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Freeze-fracture immunocytochemistry for intracellular localization of serotonin in mast cells stimulated with compound 48/80

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Abstract Changes of intracellular localization of serotonin in rat mast cells were examined by freeze-fracture immunocytochemistry, to prevent the translocation of the serotonin antigen. Rat peritoneal cells including mast cells were stimulated in vitro with compound 48/80, at 17° C for 0, 30 or 60 s for exocytosis to occur. The mast cells were fixed, quickly frozen and freeze-fractured to expose the antigen on the fractured surface. They were immunostained with serotonin antibody, and the immunoreactions on the fractured surface were examined on ultrathin sections by electron microscopy. Unstimulated mast cells exhibited serotonin localization mostly in each intragranular matrix. In contrast, mast cells stimulated for 30 s exhibited increased serotonin in their intergranular cytoplasm. Mast cells showed more distinct immunoreactions in the cytoplasm where degranulation would be promoted after 60 s. It is suggested that intracellular release of serotonin occurred in the stimulated mast cells.

Key words Mast cell · Compound 48/80 · Exocytosis · Freeze-fracture immunocytochemistry

Introduction

Secretory granules of rat mast cells contain much heparin proteoglycans and various chemical mediators such as histamine and serotonin [1, 3, 11, 16]. However, it has been suggested that coexisting histamine and serotonin have divergent roles in delayed hypersensitivity with discrepancy between their corporate functions [4]. Differential release of serotonin and histamine is promoted by the tricyclic anti-depressant drug, amitriptyline, which prevents exocytosis of secretory granules and histamine release, while serotonin is released without exocytotic mechanism [16] and it has thus been suggested that mast cells have an unknown mechanism for the release of serotonin. It has also been reported that a varying proportion of granules released serotonin intracellularly [2a], but there is little work on the ultrastructural localization of serotonin in stimulated mast cells.

Recently, freeze-fracture immunocytochemistry has been used in electron microscopy because of its advantages in avoiding the translocation of antigens [5]. We used this method to investigate ultrastructural localization of serotonin in mast cells stimulated with compound 48/80.

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Materials and methods

Twenty-five male Wistar rats weighing 240–280 g were purchased from Japan SLC (Shizuoka, Japan). The rats were anaesthetized with ether and bled out from bilateral carotid arteries. Then 10 ml of Dulbecco's modified Eagle's medium (Flow Laboratories, USA) containing 10 unit/ml of heparin and 0.1% bovine serum albumin (BSA) was injected into each peritoneal cavity. After gentle massage of the abdomen, peritoneal fluid was pipetted [2b, 7, 9]. The peritoneal fluid of each rat was mixed, and centrifuged at 1000 rpm for 10 min. The supernatant was then discarded and the centrifuged peritoneal cells were collected. To produce degranulation of mast cells, they were stimulated with 10 μg/ml of compound 48/80 (Sigma, USA), which has often been used as a mast cell stimulator, at 17° C for 30 or 60 s [1, 9, 15, 16]. Unstimulated must cells were designated as resting cells.

For freeze-fracture immunocytochemistry, mast cells were immediately fixed in 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) for 1 h, embedded in 30% BSA, and gelatinized by crosslinking with 2,5% GA in PB for 8 h. The gelatinized BSA were cut into small pieces, immersed with 30% glycerol in PB and quickly frozen in isopentane-propane mixture (−193° C) cooled with liquid nitrogen. Frozen specimens were transferred to a tissue homogenizer filled with liquid nitrogen and repeatedly fractured into fine fragments [5, 8]. The specimens were washed in 0.1 M phosphate buffered saline (PBS) containing lysine, and incubated with rabbit anti-serotonin serum (Chemicon, USA) diluted at 1:40 for 1 h at 37° C, while control preparations were treated with normal rabbit serum. After washing in PBS, they were treated with goat anti-rabbit IgG coupled with peroxidase (Cappel, USA) for 1 h at 37° C, and then visualized by routine diaminobenzidine method. They were washed in PBS, and postfixed with 1% osmium te-

troxide in PB for 1 h. They were washed in PBS, dehydrated in a graded series of ethanol, immersed with propylene oxide and embedded in Epok 812. One micrometer thick sections of the specimens were examined under a light microscope. Ultrathin sections were cut with an ultramicrotome, mounted on copper grids and examined without electron staining in a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV, while others were examined after double staining with uranyl acetate and lead citrate.

Results

Figure 1 illustrates light and electron micrographs of resting mast cells revealed by freeze-fracture immunoytochemistry. A thick section of resting mast cells shows many fine fragments of gelatinized cells (Fig. 1a) and immunoreactions of the fractured surface were examined by electron microscopy. Resting mast cells in the immunocontrol preparation did not show remarkable immunoreaction for serotonin (Fig. 1b). In contrast, the resting mast cells incubated with anti-serotonin serum showed distinct immunoreactions mostly in their intragranular matrices on the fractured surface (Fig. 1c–e). The specimens without double staining exhibited serotonin in their intragranular matrices (Fig. 1c). Doubly stained mast cells also presented the boundary of the immunoreactions (Fig. 1d–e).

