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Ultrastructural characteristics of rat peritoneal mast cells undergoing differential release of serotonin without histamine and without degranulation

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Summary. Rat mast cells pretreated with the tricyclic antidepressant drug amitriptyline and stimulated with compound 48/80 secreted 60% of the total serotonin present in the cells, but only 15% of histamine, another amine stored in the same granules. Ultrastructural studies demonstrated that mast cells undergoing such differential release do not exhibit classical degranulation by compound sequential exocytosis. However, there were changes in granule shape and size, as well as alterations in many morphometric parameters consistent with secretion. Storage granules lost their homogeneity, exhibited greatly reorganized matrix and were surrounded by clear spaces which were often associated with small (0.1–0.01 μm) cytoplasmic vesicles, some of which contained electron-dense material. Secretory granules often had bud-like protrusions or were fused together in series. Quantitative autoradiography localized ^{3}H-serotonin outside the storage granules, close to small vesicles, while staining with ruthenium red demonstrated that vesicular structures associated with differential release were not endocytotic. These results suggest that amitriptyline may inhibit regular exocytosis and permit at least serotonin to be moved selectively from storage granules to the cytosol or small vesicles from which it is eventually released.

Key words: Mast cell – Mediator release – Serotonin – Histamine – Exocytosis – Differential – Rat (Sprague-Dawley)

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Biochemical studies have shown that serotonin can be released differentially, without concomitant histamine, from rat peritoneal mast cells preincubated by the tricyclic antidepressant, amitriptyline before stimulation with the classical mast cell secretagogue, compound 48/80 (C48/80) or immunoglobulin E (IgE) and antigen (Theoharides et al. 1980). Such differential release was confirmed independently and shown to occur also when secretion was triggered by various neuropeptides (Carraway et al. 1984), while light-microscopic observations indicated that it could proceed without overt degranulation (Theoharides et al. 1985). The current study is concerned with the ultrastructural characteristics of mast cells during differential release.

Serotonin uptake and storage occurs in mast cells, platelets, enterochromaffin cells and serotonergic neurons and is mediated by a specific two-step transport mechanism (Rudnick 1987): a) at the plasma membrane for co-uptake of serotonin with Na^+ and Cl^- and countertransport of K^+, inhibited by tricyclic drugs such as imipramine; b) at the perigranular membrane due to an H^+ transmembrane potential for transport of serotonin and other biogenic amines, inhibited by reserpine.

Stimulation of serotonergic neurons leads to a non-cytotoxic Ca^{++}-dependent parallel release of serotonin and serotonin-binding proteins, of which two forms have been identified in rodent mast cells and may participate in the differential transport of serotonin (Tamir et al. 1982). One of these is a glycoprotein associated with cell membranes, while the other appears to be free in the granule core.

Differential release could occur by a vesicular shuttle between storage granules and plasma membrane, as shown in guinea-pig basophils (Dvorak et al. 1980), or could be mediated by specific transport pumps (Rothman 1985). These considerations prompted the investigation of the ultrastructural changes associated with dif-

ferential release of serotonin from rat peritoneal mast cells activated in vitro by C48/80, followed pretreatment with amitriptyline.

Materials and methods

Mast cell collection and purification

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing about 400 g were sacrificied in a CO_2 atmosphere generated by dry ice and were guillotined. Fifteen milliliters of Locke's solution, pH 7.2, buffered with 10 mM HEPES (Theoharides and Douglas 1978), were injected intraperitoneally and the peritoneal cavity was massaged for 90 s. Peritoneal fluid was then withdrawn and collected in plastic tubes (polypropylene tubes #20053, Falcon Plastics, Oxnard, CA). After collection, cells from a total of 10 rats were mixed, washed once by centrifugation at $180 \times g$ for 5 min and resuspended in Locke's solution to a final volume of 10 ml.

Mast cells were purified using 24% Metrizamide (Sigma, St. Louis, MO) as described previously (Theoharides et al. 1982). Mast cell purity was generally greater than 90%, as assessed by toluidine blue (0.25%, pH 2.3) staining and viability was generally greater than 95%, as judged by trypan blue exclusion.

Drug incubation for differential release

Purified mast cells collected from 10 rats were resuspended in Locke's solution after washing, and 0.2 ml of suspension containing about 10^5 mast cells was placed in each tube.

All tubes were placed in an ice bath and amitriptyline (Elavil, Siegfried-Ganes Chemical, Carlstadt, NJ) was added where indicated to give a final drug concentration of 5×10^{-5} M (Tamir et al. 1982). The tubes were then incubated for 10 min at 37° C with agitation in a water bath. The mast cell secretagogue, C48/80 (Sigma, St. Louis, MO), was added to certain tubes for a final concentration of 0.1 μg/ml. Each experiment included control samples treated with amitriptyline alone or tubes that had not been treated with either amitriptyline or C48/80. Mast cells were handled as described in each of the conditions listed below.

