

Involvement of Arachidonic Acid in Nonimmunologic Production of Superoxide in Mast Cells

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Key Words

Superoxide · Mast cells · Phospholipase A₂ · Arachidonic acid

Abstract

Background: A number of different molecules are known to be involved in the signal pathway to release histamine from mast cells, among which arachidonic acid (AA) is one of the key mediators. On the other hand, we found that the application of compound 48/80, a typical histamine liberator, generated superoxide in mast cells. In the present study, we investigated the mechanism of superoxide production in mast cells with respect to AA signaling in conjunction with a fine structural analysis. **Methods:** Superoxide production was monitored by chemiluminescence in rat peritoneal mast cells and their subfractions after various treatments. For scanning electron microscopy, the conditions for fixation and freeze-fracture were optimized to get natural fine images. **Results:** Compound 48/80 induced superoxide production in the isolated mast cells and some of their subfractions possibly through intracellular increase in Ca²⁺ concentration, activation of cytosolic phospholipase A₂, and release of AA. **Discussion:** The present results indicate the critical role of AA in the signal pathway to generate superoxide from mast cells in response to compound 48/80.

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Introduction

Superoxide radical (O₂⁻) and its potent by-products, such as singlet oxygen and hydroxyl radical, appear to play a major role in the microbicidal activity of phagocytic cells, such as neutrophils and macrophages [1, 2]. The absence of superoxide production may be responsible for defective killing of bacteria by polymorphonuclear leukocytes in chronic granulomatous diseases [3, 4]. In addition to the normal role of superoxide in host defenses, excess superoxide has been implicated in the pathogenesis of respiratory failure caused by paraquat poisoning [5]. Thus, its production is strictly regulated in phagocytic cells.

Superoxide is also produced by mast cells, which play a central role in immunoglobulin E (IgE)-dependent immediate hypersensitivity and inflammatory reaction by secreting a chemical mediator of histamine stored in their granules [6–8]. Our previous experiments showed that compound 48/80, a potent stimulator of histamine release, induced superoxide production in mast cells [8]. In addition, exogenously added arachidonic acid (AA) at a high dose strongly induced superoxide production in the cells [8]. AA is released from mast cells by nonimmunologic stimuli of compound 48/80, concanavalin A, calcium ionophore A23187, or lipopolysaccharide and by a natural immunologic stimulus such as allergen/anti-IgE

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reaction [9]. Thus, superoxide production in mast cells may be regulated by AA release. In the present study, we investigated whether a physiological concentration of AA could induce superoxide production in mast cells, and we examined the mechanism of superoxide production and its significance in mast cells in conjunction with their structural changes.

Materials and Methods

Materials

The following chemicals were purchased from commercial sources: Percoll (Amersham Pharmacia Biotechnology, Little Chalfont, Buckinghamshire, UK); 2-methyl-6-(*p*-methoxy-phenyl)-3,7-dihydroimidazo(1,2-a)pyrazin-3-one (MCLA; Tokyo Kasei Kogyo, Tokyo, Japan); compound 48/80 (Sigma, St. Louis, Mo., USA); manoalide (WAKO Pure Chemical Industries Ltd., Osaka, Japan); arachidonyl trifluoromethylketone (AACOCF₃; BIOMOL Research Labs., Inc., Plymouth Meeting, Pa., USA); potassium glutamate (Nacalai Tesque, Kyoto, Japan); glutaraldehyde (Nisshin EM Co. Ltd., Tokyo, Japan); osmium tetroxide (Nisshin EM Co. Ltd.), DOTITE type D-550 silver paint (Nisshin EM Co. Ltd.); superoxide dismutase (SOD; Sigma); AA sodium salt (Sigma); eicosapentaenoic acid (Sigma); γ -linolenic acid (Sigma); linoleic acid sodium salt (Sigma); oleic acid sodium salt (Sigma); stearic acid sodium salt (Sigma); polyvinyl formal (Sigma); chymostatin (Sigma), leupeptin (Sigma), phenylmethanesulfonyl fluoride (Sigma); tannic acid (WAKO Pure Chemical Industries Ltd.); adenosine 5'-triphosphate (ATP) disodium salt (Sigma); β -escin (Nacalai Tesque), and chitosan (WAKO Pure Chemical Industries Ltd.).

For AA release experiments, the 50% inhibition concentrations (IC₅₀) of manoalide against phospholipase A₂ (PLA₂) from *Naja naja* venom, bee toxin, and human synovial fluid are 1.7, 0.05, and 0.2 μ M, respectively [10–12]. The IC₅₀ values of AACOCF₃ against calcium ionophore-challenged U937 cells and platelets are 8 μ M per 2×10^6 cells/ml⁻¹ and 2 μ M per 4×10^7 cells/ml⁻¹, respectively [13].

Isolation of Mast Cells

Mast cells were collected by rinsing the peritoneal cavities of 300-gram male Wistar rats with 10 ml of physiological salt solution (PSS; 154 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 5 mM D-glucose, 5 mM Hepes-NaOH/pH 7.4) as described in our previous paper [8]. The crude cell suspensions consisted of 4–5% mast cells, 15–20% eosinophilic leukocytes, and 80–85% mononuclear cells. Neither neutrophilic polymorphonuclear leukocytes nor platelets were present in normal rat peritoneal fluid. In addition, no platelets were observed by phase-contrast microscopic examination of the original peritoneal cell suspensions. The crude cell suspensions were centrifuged at 55 g for 8 min at 4°C; the supernatants were removed, and the cell pellets were resuspended in 1 ml of PSS and 3 ml of Percoll buffer (90% Percoll, 154 mM NaCl, and 10 mM Hepes-NaOH/pH 7.4). Four-milliliter aliquots of the Percoll cell suspensions were layered over 1 ml of PSS and centrifuged at 265 g for 15 min at 4°C, and the supernatants containing interface layers were removed by aspiration. The interface layers containing no mast cells were also used in the present study. The cell pellets were washed once with PSS and resus-

pended in PSS. Thus, final suspensions contained more than 90% mast cells as visualized by the results of toluidine blue staining of the mast cell granules. Cell viability was always more than 90% as judged by the results of trypan blue exclusion tests.

Measurement of Chemiluminescence

Superoxide production by isolated mast cells was measured by an increase in chemiluminescence from MCLA, a luciferin analog, which reacts with superoxide with high specificity, as previously reported [8]. Mast cell suspensions (1×10^5 cells/ml) were preincubated for 10 min at 37°C in the presence of 0.4 μ M MCLA in order to stabilize background chemiluminescence. Compound 48/80 or various fatty acids were then added to the preincubated cell suspensions, and MCLA-generated chemiluminescence was monitored using a Lumicounter ATP-237 (Advantec Co., Ltd., Dublin, Calif., USA). It was determined that compound 48/80 and fatty acids were not cytotoxic at 1.0 μ g/ml and 10 μ M, respectively, as determined by measurement of lactic dehydrogenase release. Under these conditions, backgrounds were less than 3% and cell viabilities were more than 90%, as determined by the results of trypan blue exclusion tests. Solutions of fatty acids were prepared just before use by dissolving them in absolute ethanol at a concentration of 10 mM and then diluting with deionized water. The final concentration of ethanol was less than 0.1%. Ethanol at this concentration did not affect superoxide production in mast cells.