In contrast, mast cells stimulated with compound 48/80 for 30 s exhibited increased serotonin in their intergranular cytoplasm (Fig. 2a–b). Cytoplasmic immunoreaction was located in perigranular regions especially. The change was also detected in doubly stained specimens (Fig. 2b). Figure 2c illustrates serotonin immunoreactions in mast cells after 60 s. The stimulated mast cells also showed increased immunoreactions in their intergranular cytoplasm, compatible with those after 30 s shown in Fig. 2a, b, and the immunoreactions were more distinct. Some liberated granules exhibited serotonin immunoreactions in the extracellular space (Fig. 2d).

Discussion

Freeze-fracture immunocytochemistry has demonstrated localization of serotonin in mast cells, which had shifted into the intergranular cytoplasm from the granule matrix after compound 48/80 stimulation. Serotonin plays dif-

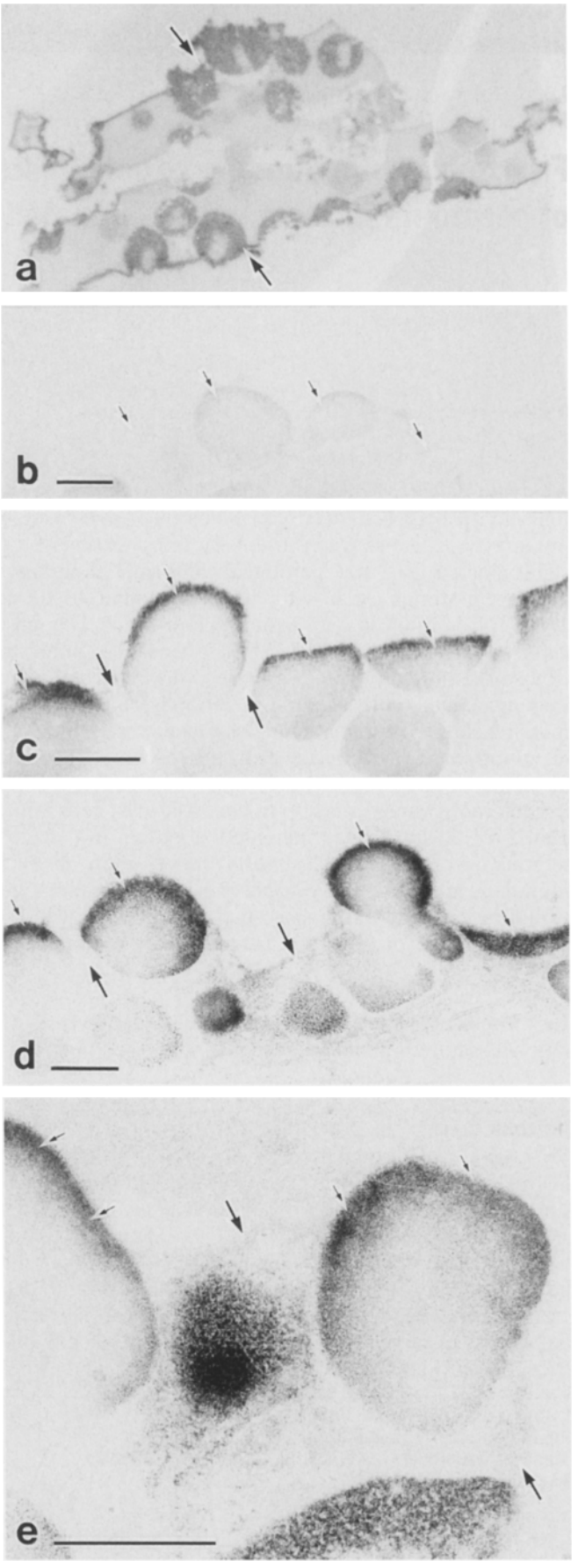

Fig. 1a–e Light and electron micrographs of resting mast cells with immunoreactions for serotonin, prepared by freeze-fracture immunoytochemistry. **a** Light micrograph of a thick section, showing cellular fragments in gelatinized albumin. The *arrows* indicate mast cells on the fractured surface. ×700. **b** Immunocytochemical control specimen without immunoreaction. The *small arrows* indicate the fracture surface. ×16100. **c–e** The fractured surface of resting mast cells with immunoreactions. (**d** and **e** were doubly stained with uranium and lead). The *large arrows* point to the intergranular cytoplasm on the fractured surface, without immunoreactions. The *small arrows* show the secretory granules with immunoreactions. **c** ×25300; **d** ×20000; **e** ×48800. *Bars*= 0.5 μm

