
Heated phospholipase A_2	10.8 ± 3.1	

* The reaction mixture contained 5 × 10^6 eosinophils in the standard salt solution, pH 7.2, and, where indicated, the calcium ionophore A23187 (10 μg/ml) or pancreatic phospholipase A_2 (2 IU/ml). In some experiments, the eosinophils were preincubated with indomethacin or ETYA at the concentrations indicated, or the phospholipase A_2 was pretreated with BPB (see Materials and Methods) or heated for 1 hour at 100 C. Incubation was for 15 minutes at 37 C. Probability values for the difference from eosinophils plus either A23187 or phospholipase A_2 are shown where significant ($P < 0.05$). LDH release was less than 5% for each of the above experimental conditions. EPO release was less than 2% when the eosinophils were incubated in buffer alone or with indomethacin (10^{-5}–10^{-6} M) or ETYA (10^{-5}–10^{-6} M).

† Mean ± SE of four experiments.

of other cellular constituents. Thus the enzyme release by A23187 appears to be due to a noncytotoxic secretory process.

Ultrastructural features similar to A23187-induced horse eosinophil degranulation are observed in mast cells stimulated by a variety of secretagogues, including the calcium ionophore A23187,[25] compound 48/80,[25] polymyxin B,[26] polylysine,[27] antigen–antibody complexes,[28] and phospholipase A_2.[29] The comparable mast cell secretory changes seen are fusion of perigranular membranes with each other and the plasma membrane, formation of intracellular channels containing swollen and less electron-dense granules, and exocytosis of the altered granules through plasma membrane pores.[25–29] The morphologic changes induced in eosinophils by phagocytosis of particulate matter such as zymosan particles,[24,30] colloidal gold,[31] *Mycoplasma*,[32] and *Candida albicans*[33] are quite different, however. Eosinophil degranulation during phagocytosis is characterized by engulfment of the particles into an intracellular vacuole, fusion of adjacent granule membranes with the vacuolar membrane, and discharge of granule contents into the phagosome.[30–33] In contrast to A23187-induced horse eosinophil secretion, there is neither bulging of the plasma membrane by peripheral cytoplasmic granules nor fusion of perigranular and plasma membranes during phagocytosis, and degranulation is not preceded by granule swelling.

The addition of highly purified porcine pancreatic phospholipase A_2 to horse eosinophils initiated degranulation with release of peroxidase, and the morphologic changes observed were compared to those induced by A23187. EPO release was inhibited by pretreatment of the phospholipase A_2 with BPB, which alkylates histidine at active sites of the protein with loss of enzyme activity.[20] Degranulation by both A23187 and phospholipase A_2 (a Ca^{2+}-dependent enzyme[23]) requires the presence of Ca^{2+} in the medium, suggesting that the ionophore A23187 may cause degranulation by a Ca^{2+}-dependent activation of endogenous phospholipase A_2.

Phospholipase A_2 may initiate secretion by conversion of surface-membrane phospholipids to their corresponding lyso-compounds, which are membrane fusogens,[34] and by the release of arachidonic acid, the subsequent metabolism of which appears necessary for degranulation. A23187-induced EPO release was inhibited by ETYA, which inhibits both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism.[35] Since EPO release was unaffected by indomethacin, which blocks only the cyclooxygenase pathway,[35] the necessary component of arachidonic acid metabolism would appear to be a lipoxygenase product or products. The hypothesis that lipoxygense products are required for degranulation depends upon the use of inhibitors, which may not be entirely specific in their action. ETYA at high concentration (> 10^{-4} M) can inhibit ^{14}C-arachidonic acid release from IgE-sensitized rat basophilic leukemic cells challenged with specific antigen, which suggests a direct inhibition of phospholipase A_2 activity under these conditions.[36] Although a lower concentration of ETYA (10^{-5} M) was employed here, a direct effect of ETYA on phospholipase A_2 activity of horse eosinophils cannot be excluded.

The findings reported here and similar findings implicating lipoxygenase products of arachidonic acid metabolism in the initiation of histamine release from mast cells[29,37] and basophils[38,39] suggest a common mechanism by which ionophore-induced Ca^{2+} influx can initiate the degranulation process. Activation of phospholipase A_2[36] and subsequent metabolism of the released arachidonic acid via the lipoxygenase pathway may be involved.

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