Serotonin release

Prior to drug treatment or exposure to C48/80, samples were incubated with ^{3}H-serotonin (^{3}H-5-hydroxytryptamine binoxalate, New England Nuclear, Boston, MA) as 20 μCi ^{3}H-serotonin/10^6 cells in 5 ml Locke's solution for 60 min at 37° C with agitation in a water bath. Cells were then washed twice by centrifugation ($180 \times g$ for 5 min) and resuspended in 1.2 ml Locke's solution. Aliquots of cell suspension (0.2 ml, containing 10^5 cells) were placed in polypropylene tubes for pretreatment with amitriptyline and stimulation with C48/80. Following the final incubation period, cells were pelleted by centrifugation as before and the supernatant fluid removed and added to 3 ml Hydrofluor (New England Nuclear). A 0.5 ml aliquot of 0.1% Triton X-100 (New England Nuclear) was added to solubilize the cell pellet, which was mixed in a vortex and added to 3 ml of Hydrofluor; all samples were counted in a scintillation counter. Serotonin release was expressed as percent of total cpm: i.e., cpm in the supernatant $\times 100$ over cpm in the supernatant + cpm in the pellet.

Histamine release

Following incubation, samples to be analyzed for histamine release were centrifuged and the supernatant fluid separated from the cell pellets. Cold Locke's solution was added to the supernatants and the cell pellets to a final volume of 1 ml. Perchloric acid (Sigma, St. Louis, MO) was then added (0.5 ml of a 40% solution, final concentration 2%) to the samples which were frozen at −20° C until they were analyzed for histamine by the fluorometric assay, as described previously (Theoharides et al. 1980). Histamine release was expressed as percent of total: i.e. supernatant/(supernatant + pellet) $\times 100$.

Electron microscopy

Biochemical determination of mediator release and electron microscopy were performed on separate aliquots of purified mast cells. Mast cells intended for ultrastructural analysis were fixed in suspension by addition of 0.2 ml of Karnovsky's modified fixative (Karnovsky 1965) containing 2% paraformaldehyde (Fisher Scientific Co.), 2.5% glutaraldehyde (EM Sciences), 6.8% sucrose (Fisher Scientific Co., Pittsburgh, PA) and 0.2 M sodium cacodylate buffer, pH 7.2 (EM Sciences, Fort Washington, PA). Erythrocytes from the same rats as above were added to some samples as a marker for the cell pellet following centrifugation. Cells were fixed in suspension for 1 h at room temperature, transferred to plastic Eppendorf tubes (0.6 ml) and centrifuged at 17000 rpm for 30 sec in an Eppendorf microfuge (Model #5414). Pelleted cells were overlaid with 0.1 M sodium cacodylate buffer (pH 7.2) and allowed to stand for 24 h at 4° C.

The following day, cell samples were osmicated with 1% OsO_4 (Polysciences, Wallington, PA) in 0.1 M sodium cacodylate buffer, pH 7.4, and stained en bloc with 2% uranyl oxalate (Fisher Scientific Co.); they were then washed and overlaid by 0.1 M s-collidine buffer, pH 7.2 (EM Sciences) and dehydrated in graded ethyl alcohol solution. Samples were then embedded in Epon 812 (EM Sciences).

Sections 1 μm thick were stained with Richardson's stain for orientation (Richardson et al. 1960). Thin (60 nm) sections were stained with 2% uranyl oxalate (20 min) and lead citrate (3 min) and were examined with a Zeiss EM 10B electron microscope.

Ruthenium red staining

Ruthenium red staining of rat mast cells was done during secretion in vitro (Lagunoff 1972). A 10 μl aliquot of a freshly prepared stock solution of 0.5% ruthenium red (EM Sciences) in a balanced salt solution (0.15 M NaCl, 0.01 M KCl, 0.1 M $CaCl_2H_2O$, 0.057 M NA_2HPO_4, and 0.37 M K_2HPO_4) was added to the cell samples after preincubation with amitriptyline and prior to stimulation with C48/80. Final concentration of ruthenium red was 0.005%. The release reaction was stopped by the addition of fixative as outlined above for electron microscopy for 1 h at room temperature. The cells were then pelleted and overlaid with fresh solution of 0.1 M sodium cacodylate buffer containing 1% paraformaldehyde, 1.25% glutaraldehyde, 3.4% sucrose and 0.75 mg/ml ruthenium red, for 5 h.

Following several cacodylate buffer rinses, the pellets were overlaid with a solution of 2% OsO_4 in 0.1 M s-collidine buffer containing 0.75 mg/ml ruthenium red and post-fixed for 2 h. Pellets were then rinsed several times in s-collidine buffer and stained with uranyl oxalate for 2 h. The pellets were dehydrated in graded ethyl alcohol solutions and embedded in Epon. Thin sections were stained and examined as described above for electron microscopy. Electron micrographs of thin sections were enlarged to $\times 25000$ and were evaluated for the total number of vesicles, as well as the percentage of stained vesicles.

Morphometric evaluation

Electron micrographs enlarged to a final magnification of $\times 12500$ were used for morphometric analysis of surface activity, total cell area, net cytoplasmic area (minus nucleus and secretory granules), and nuclear area by means of the point counting technique (Weibel 1973; Kashgarian et al. 1980). Granule alterations were assessed and the number of secretory granules and vesicles per cell were counted.

Analysis of cellular components. The analysis of cellular components was done with a Zeiss Videoplan image analysis system by use of a YX Class X, ser 4.D1, K52D program.

