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Ultrastructural study of mast cells stimulated with compound 48/80 as revealed by quick-freezing method

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Abstract The ultrastructure of mast cells stimulated with compound 48/80 was examined by quick-freezing and deep-etching (QF-DE) or freeze-substitution (QF-FS) methods. Peritoneal cells including mast cells of adult male rats were stimulated in vitro with compound 48/80 at 17° C for 0, 10, 30, 60 or 180 s. The QF-DE replicas revealed that the mast cells stimulated with compound 48/80 for 30 s decreased filamentous actin around secretory granules. In the QF-FS specimens, perigranular membranes in mast cells stimulated for 60 s formed pentalaminar structures between adjacent granules in their cytoplasm prior to degranulation. These findings suggest that preparatory states for degranulation occur in the whole cytoplasm of stimulated mast cells at early stages. Moreover, both QF-FS specimens and QF-DE replicas revealed a compact morphological appearance of discharged granules in the extracellular space, indicating the existence of considerable content within the granules. Skeletal structures in the granules were also demonstrated on QF-DE replicas prepared after extracting soluble elements from the cytoplasm. It is suggested that the granular contents associated with the skeletal structures are gradually detached from the discharged granules to ensure local concentration in the tissues.

Key words Mast cell · Compound 48/80
Ultrastructure · Quick-freezing

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Introduction

Conventional electron microscopic studies have shown that compound 48/80-induced exocytosis in rat mast cells usually begins by fusion between perigranular membrane and plasma membrane, and advances to the fusion and opening of deeply situated granules (Röhlich et al. 1971; Lagunoff 1973; Kessler and Kuhn 1975; Chi et al. 1976; Lawson et al. 1977). However, few cytoplasmic events prior to the fusion have been demonstrated morphologically in previous papers. The papers also suggested that liberated granules were rapidly destroyed after the opening of perigranular membranes into the extracellular space. However, it has been reported that mast cells are characterized ultrastructurally by many secretory granules with highly electron dense and compact appearance, while the discharged granules present a coarse, networked appearance in the extracellular space (Röhlich et al. 1971; Lagunoff 1973; Kessler and Kuhn 1975; Chi et al. 1976; Lawson et al. 1977; Oskéritzian et al. 1992).

Secretory granules in mast cells have lots of heparin proteoglycans and various chemical mediators (Tas and Berndesen 1977; Fantozzi et al. 1978; Soll et al. 1981; Befus et al. 1982; Theoharides et al. 1985; Galli 1993). Liberated granules are important in the pathological response which forms the inflammatory reaction and regulated stimulation and release of chemical mediators from the granules is required to maintain the extracellular milieu and cellular cascade reaction (Galli 1993). Morphologically, the formation of larger secretory granules in stimulated mast cells is thought to be important but there are discrepancies between conventional electron microscopic findings and the functional results of degranulation of mast cells. Recently, the quick-freezing (QF) method has been used to preserve the morphological state at the moment of cryofixation (Ohno and Fujii 1990, 1991; Naramoto et al. 1991; Ohno et al. 1992) so as to reflect in vivo ultrastructure more closely related to physiological function. The present study using the QF method describes cytoplasmic events in

stimulated mast cells prior to degranulation, and reveals no acute disintegration of the granules discharged into extracellular space. A three-dimensional existence of skeletal structures within secretory granules is described for the first time.

Materials and methods

For mast cell preparation and stimulation with compound 48/80 20 male Wistar rats weighing 240–280 g were purchased from Japan SLC (Shizuoka, Japan) and used in this study. Each rat was anaesthetized with ether and bled out from both carotid arteries. Ten millilitres of Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, USA) containing 10 U/ml of heparin and 0.1% bovine serum albumin was injected into the peritoneal cavity. After gentle massage of the abdomen, peritoneal fluid was pipetted out through a middle incision of the abdominal wall, as described in previous papers (Chandler and Heuser 1980; Pintado et al. 1984; Oskéritzian et al. 1992). The peritoneal fluid of 5 rats cooled at 4° C was mixed and centrifuged at 1000 rpm for 10 min for each experiment. The supernatant was then discarded and centrifuged peritoneal cells were collected. They were divided into several plastic tubes for the following experiments. For degranulation of mast cells, they were stimulated with 10 μg/ml of compound 48/80 (Sigma, USA) in DMEM, which has often been used as a mast cell stimulator, at 17° C for 10, 30, 60 or 180 s (Röhlich et al. 1971; Orr et al. 1972; Befus et al. 1982; Theoharides et al. 1985; Tasaka et al. 1986). Unstimulated mast cells were designated as resting.

Unfixed floating mast cells were used for light microscopic examination. A drop of resting cells was put on slides stained with toluidine blue (TB), overlaid with coverslips and immediately examined under a light microscope. Photomicrographs were taken within 30 s of staining with TB. For the examination of vital degranulation of mast cells, they were stimulated with compound 48/80 on slide glasses and stained with a drop of TB at the end of stimulation. They were also examined in the same manner as the resting mast cells.

For fluorescence microscopy in order to demonstrate the localization of actin filaments, resting or stimulated mast cells were fixed with 1% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB), pH 7.4, for 1 h. After washing in 0.1 M phosphate buffered saline (PBS) twice for 20 min, they were immersed with 30% sucrose in PB, frozen in liquid nitrogen and cut into 5-μm thick sections using a cryostat machine. For visualization of filamentous actin, the cryostat sections were treated with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Cappel, USA), diluted at 1:100 in PB at room temperature for 1 h. They were then washed in PBS and examined under a fluorescence microscope.