Chemical Skinning

In order to accelerate membrane permeability, chemical skinning of isolated mast cells was performed as described previously [14] with some modifications. Mast cell suspensions at 1×10^6 cells/ml were incubated for 3 min at 37°C in a paracytosol buffer (137 mM potassium glutamate, 4 mM MgCl₂, 3 mM ATP, 0.5 mM EGTA, 1 mM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 μ g/ml chymostatin, Hepes-NaOH/pH 6.8) including 7.5 μ g/ml of β -escin. The membrane-permeabilized cells (skinned cells) were then washed twice with fresh paracytosol buffer at 4°C. For chemiluminescence analysis, the permeabilized cells were used in the presence of paracytosol buffer containing MCLA as described above.

Preparation of Mast Cell Crude Extracts and Their Soluble and Membrane-Rich Fractions, and Isolation of Mast Cell Granules

Preparation of mast cell crude extracts and subfractionation of the extracts were carried out basically as previously described [15], with some modification. Briefly, 1×10^6 mast cells were suspended in 1 ml of the paracytosol buffer and sonicated for 35 s at 4°C at intervals of 5 s. We used the sonicated cell suspensions as mast cell crude extracts containing whole cell components. Intact granules were isolated from mast cells according to the method of Henderson and Kaliner [16] with a slight modification. Large cellular debris was removed from the crude extracts by centrifugation at 400 g for 5 min at 4°C. The granule-containing supernatants were centrifuged at 2,200 g for 10 min at 4°C, and the granular pellets were resuspended in the paracytosol buffer. The supernatants were further centrifuged at 250,000 g for 20 min at 4°C. Thus, we obtained supernatants as soluble fractions and pellets as membrane-rich fractions of mast cells.

Preparation of Two Types of Granule Fractions

Mast cells (1×10^5) were treated with compound 48/80 at 1 μ g/ml for 1 min and centrifuged at 265 g for 15 min at 4°C, and then

granule-containing supernatants were obtained. Bursting of granules was carried out by transient exposure of granules to deionized water.

Low-Voltage Scanning Electron Microscopy

For imaging *in vivo* materials using a scanning electron microscope (EM, JSM-6300F, JEOL), 2.5% glutaraldehyde solution (2.5% glutaraldehyde, 5 mM Hepes-NaOH/pH 7.4) was peritoneally injected into rats that had been pretreated with peritoneal injection of compound 48/80 at 1 µg/ml. After abdominal opening, approximately 1 cm of intestine was resected and fixed with fresh 2.5% glutaraldehyde solution for 60 min at room temperature (RT). After thoroughly washing with Hepes buffer (5 mM Hepes-NaOH/pH 7.4), the samples were refixed with 1.0% osmium solution (1.0% osmium tetroxide, 5 mM Hepes-NaOH/pH 7.4) for 60 min at 4°C. The samples were rinsed with Hepes buffer, dehydrated through an ethanol series (25, 50, 70, 90, and 100%), and exposed to a *t*-butanol series (50, 70, 90, and 100%) before freezing. Then the samples immersed in 100% *t*-butanol were congealed at 4°C and freeze-dried at -80°C overnight in a high-vacuum chamber of a lyophilizer (JFD-300; JEOL, Tokyo, Japan). After freeze-drying, the samples were attached to sample stages with silver paste (DOTITE type D-550, silver paint) and coated with gold to an average covering thickness of 2 nm using an evacuated sputter coating apparatus (JEC-6300F; JEOL). Scanning EM images were taken at a very low voltage (3 kV) to obtain more detailed and actual images of sample structures. On the other hand, isolated mast cells and granules were placed on coverslips that either had not been coated with or had been coated with 7.5 mg/ml of polyvinyl formal dissolved in 1,2-dichloroethane. The samples were fixed and dehydrated as described above, and their surface structures were observed.

Scanning EM Images of Freeze-Fractured Cell Surfaces

To obtain freeze-fractured cell surface images, isolated mast cells were suspended in 2.5% glutaraldehyde solution and kept at RT for 60 min. After washing with Hepes buffer, the fixed cells were suspended in 10% gelatin solution at 37°C. The suspensions were mixed with an equal volume of chitosan solution (2% chitosan, 0.5% CH₃COOH) with no bubbles and poured onto glass plates to make flattened masses. After cooling on ice, the masses were further fixed with 2.5% glutaraldehyde solution for 60 min at RT, washed 3 times with Hepes buffer, and exposed to a dimethyl sulfoxide (DMSO) series (25 and 50%) for 30–60 min. Fifty percent DMSO was dropped on a metal stage that had been prefrozen with liquid nitrogen, and then the flattened mass was inserted into the drop just before it was completely frozen. After complete freezing, the mass was crashed into two pieces with a razor and a hammer, and the pieces were immersed again in 50% DMSO solution. The thawed pieces with DMSO were washed with Hepes buffer, refixed with 1.0% osmium solution for 60 min at 4°C, and immersed in 0.1% osmium solution for 3 days at 4°C to remove fixed cytoplasm, which interferes with observation of the intracellular structure. After washing with Hepes buffer, the samples were immersed in tannic acid solution (1.0% tannic acid, 5 mM Hepes-NaOH/pH 7.4) for 120 min at 4°C and washed again with Hepes buffer. The final fixation of the samples was done with 1.0% osmium solution for 60 min at 4°C. The samples were dehydrated, lyophilized, and coated with gold as described above.

Statistical Analysis

Prior to statistical analysis, each experiment was performed repeatedly at least five times. The results are expressed as means ± SE. Statistical analysis was performed using Student's *t* test. *p* values less than 0.05 or 0.01 were considered statistically significant.

Results

Morphological Change of Mast Cells after Treatment with Compound 48/80

Figure 1a shows resting (top) and activated mast cells (bottom) on the intestinal serosa of the rat. Resting mast cells showed flattened shapes and were attached to extracellular matrix of intestinal serosa using their abundant microvilli (fig. 1a, top). When mast cells were activated by treatment with compound 48/80, the cells showed a remarkable decrease in microvilli on their surfaces accompanying degranulation (fig. 1a, bottom).

Figure 1b shows scanning EM images of isolated mast cells which were either stimulated with or not stimulated with compound 48/80. Like *in vivo*, resting mast cells had a number of microvilli (fig. 1b, top), while compound 48/80-treated cells showed a marked decrease in microvilli accompanying degranulation (fig. 1b, bottom).

Superoxide Production by Activation of cPLA₂ in Mast Cells in Response to Compound 48/80

Upon exposure to compound 48/80 (1.0 µM), isolated rat peritoneal mast cells immediately produced superoxide, which was measured by detecting luminescence generated by a specific reaction of this anion with MCLA (fig. 2a). The production of superoxide occurred as early as 1 min after exposure to compound 48/80, peaked at 3–5 min, and gradually decreased toward the basal level. Luminescence completely disappeared when SOD, a specific superoxide scavenger [17], was added either prior to or during the reaction (data not shown). In addition, luminescence was not detected in mast cell-free suspensions even after stimulation with compound 48/80 in the presence of MCLA (data not shown).