Cell area. Cell area was determined by the formula:

$$A_V = d^2 P_T$$

where d is the distance between points on the curvilinear grid (adjusted for magnification) and P_T is the number of points within the plasma membrane.

Granule size. Granule size was estimated by dividing the total granule area by the number of granules.

Nuclear area. The area of the nucleus was determined by dividing the number of points within the nucleus divided by those in the whole cell and was expressed as a percentage of the total cell area.

Cytoplasmic area. The area of the cytoplasm was determined by adding the total area occupied by granules and the area occupied by the nucleus and subtracting from the total cell area, in the same manner as the nuclear area above. The perimeters of the cells were determined by the equation:

$$B_L = I_i \times d,$$

where I_i is the number of intersections of the grid with the plasma membrane (Weibel 1973).

Surface activity. Surface activity was determined by calculation of the surface density (Sv) or the μm^2 of plasma membrane per μm^3 cell volume, and was calculated from the following formula:

$$Sv = 4/\pi \times I_i/d \times P_T,$$

where d is the distance between the lines on the curvilinear grid (adjusted for magnification), I_i is the number of intersects with the plasma membrane, and P_T, the number of points within the cell (Weibel 1973; Kashgarian et al. 1980).

Golgi complex analysis. Analysis of the Golgi complex was done on a set of electron micrographs enlarged to a magnification of $\times 30000$. Cell area was determined by planimetry. The surface area of the Golgi was calculated according to the formula:

$$Sv_{Golgi} = 4/\pi \times I_i/d \times P_T,$$

where I_i is the number of intersections of the curvilinear grid with the membrane stacks and P_T is the number of points covered by the area of the entire electron micrograph. This value was divided by the total cell area to give the surface area of the Golgi apparatus per μm^2 cell area.

Cytosolic area. Net cytosolic area was derived by subtracting the area of the nucleus and the collective area of all granules from the total cell area. The number of vesicles was adjusted to the measured net cytoplasmic area (Dvorak et al. 1980).

Autoradiography

Samples of cells were incubated with ^{3}H-serotonin (1 μCi per 10^6 cells) for 45 min, washed, preincubated with amitriptyline and stimulated with C48/80 as described above. Release was stopped by the addition of fixative and cell pellets were fixed, osmicated, dehydrated and embedded in Epon, as was done for electron microscopy (Tamir et al. 1982). Sections of cell pellets were then processed for electron-microscopic autoradiography with Ilford L4 emulsion (Polysciences, Wallington, PA). Following development with D19, micrographs of samples were enlarged to $\times 15000$ for analysis.

Quantitation of grain density of cells. Further analysis of autoradiographs was done by photographing areas of the cell cytoplasm at $\times 25000$ and blindly evaluating the location of each grain in the electron micrograph with respect to its association with granules and vesicles, or over areas of the cytosol by use of probability circles with a radius of 1.5 times the half distance (HD: the distance from a radioactive line within which 50% of the developed silver grains fall). This was sometimes a difficult process since the vesicles were 10–100 nm in diameter and the radiographic grains were sometimes quite large. The HD used in localization of grains to the vesicles was between 120–150 nm.

Statistical analysis

Statistical analysis for significant differences among groups was performed by the Student's *t*-test. The Mann Whitney U test for nonparametric data was used to analyze the data in Tables 3 and 5.

Results

Biochemical analysis of mast cells undergoing differential release of serotonin

Mast cells pretreated with amitriptyline before stimulation with C48/80 released more serotonin than histamine, in keeping with previous findings (Theoharides et al. 1980, 1985).

Granule changes in mast cells undergoing differential release of serotonin

Extensive morphometric analysis of mast cells was undertaken to determine granular, cytoplasmic and surface changes during differential release. Control mast cells (Fig. 1A) showed normal morphology, while mast cells treated with amitriptyline alone showed only minor granule changes (Fig. 1B, *arrowhead*). In contrast, those cells incubated with C48/80 alone displayed morphological changes indicating degranulation by compound sequential exocytosis (Fig. 1C). These included granule swelling, loss of granule density, fusion of granules and formation of large exocytotic cavities that contained swollen granule matrices of decreased density (Fig. 1C, *asterisk*). Finally, those mast cells pretreated with amitriptyline and then stimulated with C48/80 contained granules with very little swelling and loss of density, but which were more angular and filled with material which had lost its homogeneity (Fig. 1D, *arrowheads*). Moreover, such granules had many protruding, knob-like extensions (compare Fig. 2A, control, and 2B, *arrow*, treated), which were often surrounded by clear spaces and appeared to be in communication with numerous vesicular structures (*v*) of varying sizes containing berry-like, heterogeneous electron-dense material (Fig. 2B, *arrowheads*). Some of these altered granules appeared to have fused together, which may account for the apparent increase in granule size without loss of density (Fig. 2B; Table 1, column 3). Virtually all of the cells within any given field from samples treated with amitriptyline followed by C48/80 showed some

