Virchows Archiv (1994) 425:435–438

ORIGINAL ARTICLE

Ichiro Takayama · Yasuhisa Fujii · Shinichi Ohno
Masayuki A. Fujino

X-ray microanalysis of rat mast cells stimulated with compound 48/80 in combination with quick-freezing method

Received: 9 June 1994 / Accepted: 1 September 1994

Abstract X-ray microanalysis was performed on rat mast cells prepared by quick-freezing, cryosectioning and freeze-drying (QF-FD) method, or quick-freezing and freeze-substitution (QF-FS) method. Peritoneal cells including mast cells were stimulated with compound 48/80 for 0, 10 or 30 s at 17° C, and the mast cells stimulated for 30 s started exocytosis. In X-ray spectra of the QF-FD specimen, mast cells stimulated for 10 s increased their levels of phosphorus, sodium and chlorine in the intergranular cytoplasm prior to exocytosis, and kept this increase until 30 s after stimulation. In the QF-FS specimen, where soluble elements were removed, peaks of phosphorus, sulphur and potassium could be detected as elements in X-ray spectra. Phosphorus increased and potassium decreased in intergranular cytoplasm of mast cells stimulated for 10 s, and these changes became more obvious after 30 s. However, supplemental increase of other cations such as sodium could not be detected in the QF-FS specimens.

Key words Mast cell · Quick-freezing
Compound 48/80 · X-ray microanalysis

Introduction

Conventional electron microscopic studies have reported that compound 48/80-induced exocytosis in rat peritoneal mast cells starts from fusion between perigranular membrane and plasma membrane [2, 6, 7, 16]. Few cytoplasmic events prior to the fusion can be demonstrated in such exocytosis. Actin filaments exist in resting mast cells [15, 18], and may prevent membrane fusion [5, 12] and recently it was demonstrated that disassembly of actin filaments in stimulated mast cells was followed by fusion between adjacent granule membranes in the entire cytoplasm [17]. These morphological findings suggest that preparatory states for degranulation occurred physiologically in the whole cytoplasm at an early stage of exocytosis. It has been pointed out that calcium influx also plays an important role as a trigger for exocytosis in mast cells [8, 14, 18]. However, further general changes of cytoplasmic elements in the stimulated mast cells have not yet been investigated.

X-ray microanalysis has been utilized to analyse changes of cytoplasmic elements which are related to dynamic processes such as exocytosis in biological specimens [20, 21]. We performed X-ray microanalysis in combination with quick-freezing (QF), cryosectioning and freeze-drying (FD) methods, which did not permit significant artefactual shifts of elements in the specimens in order to study cytoplasmic elements in mast cells stimulated with compound 48/80. We also used QF and freeze-substituted (QF-FS) specimens for X-ray microanalysis as the QF-FS method has been used widely in electron microscopy where elements associated with cellular architecture are less altered than soluble electrolytes in the QF-FS specimens [21]. We expected that X-ray spectra of the QF-FS specimens would give clues about cytoplasmic changes during exocytosis. We examined some elemental shifts which might promote mast cell exocytosis.

Materials and methods

Twenty-five male Wistar rats weighing 240–280 g were purchased from Japan SLC (Shizuoka, Japan). Each was anaesthetized with ether and bled our from both carotid arteries. Ten millilitres of Dulbecco's modified Eagle's medium (Flow Laboratories, USA) containing 10 unit/ml heparin and 0.1% bovine serum albumin was injected into the peritoneal cavity. After massage of the abdomen, the peridoneal fluid was pipetted out as described elsewhere [13, 16, 17]. The peritoneal fluid of each rat was mixed and centrifuged at 1000 rpm for 10 min. Then the supernatant was discarded, and centrifuged peritoneal cells were collected. For exocytosis to occur, they were stimulated with 10 μg/ml compound 48/80 (Sigma, USA) at 17° C for 0, 10 or 30 s [1, 17, 19]. Unstimulated

I. Takayama (✉) · M.A. Fujiino
First Department of Medicine, Yamanashi Medical University, Tamaho, Yamanashi 409-38, Japan