We previously demonstrated that PLA₂ was closely associated with superoxide production in mast cells. A PLA₂ inhibitor, manoalide, efficiently suppressed superoxide production in rat peritoneal mast cells stimulated by compound 48/80 [8]. When mast cells were preincubated for 10 min in the presence of two types of PLA₂ inhibitors, manoalide (a general inhibitor of PLA₂) and AACOCF₃ (a specific inhibitor of cPLA₂), production of superoxide by compound 48/80-activated mast cells was remarkably suppressed dose-dependently by these inhibi-

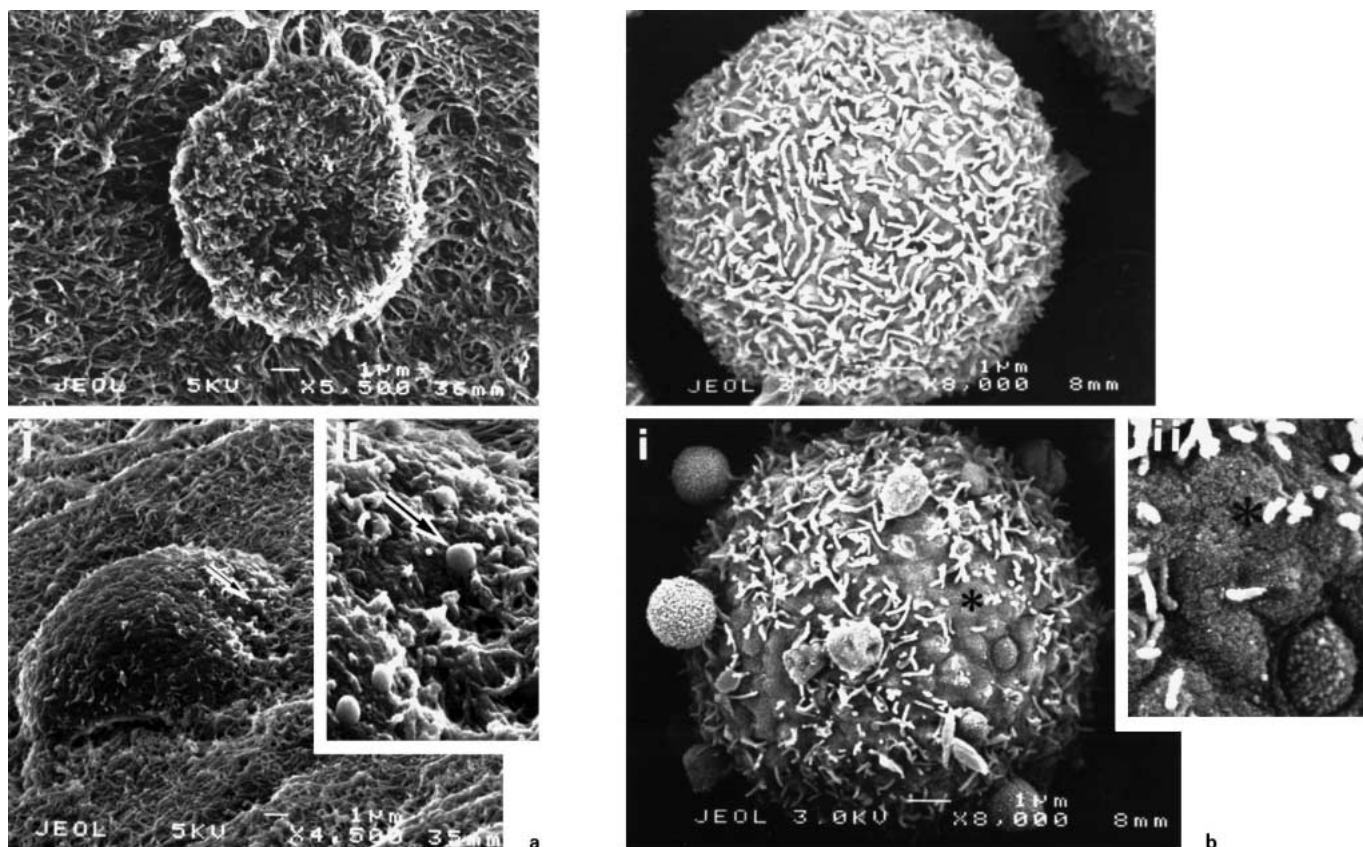


Fig. 1. Morphological change of mast cells induced by a nonimmunological stimulus of compound 48/80. **a** Resting mast cells morphologically differ from activated counterparts in the rat peritoneal cavity. Mast cells adhering to the serosa of the intestine were fixed after treatment with (bottom) or without compound 48/80 at 1.0 μg/ml (top). Resting mast cells are flat in shape with a number of microvilli on their surfaces (top). In contrast, activated mast cells showed

a remarkable decrease in microvilli with degranulation (bottom). **b** Morphological appearance of isolated rat peritoneal mast cells either treated with or not treated with compound 48/80. In contrast to resting mast cells (top), compound 48/80-treated cells showed dramatic morphological changes such as a marked decrease in microvilli and conspicuous degranulation (bottom). Bottom ii is the enlarged image of i. Scale bars: 1 μm.

tors (fig. 2b). By pretreatment of mast cells with 15 μM AACOCF₃, superoxide production was completely abolished in the cells. Neither manoalide (100 nM) nor AACOCF₃ (15 μM) were cytotoxic to mast cells, as judged by the results of trypan blue exclusion tests (data not shown).

In order to examine the effects of AACOCF₃ on the morphology of mast cells, we ultramicroscopically observed the outsides and insides of compound 48/80-stimulated mast cells that had been pretreated with AACOCF₃. As shown in figure 2c, pretreatment with AACOCF₃ markedly reduced degranulation induced by compound 48/80. These results indicated that cPLA₂ has a significant influence on degranulation as well as on superoxide production by mast cells.

Significance of Intracellular Ca²⁺ in Superoxide Production

It is well known that a micromolar amount of calcium is sufficient to activate cPLA₂. We therefore examined whether an intracellular increase in Ca²⁺ would induce superoxide production in membrane-permeabilized mast cells. Indeed, 0.25 μM Ca²⁺ induced superoxide production in mast cells, showing a peak at 2 min after Ca²⁺ exposure (fig. 3a). The superoxide production induced by 0.25 μM Ca²⁺ was dramatically inhibited by 5–15 μM AACOCF₃, indicating that an intracellular increase in Ca²⁺ may control the activity of cPLA₂ (fig. 3b). In addition, when isolated mast cells were incubated in a Ca²⁺-free medium, superoxide production never occurred even with stimulation by compound 48/80 (data not shown).