For electron microscopy the centrifuged unstimulated or stimulated mast cells were prefixed with 2.5% GA in PB for 30 min. After washing in PBS, they were postfixed with 2% osmium tetroxide in PB for 30 min. They were then washed twice in PBS, dehydrated in a graded series of ethanol, immersed with propylene oxide and embedded in Epok 812. Ultrathin sections were cut with an ultramicrotome MT-2B, mounted on copper grids and doubly stained with uranyl acetate and lead citrate. They were examined in an electron microscope, Hitachi H-600.

For *freeze-substitution* mast cells unstimulated or stimulated with compound 48/80 on egg albumin covered stages were attached to copper blocks cooled in liquid nitrogen (−196° C) with a QF apparatus (JFD-RFA). After QF, they were placed in acetone containing 2% OsO_4, and kept at about −80° C for 20 h (Ohno et al. 1992). The temperature of the samples was then raised, firstly to −20° C for 2 h, then to 4° C for 2 h and finally to room temperature. They were washed twice in absolute acetone and embedded in Epok 812. The specimens were then prepared in the same manner as described above.

In the *deep-etching* technique other mast cells stimulated with compound 48/80 on egg albumin covered stages were quickly frozen with JFD-RFA as described above. The frozen specimens were carefully fractured with a scalpel in liquid nitrogen as reported before (Ohno and Fujii 1990, 1991; Naramoto et al. 1991; Ohno et al. 1992). They were deeply etched in a freeze-fracture apparatus, Eiko FD-3AS under vacuum conditions of $6–8 \times 10^{-6}$ Pa for 15–20 min at a temperature of −95° C. The deep-etched specimens on the rotary stage were first shadowed with platinum at an angle of 30° and then rotary shadowed up to the total thickness of about 2 nm. They were additionally coated with carbon at an angle of 90°. A drop of 2% collodion in amylacetate was put onto the replicas as soon as the specimens were taken out from the machine to prevent them from breaking into pieces during the following digestion procedures. The replicas coated with dried collodion were floated on household bleach, Kao Haiter, for 15–30 min to dissolve the cellular components. The replica membranes were washed in distilled water and cut into small pieces with a pair of scissors. They were mounted on Formvar-filmed copper grids and immersed in amylacetate solution to dissolve the dried collodion.

To observe skeletal structures in mast cells, deep-etched replicas were prepared after removing soluble elements from their cytoplasm. The mast cells unstimulated or stimulated with compound 48/80 were prefixed in 2% paraformaldehyde in PB for 15 min. After washing in PB, they were treated with 0.5% saponin in PB for 10 min to remove cytoplasmic soluble proteins, and postfixed in 0.25% GA in PB for 30 min. They were immersed with 10% methanol and quickly frozen with JFD-RFA. Then deep-etched replicas of these specimens were prepared in the same way as described above. All of the replicas were observed in electron microscopes, Hitachi H-600 and H-8100. Some stereo-pictures were taken at tilting angles of ±5°. Electron micrographs were printed from the inverted negative films.

Results

Examination of floating fresh resting mast cells revealed a relatively smooth contour of the cell surface, with most of the granules localized in the deep cytoplasm and unstained by TB (Fig. 1a). A few granules localized on the cell surface showed immediate staining with TB. As shown in Fig. 1b and c, the mast cells stimulated with compound 48/80 for about 60 s had an irregular appearing cell surface. The whole cytoplasm, with many extruding granules, was heavily stained with TB. Some stimulated mast cells were observed in the stage of severe degranulation (Fig. 1c) where the granules discharged from the mast cells were heavily stained with TB like those on the surface (Fig. 1b, c).

Figure 2 illustrates fluorescence reactions of FITC-conjugated phalloidin in resting or stimulated mast cells. The whole cytoplasm of resting mast cells had a strong and uniform fluorescence to phalloidin (Fig. 2a). In contrast, the mast cells stimulated with compound 48/80 for 10 s showed decreased fluorescence in their cytoplasm before the occurrence of degranulation (Fig. 2b). It is notable that a decrease in the intensity of fluorescent reactions was observed not only in peripheral regions of the stimulated cells but also in their deeper cytoplasm. Fluorescence of FITC-conjugated phalloidin disappeared in most of the mast cells stimulated for 60 s, when their cytoplasm became more coarse and heterogeneous (Fig. 2c).