Y. Fujii · S. Ohno
First Department of Anatomy, Yamanashi Medical University, Tamako, Yamanashi 409-38, Japan

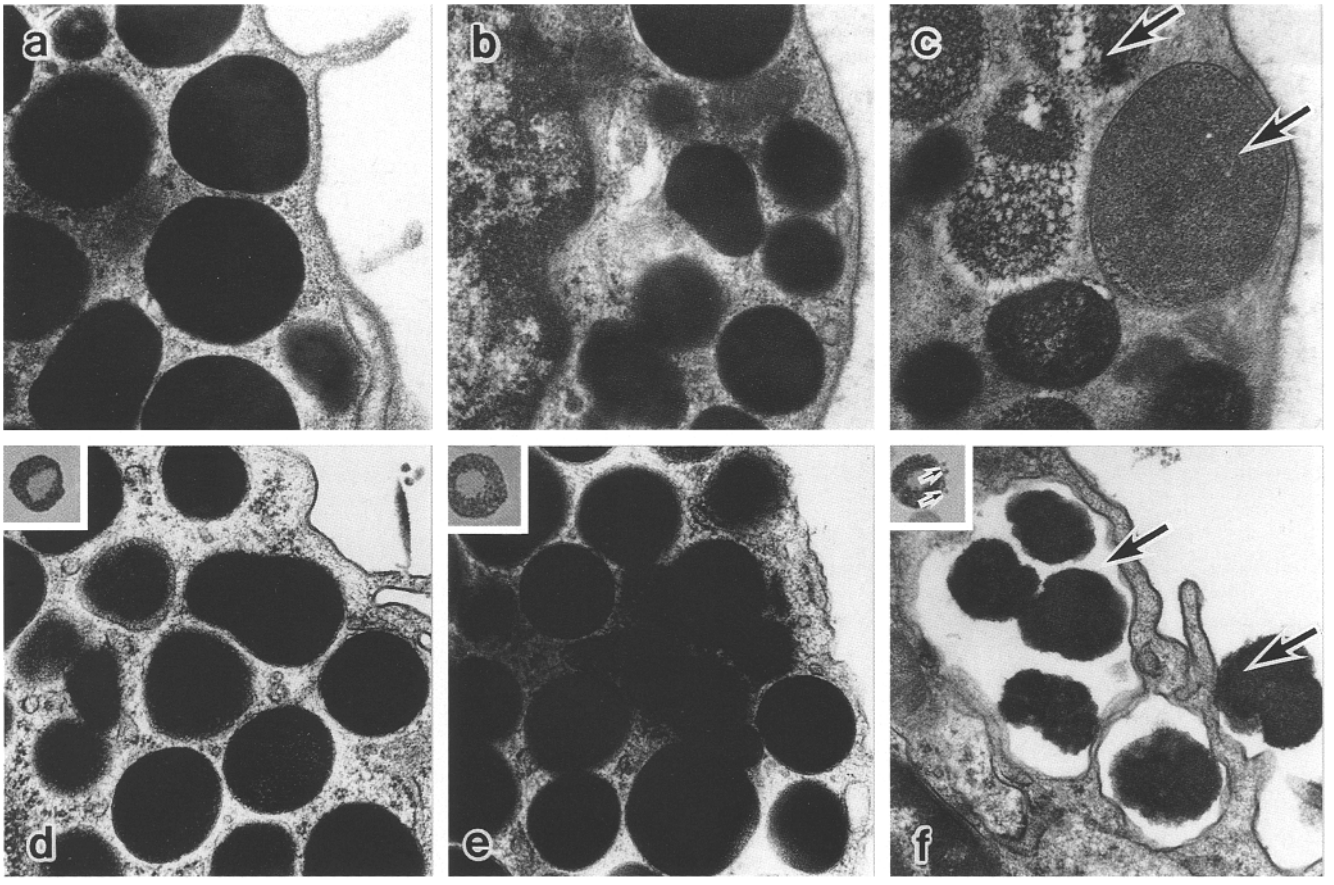

Fig. 1a–f Light and electron micrographs of mast cells at different stages of compound 48/80-induced degranulation. **a–c** The con ventional procedure. **d–f** The quick-freezing and freeze-substitution (QF-FS) procedure. **a** and **d** Resting mast cells. **b**, **e** Mast cells stimulated for 10 s. **c**, **e** Mast cells stimulated for 30 s, which start degranulation of their peripherally located granules (*arrows*). **a–c** ×12300. **d–f** ×18600. *Insets* Light micrographs of mast cells at each stage, which were prepared by the QF-FS method. *Small arrows* indicate liberated secretory granules. ×900

mast cells were designated as resting cells. Compound 48/80 stimulation was stopped by QF for electron microscopy (EM) and X-ray microanalysis, or by fixation for conventional EM to detect released secretory granules [17].

For conventional EM, specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min, washed in phosphate buffered saline (PBS), and postfixed with 2% osmium tetroxide (OsO_4) in PB for 30 min. They were washed in PBS, dehydrated in a graded series of ethanol, immersed with propylene oxide and routinely embedded in Epok 812. Ultrathin sections were cut, mounted on copper grids and doubly stained with uranyl acetate and lead citrate. They were examined by EM, Hitachi H-600 at an accelerating voltage of 75 kV.

For EM and X-ray microanalysis of QF-FS specimens, mast cells treated on egg albumin stages were attached to copper blocks cooled in liquid nitrogen (−196° C) and quickly frozen. Then they were placed in pure acetone containing 2% OsO_4 at −80° C for 20 h. 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 [11]. They were washed in absolute acetone and embedded in Epok 812. Thick sections of the QF-FS specimens were stained with toluidine blue and examined under a light microscope. Ultrathin sections were cut and examined as described above.