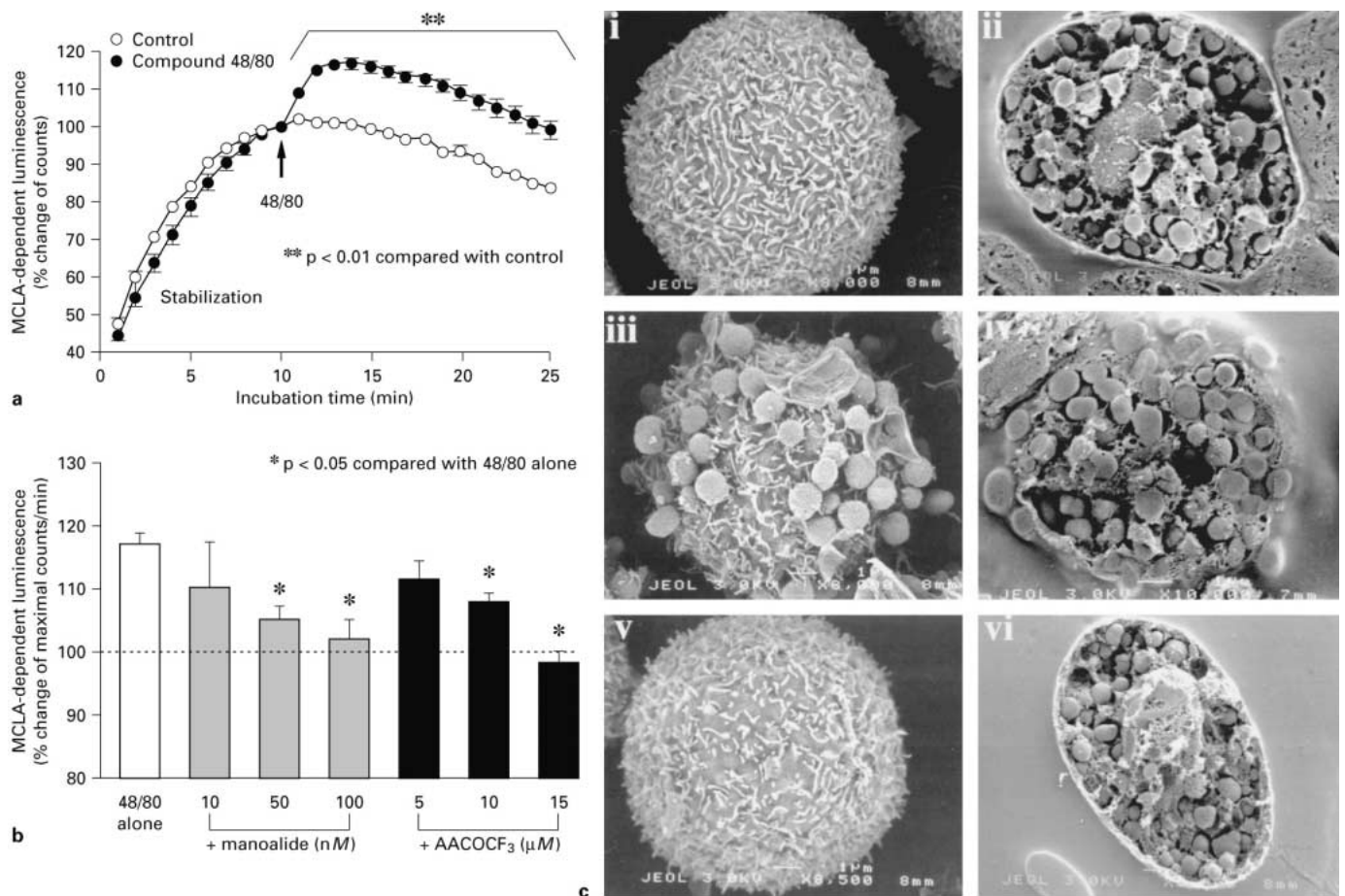


Fig. 2. cPLA₂ is closely associated with superoxide production in rat peritoneal mast cells stimulated by compound 48/80. **a** Time course of superoxide production in isolated rat peritoneal mast cells treated with or not treated with compound 48/80. Isolated rat mast cells were preincubated for 10 min in the presence of MCLA and then either treated with or not treated with compound 48/80 at 1.0 μg/ml. Results represent the means ± SE from five experiments. **b** Suppressive effects of PLA₂ inhibitors on production of superoxide in rat peritoneal mast cells stimulated by compound 48/80 at 1.0 μg/ml. A nonspecific PLA₂ inhibitor, manolide, and a specific cPLA₂ inhibitor, AACOCF₃, dose-dependently suppressed superoxide production

in mast cells stimulated by compound 48/80. Results represent the means ± SE from five experiments. **c** Antagonistic effects of AACOCF₃ on compound 48/80 induced morphological changes in mast cells. Even and uneven numbers show inner and outer appearances of mast cells, respectively. Resting intact mast cells (i and ii), compound 48/80-stimulated mast cells (iii and iv), and compound 48/80-stimulated mast cells that were pretreated with 15 μM AACOCF₃ (v and vi) were observed under a scanning EM. When mast cells were pretreated with AACOCF₃, compound 48/80 failed to induce degranulation of the cells.

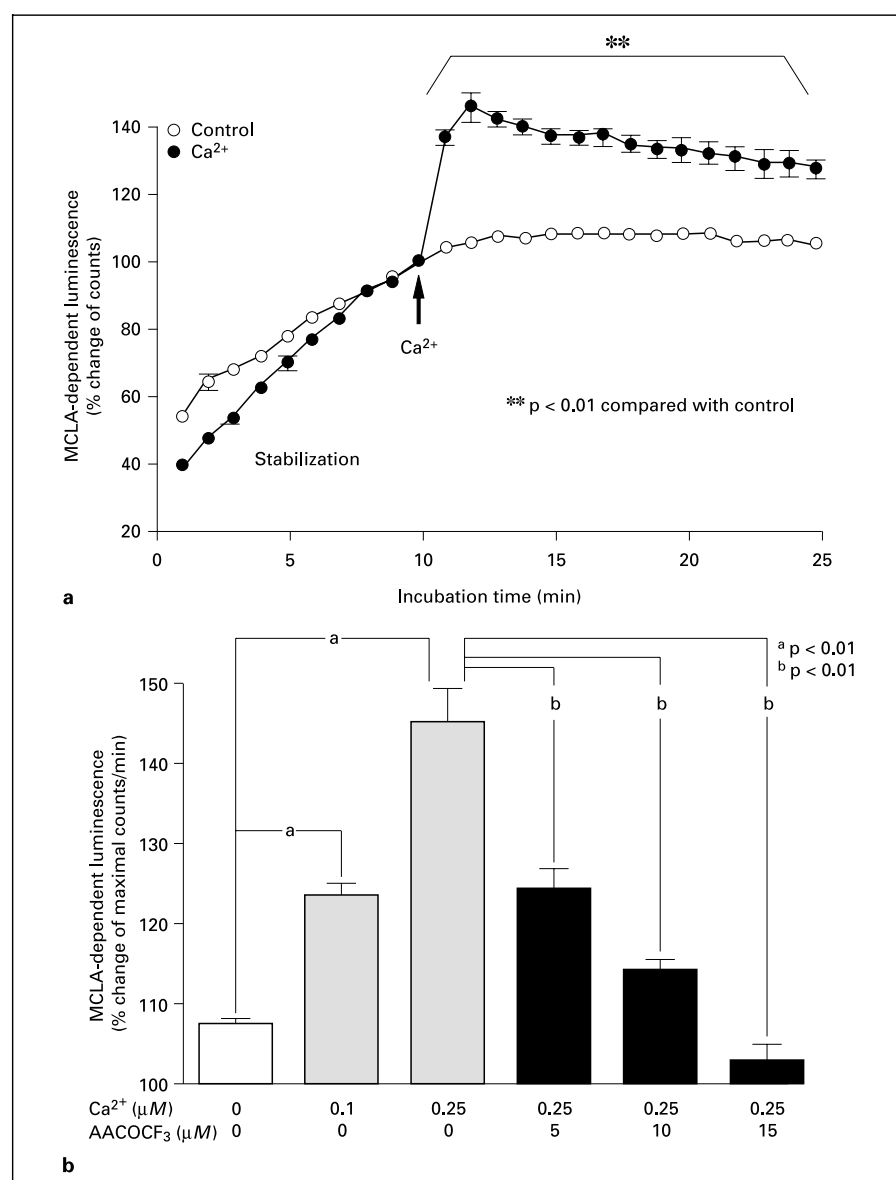
These results indicate that Ca²⁺ is necessary to activate cPLA₂ and that activated cPLA₂ mediates induction of superoxide production by compound 48/80.