Figure 3 illustrates electron micrographs of conventional ultrathin sections of peritoneal mast cells at different stages of compound 48/80-induced degranula-

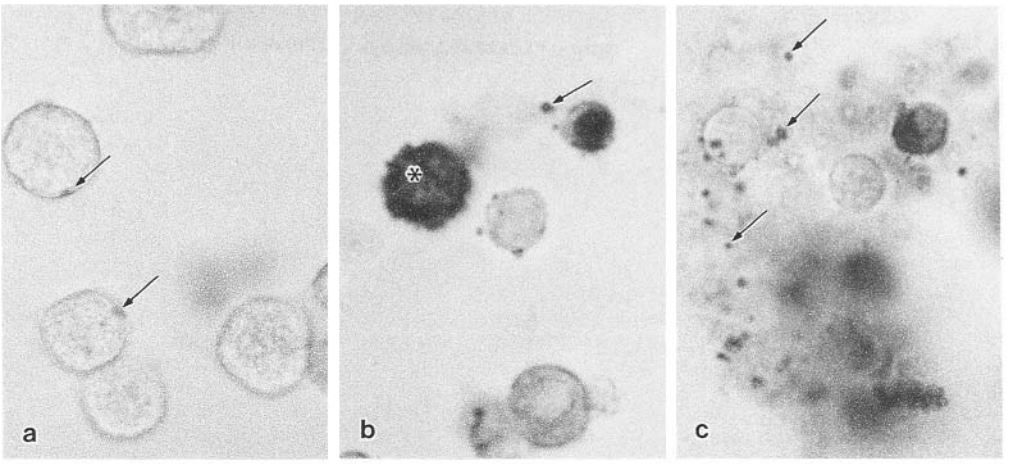

Fig. 1a–c Light micrographs of floating fresh mast cells stained with toluidine blue (TB). **a** Resting mast cells with a few granules on the cell surface (*arrows*). **b**, **c** Mast cells stimulated with compound 48/80 for about 60 s. Peripherally located (*asterisk*) or discharged (*arrows*) granules are heavily stained with TB. ×640

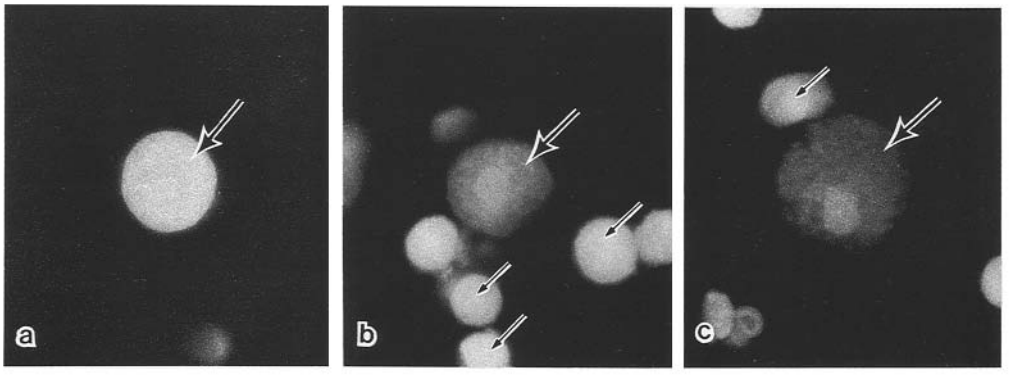

Fig. 2a–c Fluorescent reactions of fluorescein isothiocyanate (FITC)-conjugated phalloidin. **a** Resting mast cell (*arrow*). **b**, **c** Mast cells stimulated with compound 48/80 for 10 s (**b**) or 60 s (**c**) in the centre. Both fluorescent reactions were distinctly decreased by the stimulation (*arrows*). Small round cells are macrophages maintaining the same reaction intensity (*small arrows*). ×640

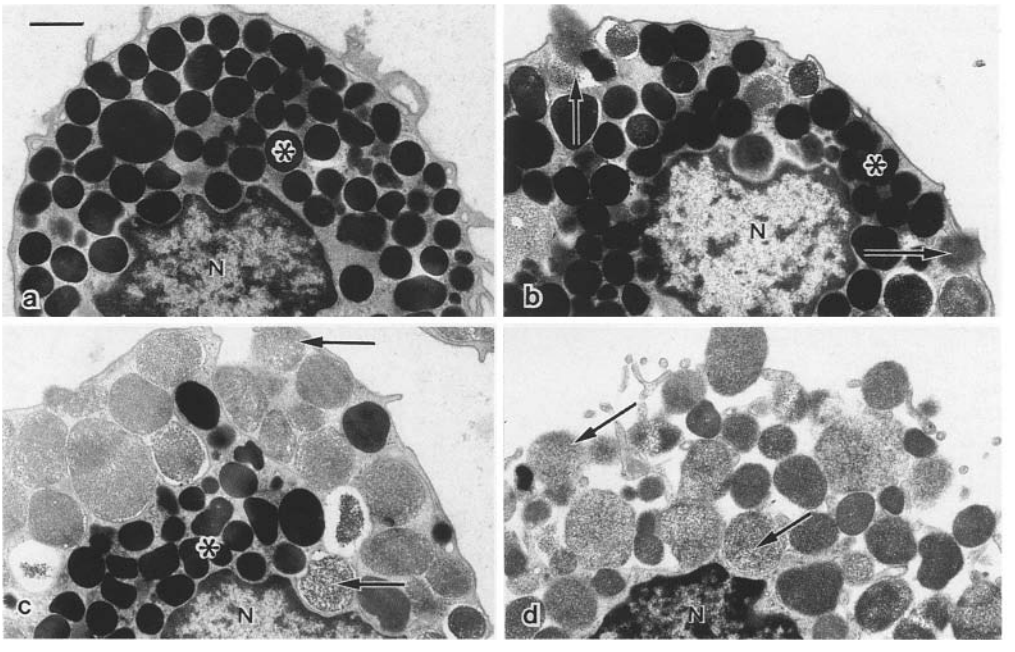

Fig. 3a–d Electron micrographs of conventional ultrathin sections at different stages of compound 48/80-induced degranulation. **a** A resting mast cell. **b–d** Mast cells stimulated with compound 48/80 for 30 s (**b**), 60 s (**c**) or 180 s (**d**). *Asterisks* indicate intracellular granules with compact and osmiophilic appearance, while *arrows* indicate liberated granules in extracellular space with loose appearance. (*N* nucleus, *bar* 1 μm.) ×7200