For energy-dispersive X-ray microanalysis fresh unfixed mast cells were plunged into a liquid isopentane-propane mixture (−193° C) cooled in liquid nitrogen [10]. Then 0.2 μm semithin sections of the frozen specimens, cooled at −130° C, were cut using a ultramicrotome equipped with a cryosectioning apparatus. The semithin sections were mounted on nylon grids and freeze-dried in a freeze-fracture apparatus under vacuum conditions of $6–8\times10^{-6}$ Pa for 30 min at −95° C. They were coated with carbon to reinforce the specimens. The QF-FS sections were also mounted and coated with carbon [9, 10]. Mast cells could be easily distinguished from other peritoneal cells by their larger size and appearance.

X-ray microanalysis was performed in a Hitachi H-8100 EM equipped with an energy-dispersive X-ray microanalysing system, Kevex Delta. Intergranular cytoplasm of different mast cells (about 15 areas for each specimen) were examined at an accelerating voltage of 200 kV, for 100 s analysing time and with 20 nm of beam spot diameter. Obtained X-ray spectra were evaluated quantitatively with a software Quantex version 6 (Fisions Instruments/Kevex, USA), which could separate some overlapped X-ray peaks mathematically. The results were represented as weight percents of cytoplasmic elements. In QF-FS specimens, some elements such as chlorine, which originated not only from mast cells but also from the resin or fixative, were used for quantitative evaluation; neglect of these elements might cause confusion although they were omitted from the final adjustment and changes in cytoplasmic elements in QF-FS specimens were finally represented as percentages of phosphorus, sulphur and potassium by recalculation among these three elements. Elements with atomic numbers under ten could not be detected.

Results

Figure 1 illustrates light and EM appearances of the mast cells. Resting mast cells had a smooth contour of the cell surface and many secretory granules (Figs. 1a, d). The granules showed staining with toludine blue by light mi-