Superoxide Production in Mast Cells Stimulated with AA

Since cPLA₂ is thought to specifically release AA from cellular phospholipids, we investigated the effects of AA on superoxide production in mast cells. When AA was added at a noncytotoxic concentration of 10 μM to sus-

pensions of isolated mast cells, superoxide production reached a maximal level as early as 2 min after the addition of AA, and the superoxide level was much higher than that induced by compound 48/80 stimulation (fig. 1b, 4a). This induction of superoxide production by AA occurred dose-dependently and was completely abolished by the addition of SOD at a dose of 100 U/ml (fig. 4b, c). Interestingly, scanning EM images of AA-stimulated cells showed abundant microvilli on the cell surfaces and no degranulation (fig. 4d). These results indi-

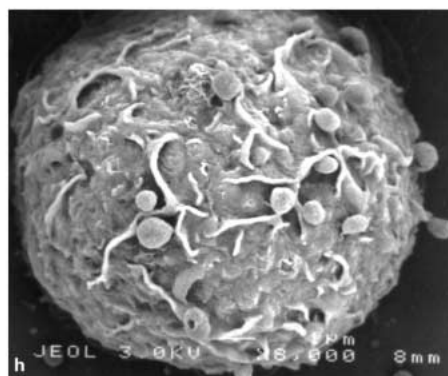
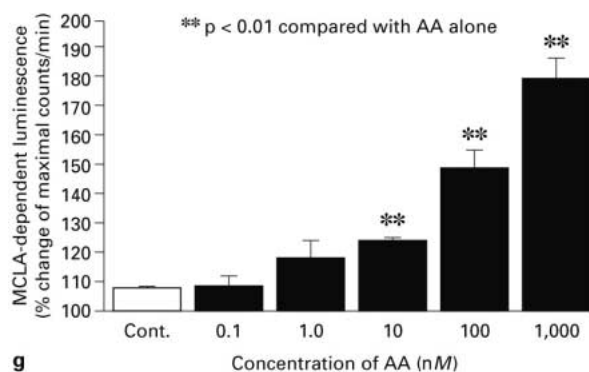
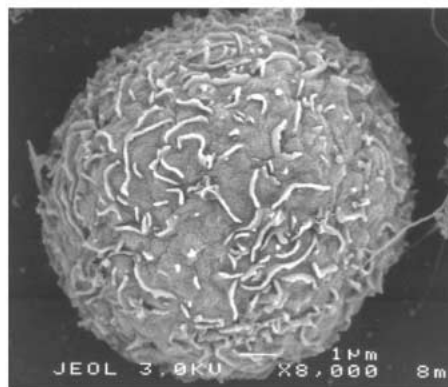
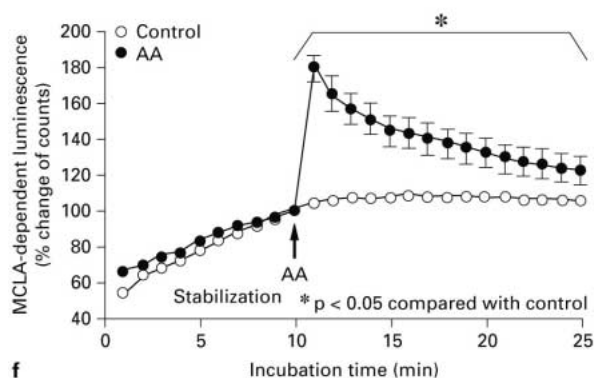
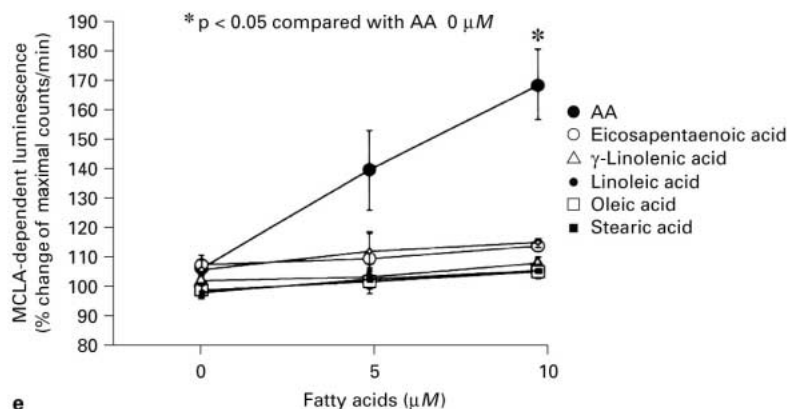
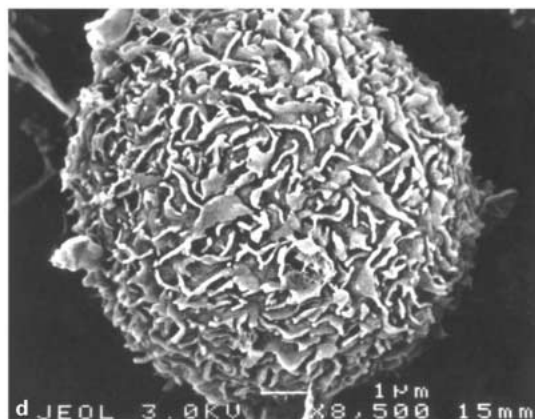
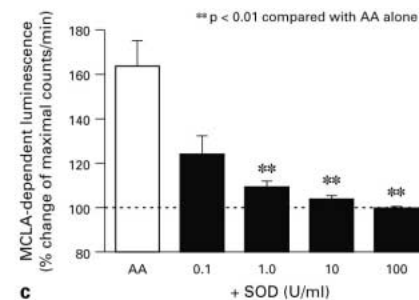
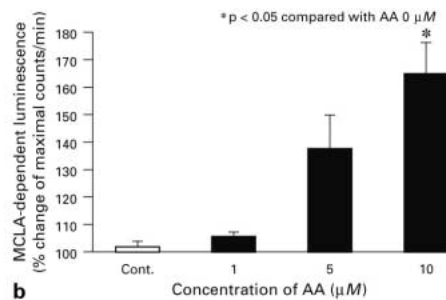
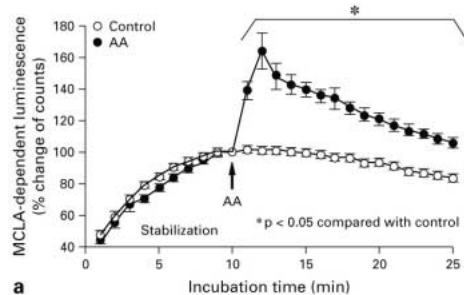
Fig. 3. Ca^{2+} induced superoxide production in membrane-permeabilized mast cells. **a** Time course of superoxide production in membrane-permeabilized mast cells after addition of $0.25 \mu\text{M}$ CaCl_2 . Membrane permeabilization of isolated rat peritoneal mast cells was performed by transient incubation of the cells with β -escin at $7.5 \mu\text{g/ml}$. The membrane-permeabilized cells (1×10^5 cells/ml) were preincubated for 10 min in the presence of MCLA, and then Ca^{2+} at $0.25 \mu\text{M}$ was added to the cell suspensions. **b** Effects of AACOCF₃ (5 – $15 \mu\text{M}$) on $0.25 \mu\text{M}$ Ca^{2+} -induced superoxide production in membrane-permeabilized mast cells. Ca^{2+} -induced production of superoxide in the cells was completely inhibited by AACOCF₃ at $15 \mu\text{M}$.