tion. Resting mast cells had a smooth contour of their surface and many secretory granules, ultrastructurally characterized by a uniform and compact appearance (Fig. 3a). They showed the same morphology as granules in cells stimulated with compound 48/80 for 10 s (data not shown). In contrast, mast cells stimulated for 30 s had irregular cell surface contours and showed degranulation of peripherally located granules (Fig. 3b). Liberated granules in the extracellular space showed distinct expansion and had morphological loose networks, as reported previously (Röhlich et al. 1971; Lagunoff 1973). They were easily distinguished from com-

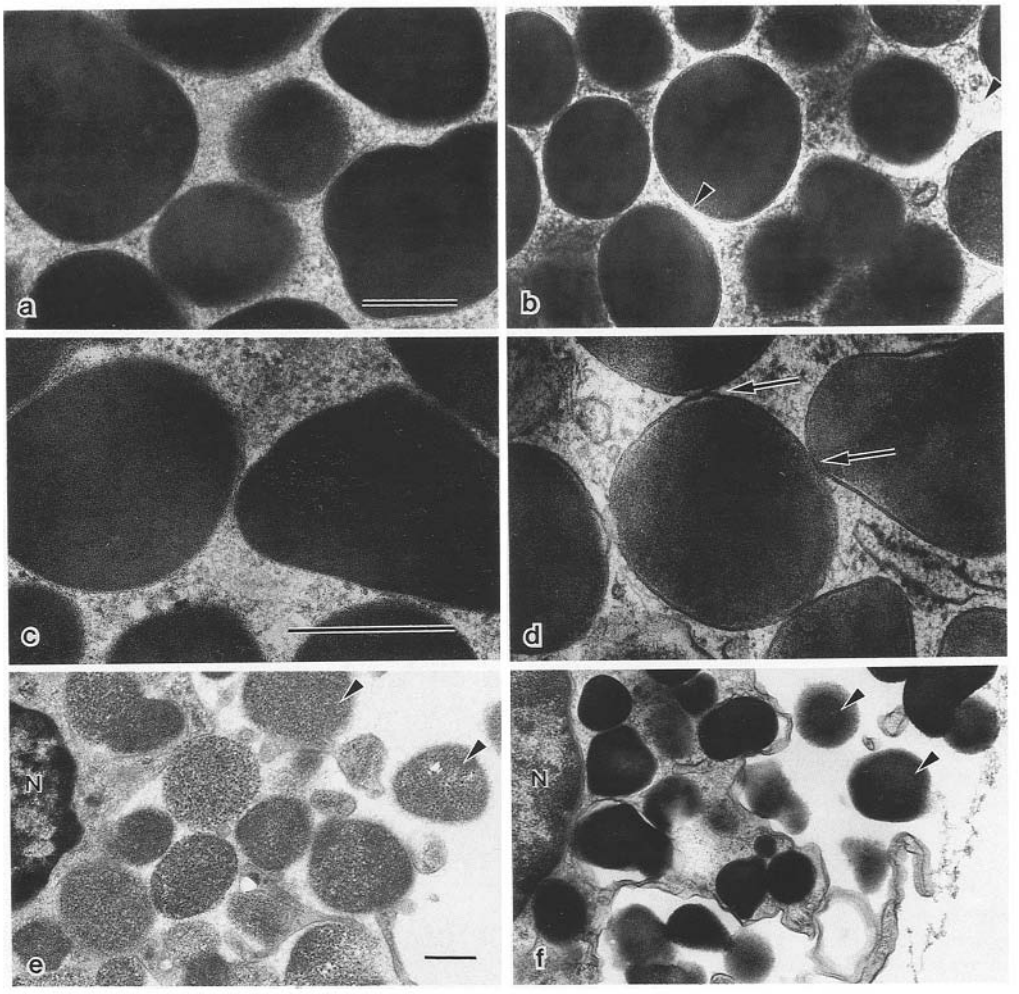

Fig. 4a–f Higher magnified electron micrographs of the stimulated mast cells. **a**, **c** and **e** were prepared by the conventional procedures, and **b**, **d** and **f** were by the quick-freezing-freeze substitution (QF-FS) method. **a**, **b** Mast cells stimulated for 10 s. *Arrowheads* in **b** indicate low electron dense areas around the granules. ×25600. **c**, **d** Stimulation for 60 s. *Arrows* in (**d**) indicate the perigranular membranes which fuse each other to form pentalaminar structures. ×44600. **e**, **f** Stimulation for 180 s. *Arrowheads* indicate liberated granules. (*N* nucleus, *bar* 0.5 μm.) ×13700