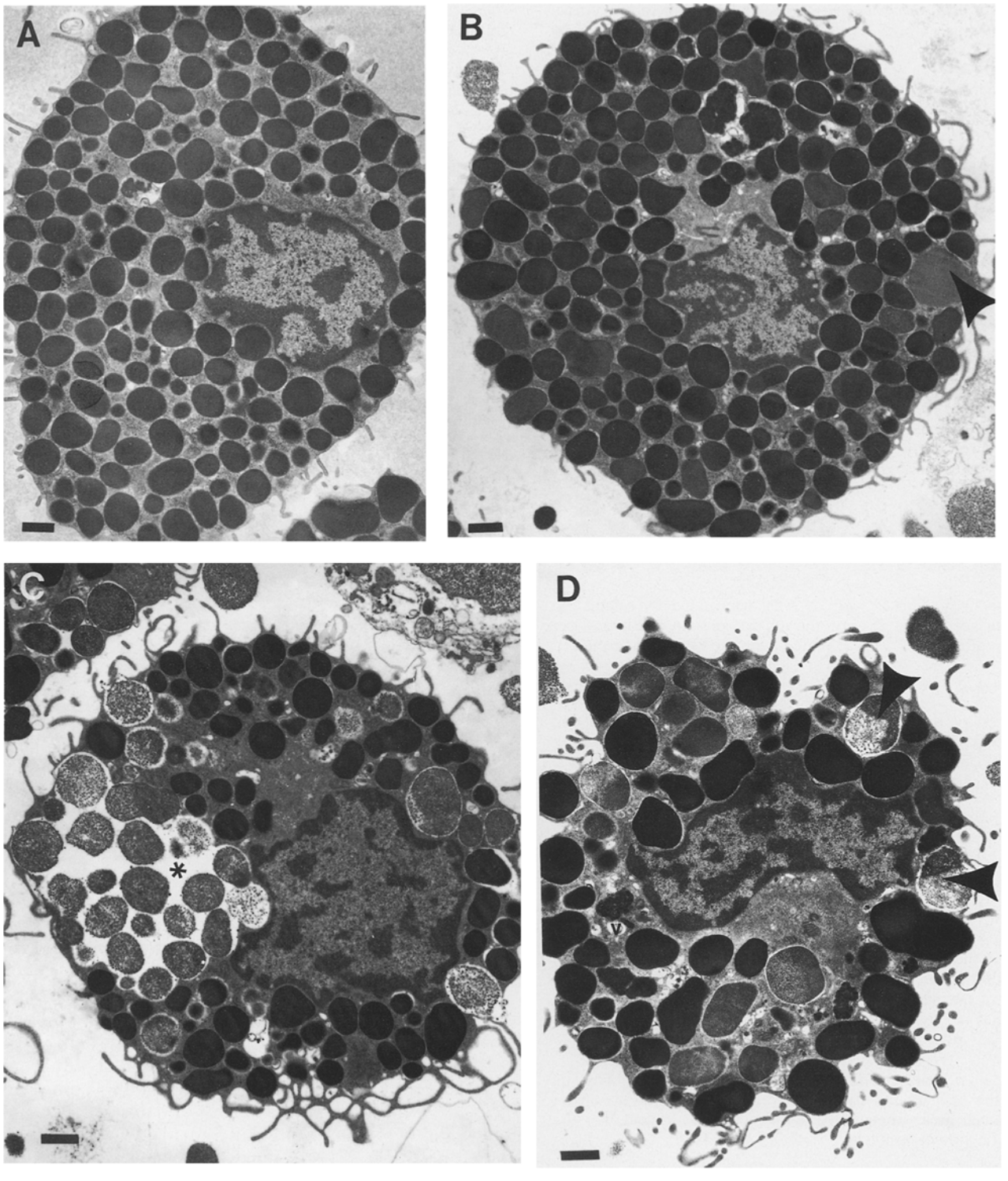

Fig. 1. A Electron micrograph of control mast cell. Note dense, spherical granules of uniform size, with very few mitochondria, vesicles, or Golgi elements and short filopodia. ×7500. *Bar* = 1 μm. **B** Mast cell pretreated with amitriptyline alone. Note large irregular granules with low density (*arrowhead*). ×7500. *Bar* = 1 μm. **C** Mast cell stimulated by C48/80. Many cytoplasmic granules are swollen and of lower density. Note large exocytotic cavity at left (*asterisk*), the result of fusion of perigranular membranes of numerous granules with each other and plasmalemma. Also note activated cell surface. ×7500. *Bar* = 1 μm. **D** Mast cell pretreated with amitriptyline, followed by C48/80. Highly activated cell surface with most filopodia in cross section. Note granules with low density heterogeneous content (*arrowheads*) and cytoplasmic vesicles (*v*). ×7500. *Bar* = 1 μm