cate that superoxide production is not due to degranulation of mast cells. Next, we examined whether other unsaturated and saturated fatty acids also induce superoxide production in mast cells like AA. Although the tested fatty acids have similar carbon numbers to that of AA, they had no effects on superoxide production in mast cells (fig. 4e). We also examined whether various inhibitors of cyclooxygenase and lipoxygenase affect superoxide production in mast cells stimulated with AA. Neither cyclooxygenase inhibitors (indomethacin and acetylsalicylic acid) nor a 5-lipoxygenase inhibitor (ardisiaquinone A) affected superoxide production in AA-stimulated mast

cells (data not shown). These results indicate that superoxide is not produced during the oxidative metabolism of AA.

It is still not clear whether exogenous AA acts on the cell surface or in the cytosol of mast cells. We therefore used membrane-permeabilized mast cells to efficiently compel exogenous AA to enter the cells. When AA ($1.0 \mu\text{M}$) was added to suspensions of membrane-permeabilized mast cells, production of superoxide was detected as early as 1 min after the addition of AA (fig. 4f). The level of superoxide production in membrane-permeabilized cells was nearly the same as that in intact mast



cells stimulated with 10 μ M AA (fig. 4a, f). Exogenous AA at a very low concentration (1.0 nM) efficiently induced superoxide production (fig. 4g). It has been reported that compound 48/80 caused AA release within 30 s at a rate of 0.22 nmol/min per 10^6 mast cells [9]. These results strongly indicate that a very low concentration of AA released from mast cells may stimulate superoxide production in the cells themselves. Membrane-permeabilized mast cells morphologically resembled compound 48/80-treated cells, showing a remarkable decrease in microvilli on their uneven surfaces (fig. 4h, top). This means that permeabilization causes membrane perturbation with advance in ion permeability and fluidity, like cell activation by compound 48/80. Interestingly, when membrane-permeabilized cells were exposed to AA, degranulation was observed on the cell surfaces (fig. 4h, bottom). However, such degranulation was not observed in the case of intact mast cells treated with AA alone (fig. 4d).

Fig. 4. AA induced superoxide production in rat peritoneal mast cells. **a** Time course of superoxide production in rat peritoneal mast cells treated with AA at 10 μ M. AA caused a more prominent increase in superoxide production in mast cells (1×10^5 cells/ml) than did other stimulators, such as compound 48/80, and Ca^{2+} (see fig. 1b, 3a). **b** Dose-dependent enhancing effects of AA on superoxide production in mast cells. Cont. means buffer-treated cells. **c** Effect of SOD on superoxide production in rat peritoneal mast cells stimulated by AA at 10 μ M. SOD at 100 U/ml completely eliminated superoxide produced by mast cells stimulated with 10 μ M AA. **d** Appearance of rat peritoneal mast cells treated with AA at 10 μ M. A remarkable increase in microvilli was observed on the cell surface. Scale bar: 1 μ m. **e** Effects of various fatty acids on superoxide production in rat peritoneal mast cells. Unsaturated fatty acids (AA, eicosapentaenoic acid, γ -linolenic acid, linoleic acid, and oleic acid) and a saturated fatty acid (stearic acid) were tested at 1.0–10 μ M. AA alone increased superoxide production in a concentration-dependent fashion. **f** Time course of superoxide production in membrane-permeabilized mast cells stimulated by AA. Superoxide production sharply increased at 1 min after addition of 1.0 μ M AA. **g** Dose-dependent induction by AA of superoxide production in membrane-permeabilized mast cells. Permeabilized cells (1×10^5 cells/ml) were treated with AA at various concentrations ranging from 0.1 to 1,000 nM. The minimal effective concentration of AA was 1.0 nM. Cont. means buffer-treated cells. Results represent the means $\pm$ SE from five experiments. **h** Scanning EM images of membrane-permeabilized mast cells either treated with or not treated with AA. Membrane-permeabilized mast cells showed a decrease in microvilli but had no granules on their surfaces (top). Many granules appeared after the addition of 1.0 μ M AA (bottom). Results represent the means $\pm$ SE from five experiments. Scale bars: 1 μ m.

Superoxide Production in Crude Extracts of Mast Cells

For further mechanistic studies on the superoxide production in mast cells, it is important to reproduce the above observations in crude cell extracts of mast cells. We tested 5 kinds of unsaturated fatty acids (AA, eicosapentaenoic acid, γ -linolenic acid, linoleic acid, and oleic acid) and a saturated fatty acid (stearic acid) for induction of superoxide production in the crude extracts of mast cells. In contrast to intact mast cells, all of the tested fatty acids significantly induced superoxide production in the crude extracts, and AA was the most effective among the tested fatty acids (fig. 5a). This induction was, however, completely abolished by boiling the crude extracts at 105°C for 10 min (fig. 5b). These results indicate that there are some proteinaceous factors, maybe enzymes, responsible for AA-induced superoxide production in mast cells.