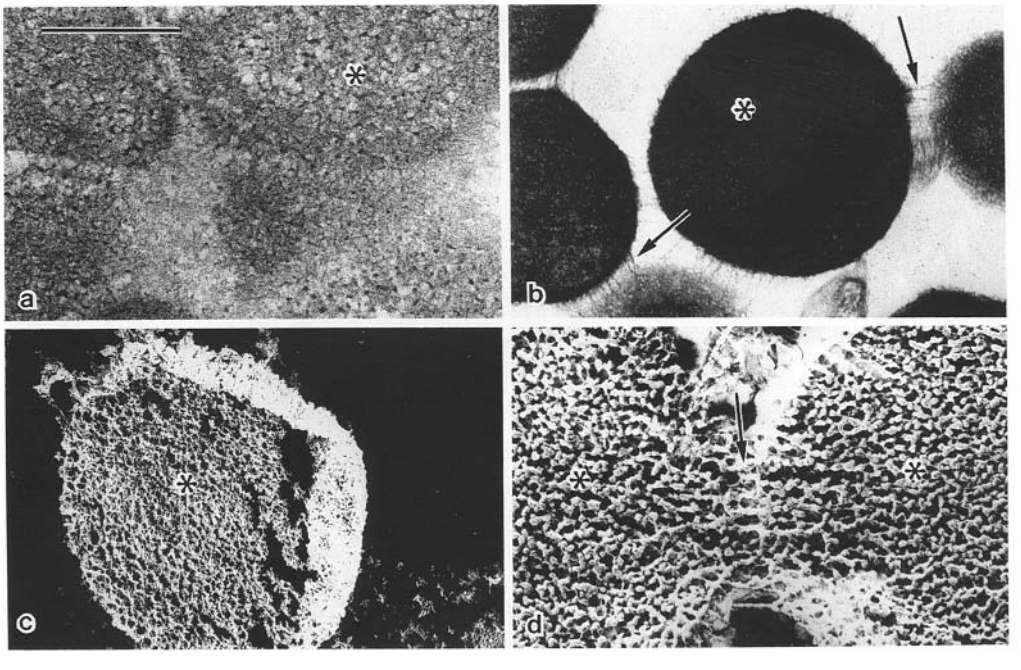

Fig. 5a–d Ultrastructural appearance of liberated granules as revealed by **a** the conventional procedures, **b** the QF-FS method, **c** QF-deep-etching (DE) replicas with fresh mast cells or **d** QF-DE replicas with saponin-treated mast cells. *Asterisks* indicate granular matrices, and *arrows* in **b** and **d** indicate interconnecting filaments between the liberated granules. (*Bar* 0.5 μm.) ×37000