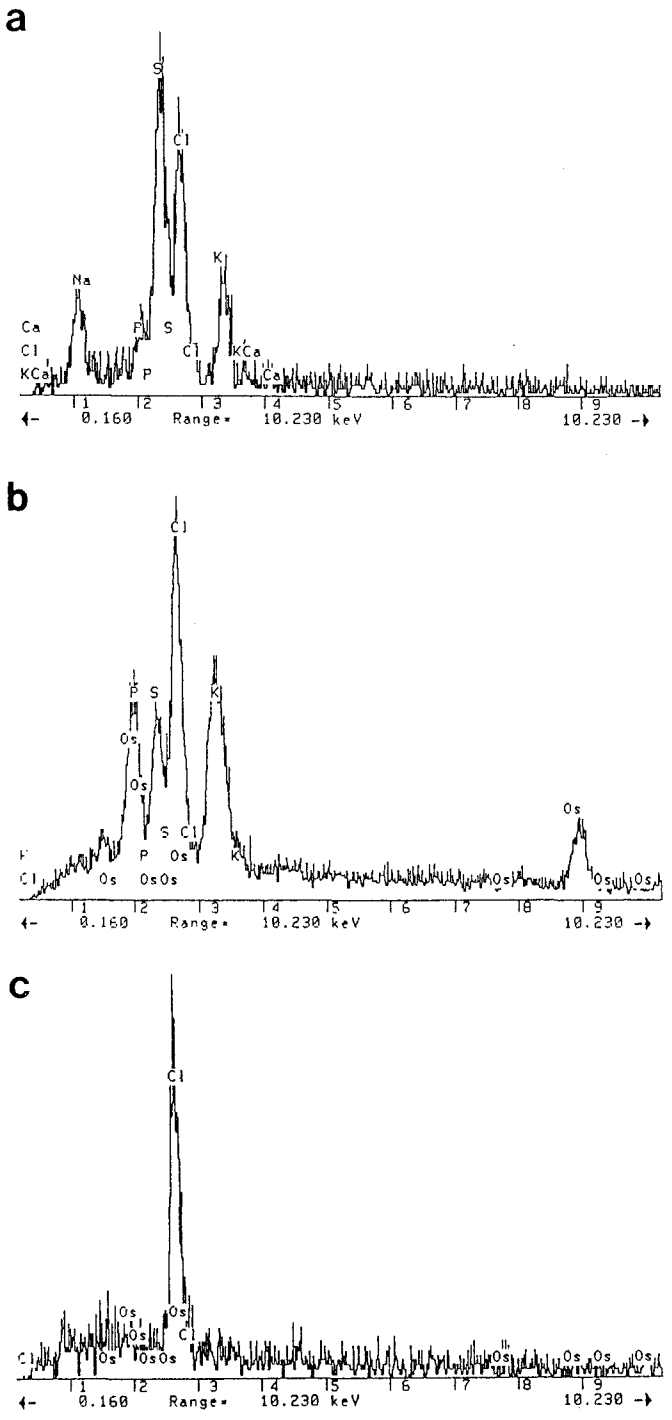

Fig. 2a–c Examples of X-ray spectra by energy-dispersive X-ray microanalysis in resting mast cells. **a** X-ray spectra examined in the QF-freeze-drying specimen. Those in the QF-FS specimen. **c** Those in background epoxy resin

croscopy (Fig. 1d, *inset*), and had compact and uniform appearance by EM. Mast cells stimulated for 10 s did not show morphological changes (Fig. 1b, e). In contrast, mast cells stimulated for 30 s showed irregular contour of the cell surface and exocytosis (Fig. 1c, f). Released secretory granules in QF-FS specimens had compact appearance (Fig. 1f), while such granules showed distinct expansion and rough network appearance in conventional EM (Fig. 1c), which was probably a morphological artefact [17]. However, they could be easily distinguished from compact and electron dense granules within the cytoplasm before exocytosis [6, 16].

Figure 2 illustrates examples of X-ray spectra obtained from resting mast cells. In QF-FD specimens, X-ray peaks of sodium, sulphur, phosphorus, chlorine, potassium and calcium were detected (Fig. 2a). X-ray peaks of the other elements were not distinguishable from background noise and hard to use for quantitative evaluation. In QF-FS specimens, X-ray peaks of sulphur, phosphorus and potassium could be identified (Fig. 2b), while they did not exist in the background resin (Fig. 2c). Chlorine was also detected in both areas (Fig. 2b, c).