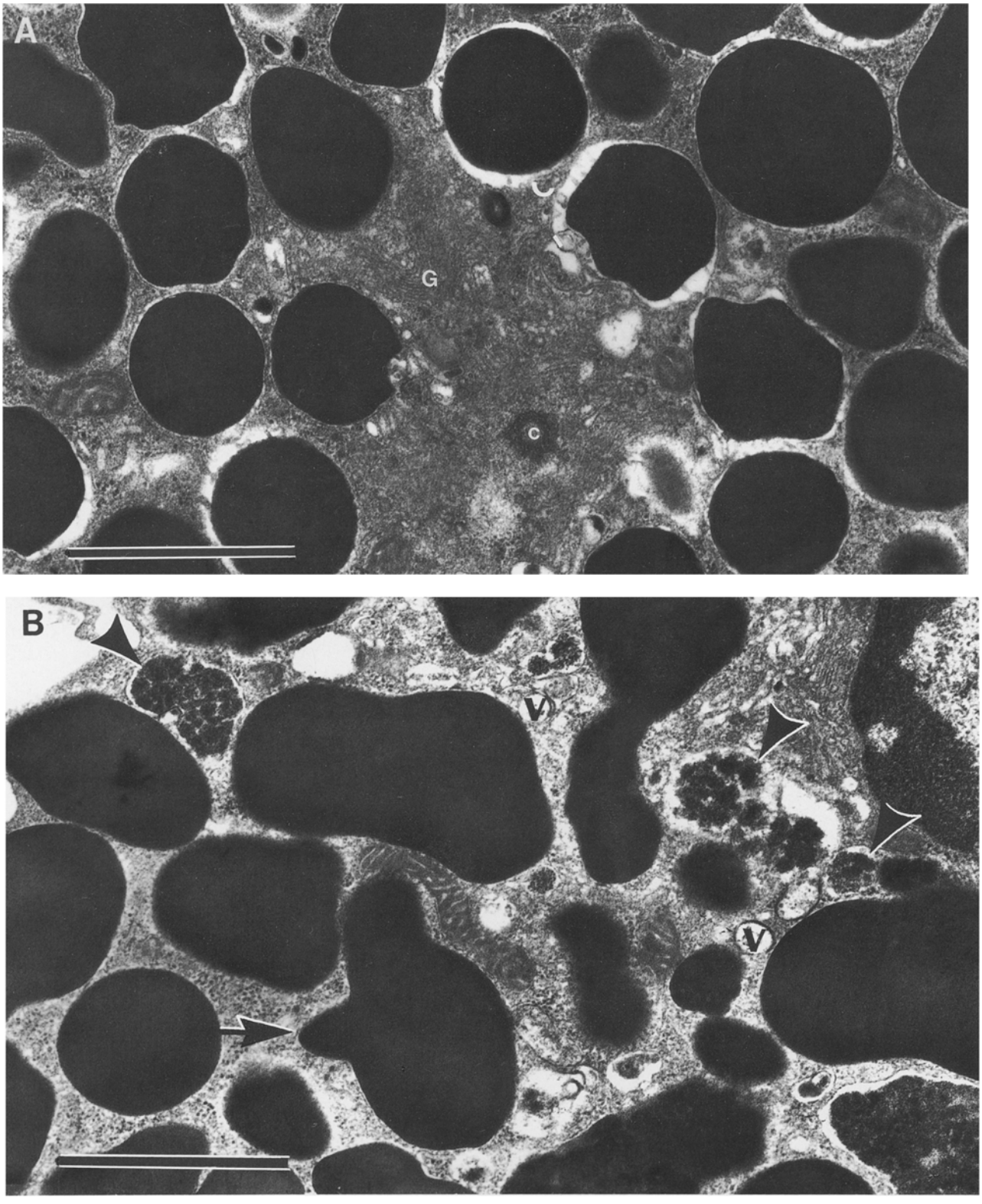

Fig. 2. **A** Part of control mast cell showing spherical granules of uniform shape, size and density. Note Golgi area (*G*) and prominent centriole (*c*). ×40000. *Bar* = 1 μm. **B** Part of mast cell pretreated with amitriptyline, followed by C48/80. Irregularly shaped granules with many knob-like protrusions (*arrow*). Electron-dense granule core forming bead-like material (*arrowhead*) associated with vesicles (*v*). ×40000. *Bar* = 1 μm

Table 1. Cytoplasmic granule and vesicle characteristics during differential secretion

Mast cell treatment	Total cell area (μm^2)	Net cytoplasmic area (μm^2)	Granule size (μm^2)	No. Vesicles/ Net total cytoplasmic area
Control	82.7 ± 3.7^a	32.1 ± 2.1	0.31 ± 2.0	0.9 ± 0.1
Compound 48/80	63.6 ± 4.4^b	22.1 ± 5.9^b	0.50 ± 0.0^b	1.9 ± 0.6^c
Amitriptyline	73.9 ± 4.7	26.3 ± 2.5	0.31 ± 0.0	$1.2 \pm 0.2^{d,e}$
Amitriptyline + C48/80	72.2 ± 3.9^c	22.7 ± 2.5^b	0.52 ± 0.1^b	2.2 ± 0.5^b

[a] Mean ± S.E., $n=15$ per treatment group from two experiments; [b] $p<0.01$ vs. control group; [c] $p<0.1$ vs. control group; [d] $p<0.05$ vs. control group; [e] $p<0.05$ vs. group treated with amitriptyline + compound 48/80

Table 2. Mast cell granule changes during differential secretion[a]

Mast cell treatment (n)[b]	Normal	Decreased density	Altered shape
Control (25)	93.4	2.5	4.1
Compound 48/80 (25)	59.4	34.5	6.1
Amitriptyline (25)	67.3	17.8	14.9
Amitriptyline + C48/80 (25)	46.8	9.0	44.2

[a] % of total
[b] (n) = number of cells examined

granule changes, even though no more than 50% of all the granules had altered shapes (Table 2). Moreover, only 9% of the granules in this latter group had decreased density as compared to about 35% in the group treated with C48/80 alone (Table 2).