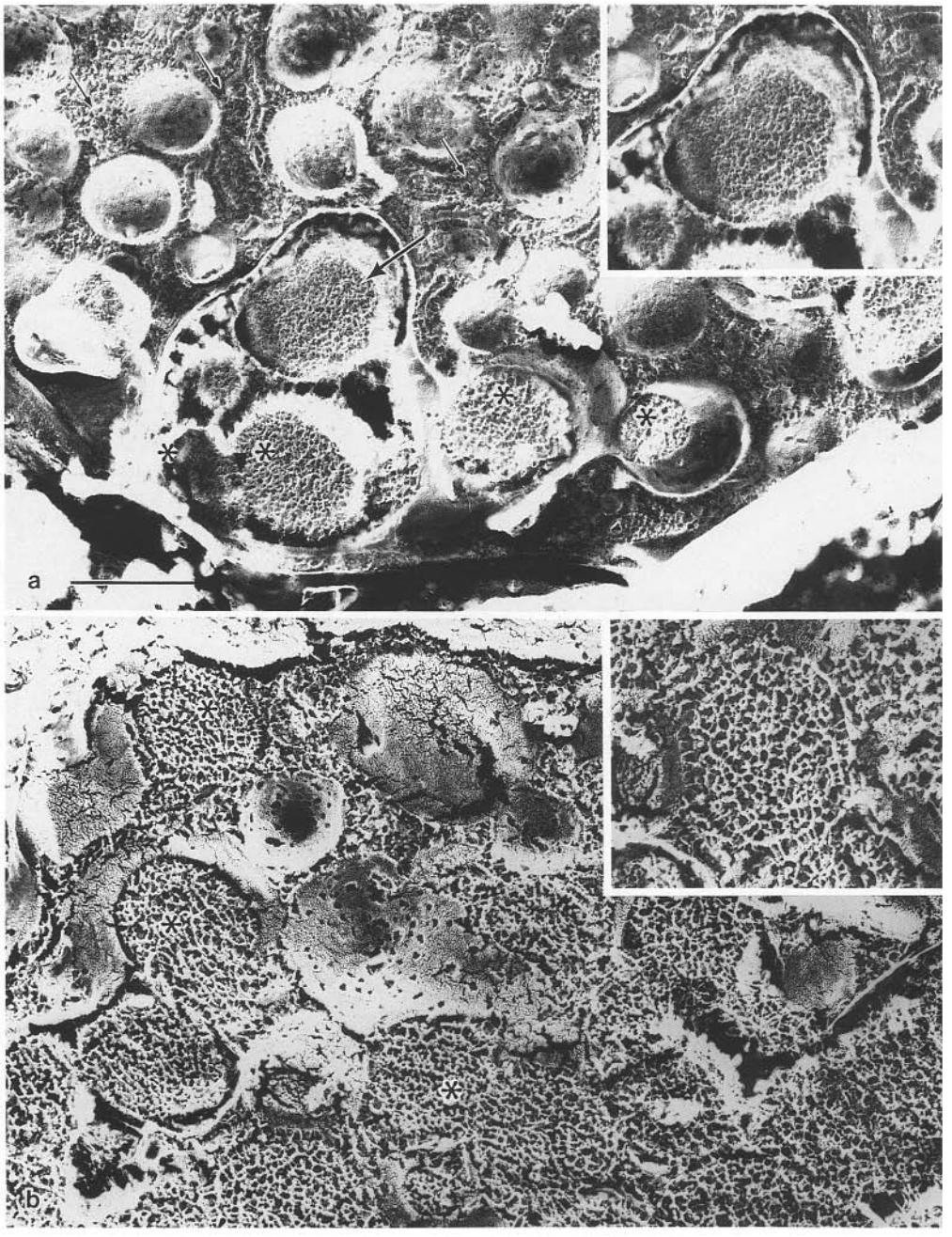

Fig. 6a, b Replica electron micrographs of degranulating mast cells stimulated with compound 48/80 for 30 s. **a** QF-DE replicas with fresh mast cells. *Inset* A higher magnified view of a granule indicated by *large arrow*. **b** QF-DE replicas with saponin-treated mast cells. *Inset*, A higher magnified view of a granule. *Small arrows* in **a** indicate cytoplasmic matrices among the granules, and *asterisks* indicate granular matrices. (*Bar*, 1 μm.) ×17400 (*inset* ×25700)

pact and electron dense granules within the cytoplasm of the stimulated mast cells. The degranulation process went on throughout the cell interior at later stages (Fig. 3c). Finally the stimulated cells were filled by a system of complicated cavities which contained a number of altered granules seen at stimulation times of 60–180 s (Fig. 3d). Conventional electron micrographs do not demonstrate detailed cytoplasmic changes in mast cells before the occurrence of degranulation.

Electron micrographs of ultrathin sections prepared by the QF-freeze substitution (FS) method do show cytoplasmic change in stimulated mast cells. Figure 4 illustrates electron micrographs of stimulated mast cells using conventional methods (Fig. 4a, c, e) and the QF-FS method (Fig. 4b, d, f). In mast cells stimulated with compound 48/80 for 10 s (Fig. 4a, b), a conventional electron micrograph shows no cytoplasmic changes (Fig. 4a), while the QF-FS method demonstrates that intergranular cytoplasm has changed into low electron dense areas especially around granules (Fig. 4b). In mast cells stimulated for 60 s (Fig. 4c, d), the QF-FS method illustrates that neighbouring granules fused to form pentalaminar membrane structures (Fig. 4d). The space between the granular contents and perigranular membranes became wider after membrane fusion. These ultrastructural changes may rarely be detected in a conventional electron micrograph, as shown in Fig. 4c.