Superoxide Production in Cell Fractions of Mast Cells

In order to determine the intracellular localization of enzymes responsible for superoxide production in mast cells, we first focused on secretory granules occupying most of the cytosolic space in mast cells. Figures 6a and b show the structures of secretory granules present in resting mast cells. Secretory granules are wrapped in membrane-like substances (fig. 6a, b, arrows). We then prepared three types of secretory granule fractions from mast cells, as shown in figure 6c. The first type contained granules released from mast cells by treatment with compound 48/80 (fig. 6d-i); the second type contained granules isolated by sonication and centrifugation (fig. 6d-ii), and the third type contained bursted granules prepared by hypotonic treatment (fig. 6d-iii). Scanning EM images showed that membrane-free granules consisted of many small granules (fig. 6d-i). The granules shown in figure 6d-ii were tightly wrapped in membrane-like substances. As shown in figure 6d-iii, the burst granules showed matrix-like structures consisting of small granules that were very similar to the constituent small granules shown in figure 6d-i. Using these preparations, we examined whether they could produce superoxide. However, none of the three types of granules produced superoxide under AA stimulation (fig. 6e). Next, we prepared soluble cytosol fractions and insoluble membrane fractions from mast cell crude extracts (fig. 6c, f-D, f-E) and tested them for their ability to produce superoxide. Superoxide production was only observed in the case of a combination of cytoplasmic and membranous fractions in the presence of AA (fig. 6f). However, further addition of isolated mem-

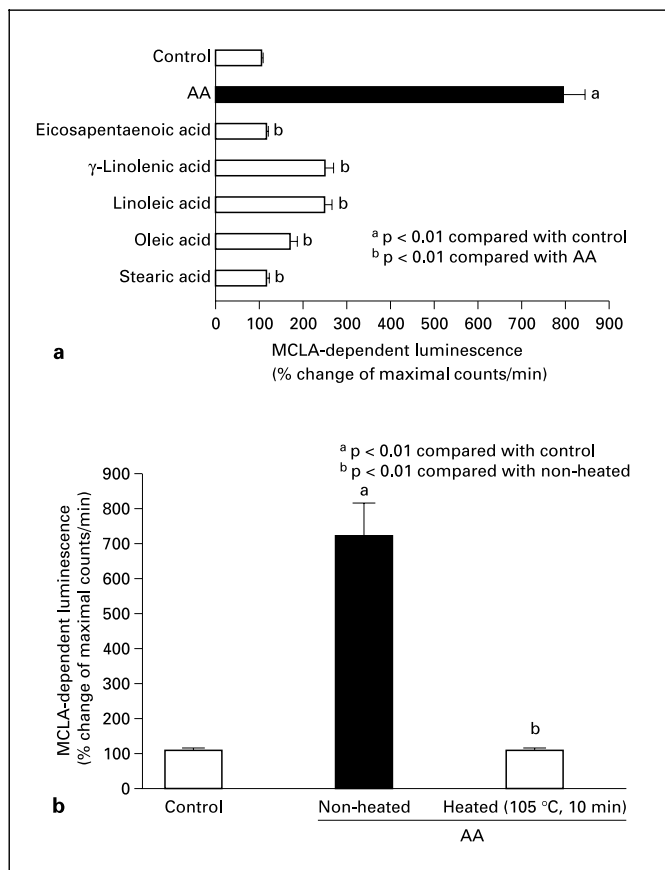


Fig. 5. Superoxide production in crude extracts prepared from rat peritoneal mast cells. **a** Effects of various fatty acids on superoxide production in mast cell crude extracts. Isolated mast cells (1×10^6 cells/ml) were homogenized by sonication. Unsaturated fatty acids (AA, eicosapentaenoic acid, γ -linolenic acid, linoleic acid, and oleic acid) and a saturated fatty acid (stearic acid) were added at $1.0 \mu\text{M}$ to unheated crude extracts. AA showed the highest induction of superoxide production among the tested fatty acids. **b** Effect of heating pretreatment of crude extracts on induction of superoxide production in the extracts by AA. Some of the crude extracts were heated at 105°C for 10 min. Heated and unheated extracts were preincubated for 10 min in the presence of MCLA, and then $1.0 \mu\text{M}$ AA was added to them. AA highly induced superoxide production in the unheated extracts but not in the heated extracts of mast cells. Results represent the means $\pm$ SE from five experiments.

brane-wrapped granules (fig. 6d-ii) to this combination showed no further enhancing effect on superoxide production (fig. 6f-G). These results indicate that enzymes responsible for superoxide production may be present in the membrane and cytoplasm of mast cells.

Discussion

Superoxide Production in Mast Cells

AA, an unsaturated fatty acid, efficiently induced superoxide production in isolated mast cells. However, other saturated and unsaturated fatty acids tested had no effect. Thus, this action is characteristic of AA. It is thought that exogenous AA may penetrate particular lipid domains of the plasma membrane and affect the protein-lipid interaction, resulting in superoxide production in cells. In the present study, we also used membrane-permeabilized mast cells to compel exogenous AA to efficiently enter the cells. AA at a very low concentration (1.0 nM) induced superoxide production in membrane-permeabilized mast cells. These findings indicate that AA is effective at the physiological concentration and acts on mast cells intracellularly but not extracellularly.

Two types of PLA_2 have been identified in mast cells: a cytosolic-type cPLA_2 [18] and a secretory-type sPLA_2 (type II) [19]. The latter requires a millimolar amount of Ca^{2+} for its activation, while activation of the former needs only a nanomolar amount of Ca^{2+} [20–23]. Compound 48/80 increases intracellular Ca^{2+} to a micromolar order in mast cells [24, our unpubl. data]. This concentration is sufficient to activate cPLA_2 in mast cells. In the present study, Ca^{2+} at nanomolar concentrations induced superoxide production in membrane-permeabilized mast cells in a dose-dependent manner with maximal induction at 250 nM , and the superoxide production was remarkably inhibited by AACOCF_3 , a specific inhibitor of cPLA_2 . Manoilide, another PLA_2 inhibitor, also decreased superoxide production. These findings indicate that a Ca signal, which causes AA liberation via cPLA_2 activation, is essential for superoxide production in mast cells. AA induced superoxide production in unheated crude extract but not in heated crude extract of mast cells, while it failed to induce superoxide production in any of the cytosol, membrane, and granule fractions of mast cells. However, superoxide production occurred when cytosol and membrane fractions were combined, but further addition of the granule fraction to the combination had no effect. Taken together, the results suggest that factors related to superoxide production, probably enzymes, are present in the membrane and cytoplasm of mast cells, and that interaction of these factors is necessary for superoxide production in mast cells.