In mast cells treated with C48/80 alone, 37% of granules showed a loss of density, while only 3% had any changes in shape. Conversely, in mast cells undergoing differential release only 9% of granules showed loss of density while 37% had altered form.

Cytoplasmic changes in mast cells undergoing differential release of serotonin

Morphometric analysis of C48/80-activated mast cells showed a decrease in both total cell area and net cytoplasmic area compared to unstimulated control mast cells (Table 1). In contrast, differential serotonin release was accompanied by negligible loss of cell area, but a significant increase in granule size and loss of net cytoplasmic area (Table 1).

The surface area of the membranes associated with the Golgi apparatus in mast cells undergoing differential release was $1.05 \pm \times 10^{-4}$ $\mu m^2/\mu m^3$ compared with $0.7 \pm 0.4 \times 10^{-4}$ $\mu m^2/\mu m^3$ in control cells ($p<0.05$). The other experimental groups, C48/80 alone and amitriptyline alone, showed no change in the Golgi area compared to control cells and there did not appear to be any difference in the number of Golgi-associated vesicles (results not shown).

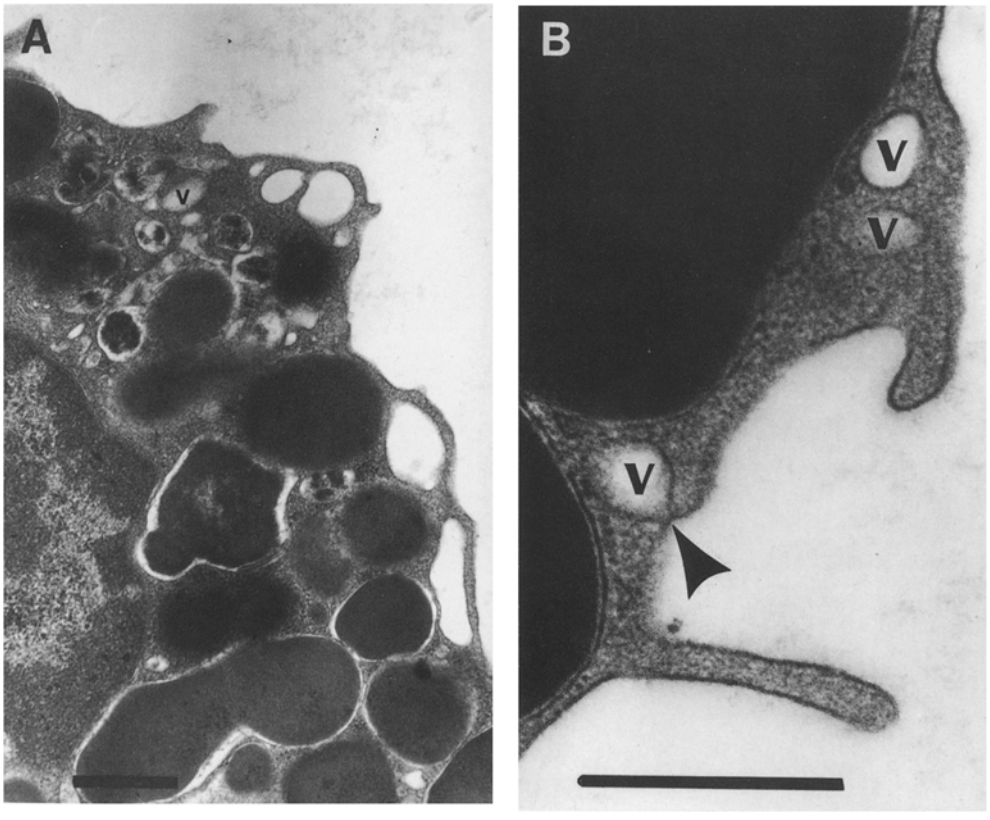

Fig. 3. A High magnification of mast cell pretreated with amitriptyline, followed by C48/80. Note extensive filopodia and cytoplasmic vesicles (*v*). ×25000. *Bar* = 1 μm. **B** Higher magnification of mast cell pretreated with amitriptyline, followed by C48/80. Note surface-associated vesicles (*v*), one of which has the "omega" shape (*arrowhead*), indicative of exocytosis. ×75000. *Bar* = 1 μm

Cytoplasmic vesicles in mast cells undergoing differential release of serotonin

Cytoplasmic vesicles (0.1–0.01 μm diameter) were increased in all experimental samples (i.e., those treated with C48/80 alone or with amitriptyline alone, as well as samples treated with amitriptyline followed by C48/80. This increase was, however, maximal in the sample undergoing differential secretion (Table 1, column 4). Vesicles in cells undergoing differential release were localized near secretory granules and occasionally appeared to arise by budding off from them (Fig. 2B, *arrow*). Vesicles were also seen in association with the plasma membrane (Fig. 3A, B) and, in rare instances, these vesicles were captured in the process of forming an "omega" shape as they were fusing with the plasma membrane (3B, *arrowhead*).