Table 1 demonstrates the weight percentages of cytoplasmic elements in the mast cells. In QF-FD specimens, mast cells stimulated for 10 s increased phosphorus and chlorine, and decreased potassium in their intergranular cytoplasm. They also increased their sodium after 30 s of stimulation. Changes in calcium, however, were hardly detected. In QF-FS specimens, mast cells stimulated for 10 s increased phosphorus and decreased potassium. They progressively increased phosphorus and further decreased potassium after 30 s stimulation.

Discussion

Resting mast cells had smooth contour of the cell surface and did not show degranulation. However, mast cells stimulated for 30 s released peripherally located granules. Liberated granules in the extracellular space showed a compact morphological appearance in QF-FS specimens, indicating existence of considerable contents. The granules regulate some aspects of the inflammatory reaction [4], and their gradual release would be reasonable as a regulatory process [17]. Conventional EM, however, produced an appearance of distinct expansion and loose network formation [1, 6, 7, 16], as shown in Fig. 1c, and suggested rapid collapse of the granules during exocytosis. In contrast, granules released in QF-FD specimens have kept a relatively rich content. The granule in the QF-FD specimens may reflect a more natural appearance than that in conventional preparations.

Energy-dispersive X-ray microanalysis has advantages for showing elemental features in biological specimens [20, 21]. The QF-FD specimen allowed examination of general elemental changes related to their functional conditions at the moment of quick-freezing [20]. Free cells in culture medium are easy to treat under controlled conditions [22], so in vitro mast cells would be useful for X-ray microanalysis. In QF-FD specimens, stimulated mast cells increased their content of electrolytes such as sodium and chlorine, and decreased potassium. These changes suggested that exchanges of extracellular and intracellular liquid might occur in the mast cells. The stimulated mast cells also increased phosphorus in their intergranular regions. It has been reported that disassembly of intergranular actin filaments promotes exocytosis in mast cells [17], requiring oxidative phosphorylation [3]. The shift of

Table 1 Percentages of the elements in the cytoplasm of mast cells, represented as mean±standard deviation (*QF* quick-freezing, *FS* freeze-substitution, *FD* freeze-drying)

Time (s)	Sodium	Phosphows	Sulphur	Chlorine	Potassium	Calcium
QF-FD						
0	4.11±3.00	11.12±13.88	58.89±14.58	13.54±6.10	11.66±4.00	0.78±0.53
10	5.69±1.75	24.13±22.16	35.95±24.61	28.32±9.85	5.24±2.40	0.69±0.56
30	11.07±6.74	21.08±17.28	36.28±25.27	24.43± 6.03	6.08±1.52	1.06±0.56
QF-FS						
0	–	15.89±3.99	62.87±3.38	–	21.24±2.67	–
10	–	20.03±5.74	66.09±5.93	–	5.89±2.65	–
30	–	41.28±4.84	58.80±4.76	–	0.91±1.04	–
back	–	0.00±0.00	0.00±0.00	–	0.00±0.00	–

phosphorus presented in the present study may be related to these biochemical events. Moreover, reorganization of membranous structure may occur in the stimulated mast cells, and the changes in phopshorus may be related to this reorganization.

In QF-FS specimens, both increase of phosphorus and decrease of potassium could be detected, when sodium had been removed from the specimens. It is interesting that considerable amounts of potassium could be detected in the QF-FS specimens which had been allowed to retain some soluble proteins, especially in resting mast cells. Furthermore, the postassium in resting cells decreased after compound 48/80 stimulation, and sodium, which was increased in QF-FD specimens, was not detected. It is widely accepted that potassium exists as a free cation in the cells and no binding proteins have been reported, so it is hard to explain these findings. Potassium binding sites, however, might be able to regulate cell volume and functions during rapid exocytosis. Further examination to purify binding proteins with biochemical techniques would be required to confirm the present finding.

The present study reflects general changes in cytoplasmic elements in the stimulated mast cells. Dramatic shift of a minor element like calcium, a trigger for exocytosis, could be hardly detected. Another physiological approach in combination with X-ray microanalysis would be required to reveal a slight shift of calcium.