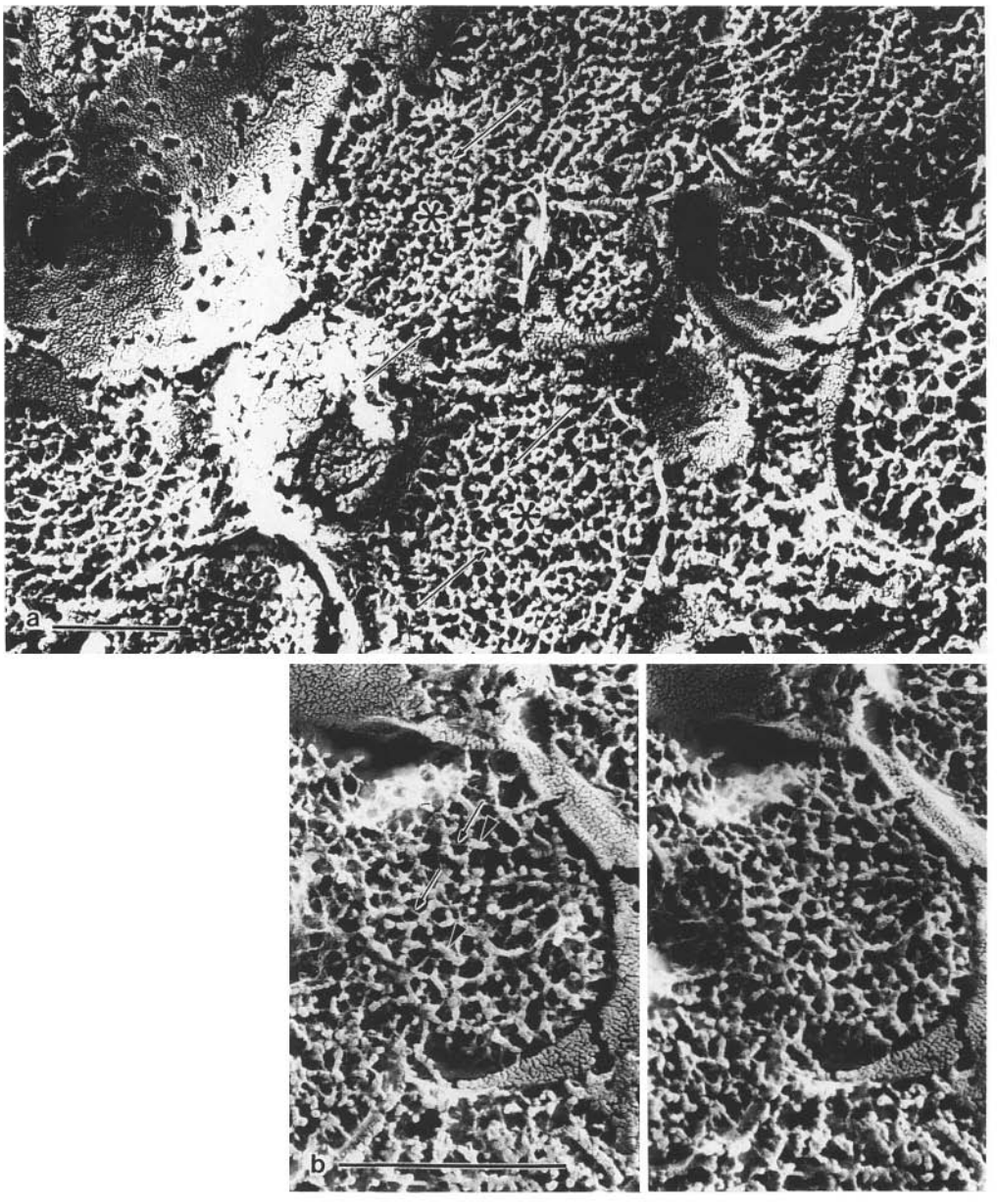

Fig. 7a, b Highly magnified view of QF-DE replicas using saponin-treated mast cells stimulated with compound 48/80 for 30 s. **a** Expanded granules (*asterisks*) indicate the existence of skeletal structures (*arrows*). (*Bar*, 0.5 μm.) × 34300. **b** Stereo-pictures of a granule demonstrate three-dimensional ultrastructures of the networks. *Arrowheads* indicate thick filaments, and *arrows* indicate thin filaments. (*Bar*, 0.5 μm.) × 62600

Figure 4e and f illustrates the ultrastructure of degranulating mast cells stimulated with compound 48/80 for 180 s. Observations on the specimens prepared by the conventional procedures confirmed that liberated granules in the extracellular space showed distinct expansion and had an ultrastructural appearance with loose networks (Fig. 4e), as reported in previous papers (Röhlich et al. 1971; Lagunoff 1973; Lawson et al. 1977; Fantozzi et al. 1978; Befus et al. 1982). They were markedly different in morphology from the compact and electron-dense granules localized within the cytoplasm, as shown in Fig. 3a. In contrast, liberated granules were filled with high electron dense contents as revealed by examination of QF-FS specimens (Fig. 4f). They were similar to the granules within the cytoplasm shown in Fig. 4a, and had no remarkable expansion as shown in Fig. 4e.

Figure 5 illustrates the ultrastructural appearance of liberated granules, as revealed by the conventional procedure (Fig. 5a), the QF-FS method (Fig. 5b), the QF-deep-etching (DE) method with fresh mast cells (Fig. 5c) and the QF-DE method with saponin-treated mast cells (Fig. 5d). Liberated granules in specimens prepared by the conventional procedure showed distinct expansion and had an ultrastructural appearance with loose networks (Fig. 5a). However, discharged granules in QF-FS specimens (Fig. 5b) and QF-DE replicas of fresh mast cells with soluble elements (Fig. 5c) had an appearance with fine networks and electron-dense contents. In contrast, liberated granules showed an ultrastructure of loose networks in QF-DE replicas of saponin-treated mast cells (Fig. 5d). Interconnecting filaments between the liberated granules could be easily detected (Fig. 5b, d).

Figure 6 illustrates replica electron micrographs of degranulating mast cells stimulated with compound 48/80 for 30 s. On examination on QF-DE replicas of unfixed fresh mast cells, the granules liberated into extracellular space had an ultrastructural appearance with fine networks and some content within the structures (Fig. 6a), which correspond to those revealed in Fig. 5b of the QF-FS specimens. Moreover, lots of cytoplasmic matrices still exist among the granules in the cytoplasm. The granules in saponin-treated mast cells had an appearance of looser networks (Fig. 6b), where the soluble contents in the granules and the cytoplasmic matrix among the granules almost disappeared when compared with those of unfixed fresh mast cells. Some granules seemed to be fused. The granules also showed distinct expansion, which had the same appearance as those liberated into extracellular space.

Figure 7a and b provides highly magnified views of granules in saponin-treated mast cells, indicating existence of skeletal structures within the granules. These three-dimensional ultrastructures were characterized with networks with short thick filaments from the intersection, while thin filaments cross-bridging between the thick filaments were also identified. Stereo-pictures demonstrate truly three-dimensional ultrastructures of the networks (Fig. 7b).

Discussion

Unfixed fresh mast cells with a few drops of TB were used to examine natural morphology of viable resting or stimulated mast cells. The cytoplasm of the resting mast cells showed little staining with TB, while secretory granules in the stimulated mast cells were stained with TB, indicating the dye affinity with heparin proteoglycans of exposed granules. The discharged granular content of the extracellular space was also stained with TB. These findings suggest that considerable amounts of granule contents were still bound within skeletal structures. They are also compatible with the findings in QF-FS ultrathin sections or QF-DE replicas, as shown in Figs. 4f, 5b and c. The granules liberated into the extracellular space had relatively rich contents, which appear different from those in conventional preparations. The liberated granules in conventional ultrathin sections have been thought to be ultrastructural artefacts from the preparation steps of fixation and dehydration (Röhlich et al. 1971; Lagunoff 1973; Kessler and Kuhn 1975; Chi et al. 1976; Lawson et al. 1977; Fantozzi et al. 1978; Befus et al. 1982; Oskéritzian et al. 1992).