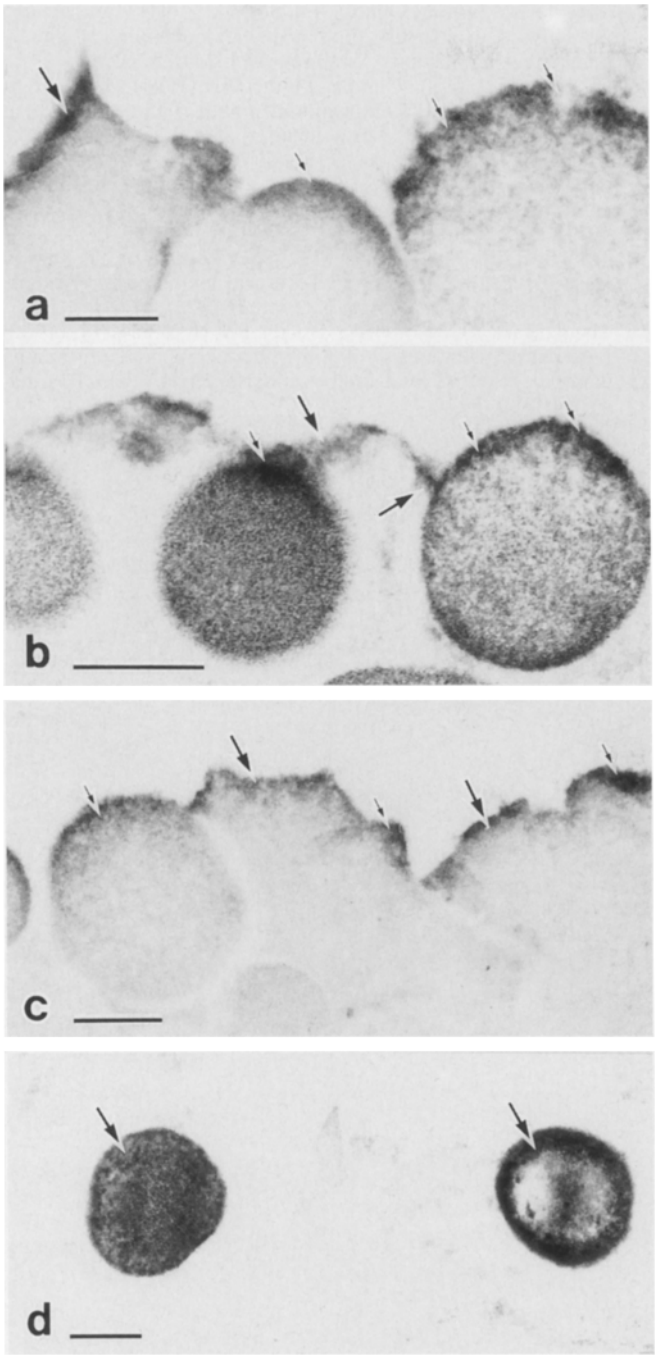

Fig. 2a–d Electron micrographs of serotonin localization in mast cells stimulated with compound 48/80 for 30 or 60 s, prepared by freeze-fracture immunocytochemistry. **a** The fractured surface of mast cells without double electron staining. ×24000. **b** The same with double uranium and lead staining. ×33700. **c** The fractured surface of mast cells stimulated for 60 s without double electron staining. ×23300. For **a–c** *arrows* indicate intergranular cytoplasm on the fractured surface with immunoreactions for serotonin. The *small arrows* indicate secretory granules with immunoreactions. **d** The liberated secretory granules in the extracellular space with dense immunoreactions (*arrows*). ×18800. *Bars*=0.5 μm

ferent biochemical roles to histamine, so the parallel release will produce a functional discrepancy [4, 16]. Marked serotonin release was not accompanied by mast cell degranulation, indicating a possible intracellular release of serotonin from the granule matrix [2]. Their morphological evidence, however, had not yet been presented. In the present study, we demonstrated the cytoplasmic events of serotonin release in stimulated mast cells by freeze-fracture immunocytochemistry [5].

It has been reported that amitriptyline inhibits histamine release from mast cells stimulated with compound 48/80, while it permits the release of serotonin [16]. Sandra et al. [10] reported that amitriptyline changed the granules in size and shape. Serotonin is localized outside the granules and under our experimental conditions was close to small vesicles. These studies suggested that a pathway of serotonin release other than exocytosis exists in rat mast cells. In the present study, however, no marked serotonin release was detected around cell organelles in stimulated mast cells, apart from the secretory granules.

It has been reported that preparatory states for degranulation occur throughout the whole cytoplasm of the stimulated mast cells in early stages [12, 13, 14], and these have important roles in the intracellular shift of serotonin in cells under experimental conditions. In addition, it has been reported that supporting skeletal structures exist within the granules and that they prevent granule contents from simple diffusion immediately after the exocytosis [12, 14]. Further studies are required to investigate the relationship between cell organelles and the shift of serotonin in the stimulated mast cells. In conclusion, the present study revealed the intracellular shift of serotonin in mast cells stimulated with compound 48/80.

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