Table 3. Surface area activation during differential secretion[a]

Mast cell treatment	Cell perimeter[b] (μm)	Cell area[c] (μm^2)	Surface clensity (S_v)[d]
Control	51.3±1.7	72.5±2.5	0.9±0.1
Compound 48/80	71.7±2.8	63.0±3.5	1.6±0.1[e]
Amitriptyline	60.4±2.3	77.6±3.0	1.1±0.1
Amitriptyline +C48/80	66.0±2.4	67.5±2.5	1.3±0.1[e]

[a] Data were calculated using the point counting technique. The combined results of two experiments are presented. Total number of mast cells examined per treatment group was 85. Numbers represent the mean±S.E.; [b] Cell perimeter $= I_i d$ where I_i is the number of lines of the curvilinear grid intersecting the cell perimeter and d is the distance between the lines of the grid; [c] Cell area $= P_T d^2$ where P_T is the number of points contained within the cell; [d] $S_v = 4/I_i d \times P_T$ to give μm^2 plasma membrane per μm^3 cell volume; [e] $p <$ 0.001 vs. control group

Surface changes in mast cells undergoing differential release of serotonin

Cells stimulated by C48/80 alone showed a significant increase in surface plasma membrane (Table 3). This was due to: a) an increase in the number of filopodia extending from the cell; and b) the formation of multiple membranous cavities (Fig. 1C). Mast cells treated with amitriptyline, followed by activation with C48/80, showed a similar increase in surface activity (Table 2, Fig. 1D). This was due primarily to an increase in cell perimeter caused by an increased number of filopodia, rather than due to the formation of membranous exocytotic cavities. Thus, differential mast-cell release of serotonin was accompanied by surface activation without degranulation.

Autoradiographic localization of serotonin in mast cells undergoing differential release

Autoradiographs of control samples showed localization of silver grains over cytoplasmic granules (Fig. 4); occasional grains found over the nucleus and extracellularly were considered to be background (results not shown). Calculation of the grain density of autoradiographs from each treatment group demonstrated that about 50% of serotonin was released in the samples stimulated with C48/80 alone, as well as in those pretreated with amitriptyline prior to C48/80 (Table 4). These results were consistent with the biochemical data.

In cells undergoing differential release, 22% of autoradiographic grains remaining in the cells appeared not to be associated with secretory granules, but rather with small cytoplasmic vesicles (Fig. 4, Table 4). The similar size of the vesicles and of the grains precludes, however, any definitive statement as to whether the grains were localized over the vesicular structures. This population

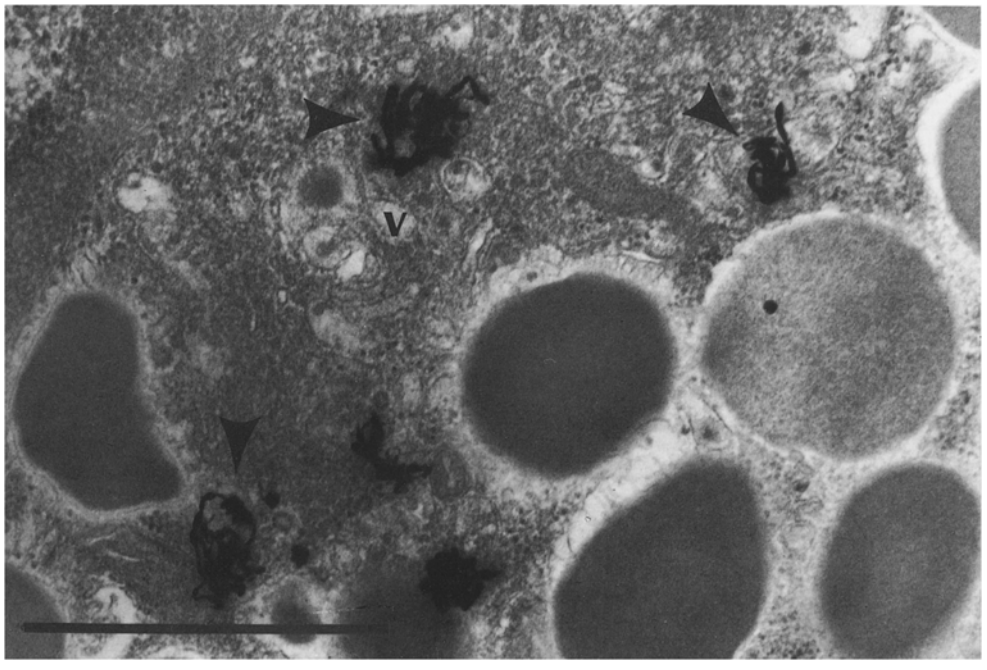

Fig. 4. Autoradiograph of mast cell pretreated with amitriptyline, followed by C48/80. Note silver grains associated with vesicular structures (*arrowhead*). ×60000. *Bar* = 1 μm

Table 4. Localization of ^{3}H-serotonin during differential secretion

Treatment (n)[a]	Total grains localized	Grains over granules[b]	Grains over vesicles[b]
Control (20)	736	659 (90%)	77 (11%)
C48/80 (10)	160	126 (79%)	34 (21%)
Amitriptyline (10)	367	330 (90%)	37 (10%)
Amitriptyline +C48/80 (20)	441	343 (78%)	98 (22%)[c]