Control of superoxide production in mast cells may be similar to that in neutrophils. In neutrophils, superoxide is produced by the NADPH oxidase system, which includes cytosol factors, such as p47 phox, p67 phox, and

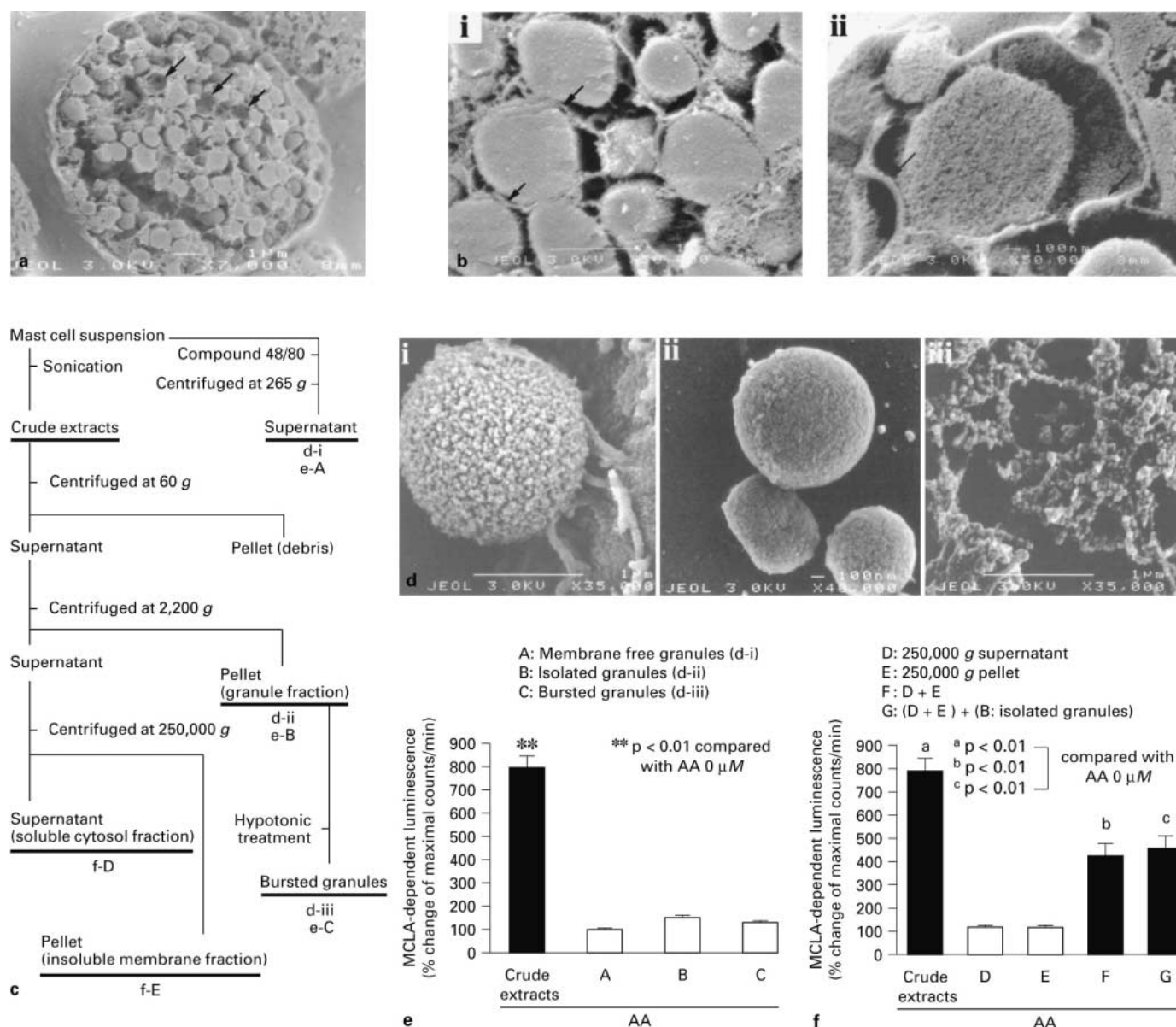


Fig. 6. Structure of granules in rat peritoneal mast cells and superoxide production in granule, cytosol, and membrane fractions from mast cells. **a** Freeze-fractured surfaces of mast cells. **b** Freeze-fractured surfaces of granules in mast cells. **a, b-i, b-ii** Arrows show the membranous structure of a mast cell granule. **c** Schema of the preparation of mast cell crude extracts and granule, soluble cytosol, and insoluble membrane-rich fractions. These samples are shown in the schema by underlines. **d** Scanning EM images of granules prepared from rat peritoneal mast cells. **d-i** Membrane-free granules secreted from mast cells treated with compound 48/80 at 1.0 $\mu\text{g}/\text{ml}$. **d-ii** Granules isolated by sonication and centrifugation of mast cell crude extracts. Isolated granules were wrapped in membrane-like substances. **d-iii** Burst granules. Burst granules consisted of many small

granules showing a matrix-like structure. Scale bars: 1 μm . **e** Effects of AA on superoxide production in three types (**d**) of granules prepared from rat intraperitoneal mast cells. These granule preparations were preincubated for 10 min in the presence of MCLA and then treated with AA at 1.0 μM . None of these granule preparations produced superoxide. Results represent the means $\pm$ SE from five experiments. **f** Effects of AA on superoxide production by mast cell subfractions. Although the soluble supernatant (D; cytosol fraction) or insoluble pellet (E; membrane-rich fraction) alone did not produce superoxide, the combination (F) of D and E showed superoxide production by stimulation with AA at 1.0 μM . However, the addition of B (isolated granules) to this combination failed to further enhance the production of superoxide (G).

small GTP binding proteins, and membrane factors, such as cytochrome b558 and flavine proteins [25–30]. In addition, AA is required for the activation of the NADPH oxidase system in neutrophils [31], and p47 phox-like protein is also present in mast cells [8]. Thus, the regulation and physiological significance of superoxide production in mast cells are very similar to those in neutrophils, except for microbicidal activity.

Regulation of Superoxide Production in Mast Cells

The level of superoxide production in intact mast cells was very low compared with that in their crude extracts. Although the reason for this difference is not clear, the following findings may be relevant. First, a pathway through the histamine H₂ receptor plays an important role in the inhibition of superoxide production in mast cells [8]. Since a large number of histamine molecules are released concomitantly with superoxide production in the process of mast cell activation, the released histamine eventually suppresses superoxide production via interaction with its H₂ receptor. Second, mast cells have many antioxidative factors. Antioxidants such as ascorbic acid and SOD, which specifically convert superoxide to H₂O₂, are included in secretory granules in mast cells, and these antioxidants are released from granules when mast cells are activated [16, 32]. Third, AA is known as a prominent inhibitor of cPLA₂ [33]. This may be a negative feedback

system for cPLA₂ activation. Thus, many kinds of suppression systems against superoxide production may work in mast cells.

Physiological Significance of Superoxide Production in Mast Cells

Since the amount of superoxide produced by activated mast cells is very low compared with that produced by activated phagocytic cells, superoxide produced by mast cells may work not as a microbicide but as a second messenger in signal transduction, as shown in the release of various cytokines in inflamed tissues [34]. Since superoxide is unstable and its half-life is very short, superoxide may not work directly as a second messenger. Hydrogen peroxide, an intermediate metabolite of superoxide, is very stable at physiological pH and can easily pass through the plasma membranes of cells. Thus, H₂O₂ may work as a second messenger in cells surrounding activated mast cells or in mast cells themselves during inflammation, as SOD is abundant in granules of mast cells. In fact, H₂O₂ works as a signal messenger implicated in cell growth, differentiation, and apoptosis of various cells [35–37]. In addition, it also affects mast cell functions such as inhibition of the degranulation responses caused by immunological and nonimmunological stimuli, and induction of interleukin-4 mRNA expression [38, 39].

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