The intensity of FITC-phalloidin reactions in the stimulated mast cells decreased in fluorescence microscopy. Mast cells with stimulation of 10 s showed distinct decrease of FITC-phalloidin reactions through the whole cytoplasm before their degranulation occurred, suggesting that the total amount of filamentous actin decreased in the stimulated mast cells. However, conventional electron microscopy has not demonstrated morphological changes in the cytoplasm of mast cells stimulated with compound 48/80 (Röhlich et al. 1971; Lagunoff 1973). It is probable that microfilament networks form continuous cages preventing the access of secretory granules to the plasma membrane. Destruction of the filaments after stimulation may allow granules to move toward the plasma membrane, as reported without ultrastructural evidence (Orr et al. 1972). Several studies also reported that mast cells had microfilaments mainly localized in their cortical cytoplasm (Röhlich 1975; Nielsen and Jahn 1984; Tasaka et al. 1986; Koffer et al. 1990), and suggested that disassembly of the cortical microfilaments prior to fusion of each membrane was required to promote the events of degranulation in stimulated mast cells (Koffer et al. 1990). From the present study, it is also stressed that actin microfilaments were decreased throughout the whole cytoplasm of the stimulated mast cells.

The cytoplasmic matrix between granules in mast cells disappeared after the stimulation of compound 48/80, which is compatible with previous reports (Orr et al. 1972; Koffer et al. 1990). However, in the present study, the disappearance of structural elements seems to occur simultaneously throughout the whole cytoplasm of stimulated cells, and membrane fusion may occur. The influx of calcium ions into mast cells is required to promote the degranulation reaction (Mongar and Schild 1958; Pintado et al. 1984; Tasaka et al. 1986) and further studies by X-ray microanalysis are indicated to reveal shifts of cytoplasmic electrolytes at an ultrastructural level (Hall 1979; Morgan 1985; Zierold and Hagler 1989; Zierold 1990, 1992a, b). The present study has demonstrated that filamentous actin decreased in the whole cytoplasm of mast cells stimulated with compound 48/80 before degranulation begins.

Decrease of the FITC-phalloidin reaction in the stimulated mast cells is shown by ultrastructural findings on QF-FS specimens or QF-DE replicas of saponin-treated mast cells, indicating that intergranular cytoplasm was changed into translucent areas and almost disappeared between adjacent granules. In addition, neighbouring granules were fused to form pentalaminar membrane structures, and the space between granular contents and granular membranes became wider after the membranous fusion. It is suggested that the cytoplasm of mast cells is altered morphologically by stimulation with compound 48/80 prior to degranulation but stimulated mast cells may show ultrastructural artefacts which can be clearly identified in QF-FS ultrathin sections (Fig. 4).

The release of granular contents from stimulated mast cells leads to further reactions in mast cells and leucocytes and functional changes of vascular permeability (Galli 1993). These phenomena are related to the formation of inflammatory reactions. To maintain local effects in tissues, it would be reasonable to have a system to release chemical mediators gradually from the granular skeletons. However, conventional electron microscopic studies have suggested that ultrastructural modi-

fication was evident in the granules which were discharged into extracellular space (Röhlich et al. 1971; Lagunoff 1973). It is well known that the granules of mast cells contain lots of heparin proteoglycans and chemical mediators, which are bound (Tas and Berndesen 1977; Fantozzi et al. 1978; Soll et al. 1981; Befus et al. 1982; Theoharides et al. 1985; Galli 1993). Released granular contents without membranes may show artefactual changes with apparent natural ultrastructures arranged in loosely organized networks in conventional ultrathin sections.

Skeletal structures in secretory granules of mast cells were clearly demonstrated by examining on QF-DE replicas of saponin-treated specimens. It has been reported that the core protein of heparin proteoglycans has a single strand structure with a thin diameter (Alberts et al. 1989). In the present study, thick skeletal networks did not correspond to the proteoglycans, though thin filaments in the networks seemed to have some correspondence with them. However, immunocytochemical analysis of these filaments will be required to identify each component. Morphologically, the skeletal structures had three-dimensional networks as basal units. It is probable that many contents including heparin proteoglycans and chemical mediators were bound to each other, as revealed on QF-FS specimens or QF-DE replicas of the unfixed fresh mast cells. However, conventional methods using ultrathin sections showed similar ultrastructure for released granules as those observed on QF-DE replicas of the saponin-treated mast cells. Conventional images of the released granules may well reflect the skeletal structures in some part. The existence of filamentous structures, which allows expansion of granules, may have a regulatory function on the release of granular contents. It is suggested that the granules of mast cells are not a simple lump, but a highly functional apparatus for secretion. Connecting filaments between the released granular contents have been demonstrated on QF-FS specimens as shown in Fig. 5b. Although further studies to explore their functional meaning will be required, they may be concerned with the regulation on release of granular contents.

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