References

1. Befus AD, Pearce FL, Gauldie J, Horsewood P, Bienenstock J (1982) Mucosal mast cells. I. Isolation and functional characteristics of rat intestinal mast cells. J Immunol 128:2475–2480
2. Chandler DE, Heuser JE (1980) Arrest of membrane fusion events in mast cells by quick-freezing. J Cell Biol 86:666–674
3. Cooke R, Kirschner M, Klymkowsky M, Mooseker M, Pollard T, Rosenbaum J, Tilney L, Weber K (1989) The cytoskeleton. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (eds) Molecular biology of the cell, 2nd edn. Garland Publishing, New York, pp 613–677
4. Galli SJ (1993) New concepts about the mast cell. N Eng J Med 328:257–265
5. Koffer A, Tatham PER, Gomperts BD (1990) Changes in the state of actin during the exocytotic reaction of permeabilized rat mast cells. J Cell biol 111:919–927
6. Lagunoff D (1973) Membrane fusion during mast cell secretion. J Cell biol 57:252–259
7. Lawson D, Raff MC, Gomperts B, Fewtrell C, Gilula B (1977) Molecular events during membrane fusion. A study of exocytosis in rat peritoneal mast cells. J Cell Biol 72:242–259
8. Mongar JL, Schild HO (1958) The effect of calcium and pH on the anaphylactic reaction. J Physiol 140:272–284
9. Naramoto A, Ohno S, Nakazawa K, Takami H, Itoh N, Shigematsu H (1991) Three-dimensional ultrastructure of glomerular injury in serum sickness nephritis using the quick-freezing and deep-etching method. Virchows Arch [A] 418:185–192
10. Ohno S, Fujii Y (1990) Three-dimensional and histochemical studies of peroxisomes in cultured hepatocytes by quick-freezing and deep-etching method. Histochem J 22:143–154
11. Ohno S, Hora K, Furukawa T, Oguchi H (1992) Ultrastructural study of the glomerular slit diaphragm in fresh unfixed kidneys by a quick-freezing method. Virchows Arch [B] 61:351–358
12. Orr TSC, Hall DE, Allison AC (1972) Role of contractile microfilaments in the release of histamine from mast cells. Nature 236:350–351
13. Oskéritzian C, Prévost M-C, Prouvost-Danon A, Gounon P, David B (1992) Ultrastructural changes in mouse peritoneal mast cells in response to L-leucine methyl ester or anti-Ig E. Res Immunol 143:893–901
14. Pintado E, Carrasco M, Goberna R (1984) Histamine release by pharmacological agents in the absence of external free Ca^{2+}. FEBS Lett 175:147–151
15. Röhlich P (1975) Membrane-associated filaments in the cortical cytoplasm of the rat mast cell. Exp Cell Res 93:293–298
16. Röhlich P, Anderson P, Uvnäs B (1971) Electron microscope observations on compound 48/80 induced degranulation in rat mast cells. Evidence for sequential exocytosis of storage granules. J Cell Biol 51:465–483
17. Takayama I, Fujii Y, Ohno S, Fujino MA (1994) Ultrastructural study of mast cells stimulated with compound 48/80 as revealed by quick-freezing method. Virchows Archiv 424: 287–294
18. Tasaka K, Mio M, Okamoto M (1986) Intracellular calcium release induced by histamine release and its inhibition by some antiallergic drugs. Ann Allergy 56:464–469
19. Theoharides TC, Kops SK, Bondy PK, Askenase W (1985) Differential release of serotonin without comparable histamine under diverse conditions in the rat mast cell. Biochem Pharmacol 34:1389–1398
20. Zierold K (1990) Cryofixation methods for ion localization in cells by electron probe microanalysis; a review. J Microsc 161:357–366
21. Zierold K (1992) Cryopreparation for electron probe microanalysis of biological cells. Electron Microsc 3:45–49
22. Zierold K, Hagler HK (eds) (1989) Electron probe microanalysis applications in biology and medicine. Springer, Berlin Heidelberg New York