[a] (n) = number of cells evaluated per group; [b] Numbers in parentheses represent percent of total; [c] $p < 0.05$ level of significance for the difference between controls and amitriptyline + C48/80 as determined by the Mann-Whitney U-test

Table 5. Ruthenium red staining during differential secretion

Experiment group	No. of vesicles present[a]	% Stained vesicles (endocytosis)	% Unstained vesicles (exocytosis)
Control	0.6 ± 0.3	20%	80%
C48/80	0.8 ± 0.9	10%	90%
Amitriptyline	0.5 ± 0.4	20%	80%
Amitriptyline +C48/80	1.1 ± 0.8[b]	4%	96%

[a] Number of vesicles ± S.E. per μm^2 of peripheral cytoplasmic area, as determined by counting vesicles only within 2 μm of the plasmalemma (n = 20 per treatment group); [b] $p < 0.05$ level of significance for difference between controls and amitriptyline + C48/80

of grains represents a residual amount of serotonin that has left the granules, but has not been released within 5 min of incubation with C48/80.

Staining of plasma membrane with the extracellular marker ruthenium red

Cells from samples undergoing compound sequential exocytosis in response to C48/80 alone demonstrated staining of plasma-membrane exocytotic cavities and secretory granules exposed to the extracellular compartment. In contrast, cells from samples undergoing differential secretion had numerous membrane-associated vesicles most of which (96%) did not stain and thus were clearly of intracellular origin (Table 5).

Discussion

Studies of neurons and chromaffin cells of the adrenal medulla have shown that substances co-stored within one compartment are secreted simultaneously in the same predetermined ratio as they are stored in that compartment (Viveros et al. 1983).

Rat peritoneal mast cells activated by C48/80 in the presence of amitriptyline undergo differential release of serotonin without a comparable release of histamine (Theoharides et al. 1980; Tamir et al. 1982; Carraway et al. 1984; Theoharides et al. 1985). Serotonin and histamine are stored together in mast-cell granules (Csaba 1971), but they are present in unequal amounts (histamine : serotonin = 10 : 1) and this ratio is retained during secretion by exocytosis. In differential mast-cell release, the ratio is altered such that the percentage of total serotonin that is released remains the same, while the percentage of total histamine released is reduced, thus bringing the amount of serotonin molecules released closer to the number of molecules of histamine released. Differential or selective serotonin release may, therefore, involve sequestration of serotonin or serotonin-binding proteins into a separate compartment (Tamir et al. 1982), possibly present in the cytoplasm, as suggested by previous investigators (Carlsson and Ritzen 1969; Gustaffson and Enerbäck 1980). Sequestration of the secreted substances in separate compartments from which they may be secreted in response to different stimuli has been suggested also for other systems (Vindrola et al. 1988; Benyon et al. 1989).

One report suggested that amitriptyline (10^{-4} M) pretreatment inhibits the release of both histamine and serotonin (Berlin and Enerbäck 1986). However, none of the conditions used would have allowed differential secretion to be observed since serotonin was injected subcutaneously, the isolated cells were preincubated with 10^{-4} M amitriptyline at room temperature and the mast cells used per ml were 10 times more cells than optimal (Berlin and Enerbäck 1986). Differential release has been confirmed independently (Carraway et al. 1984), has been shown also to occur with progesterone alone (Vliagoftis et al. 1989), and to be able to discriminate between prestored and de novo secreted products (Benyon et al. 1989).

Differential release is characterized by intracellular granule fusion and alterations in shape, which are somewhat reminiscent of the granule fusion observed in rat peritoneal mast cells exposed to polylysine (Padawer 1970). This is in contrast to the swelling and loss of granule density seen when mast cells degranulate under the influence of C48/80 alone and may involve inhibition of perigranular membrane fusion with the plasmalemma, normally seen when the cells are stimulated with C48/80 or with IgE (Röhlich et al. 1971; Lagunoff 1973; Cochrane and Douglas 1974; Lawson et al. 1977).

It has been proposed that granule volume may be regulated by the fusion of unit-sized granules into dumbbell or pear-shaped structures which then evolve into larger spherical granules (Hammel et al. 1983). The fusion of cytoplasmic granules into irregular shapes and subsequent remolding of the matrix into spherical forms described here could involve a reduction of excess perigranular membrane which may give rise to the numerous small cytoplasmic vesicles noted. Vesicle fusion with the plasma membrane would account for the increased surface area and localization of vesicles close to the plasma membrane.

The total number of vesicles and the percentage of autoradiographic grains associated with vesicles was similar in C48/80-treated and amitriptyline plus C48/80-treated cell groups. Localization of autoradiographic silver grains over vesicles smaller than 0.01–0.1 μm could

not be established because of the large size of the grains. Consequently, it is not certain whether the smaller cytoplasmic vesicles played any role in the differential release process. Differential release may, therefore, be viewed as an inhibited form of classical exocytosis in which serotonin release results from either exocytosis of small transport vesicles from which histamine is excluded or transmembranous cytoplasmic release. This latter process has been postulated for small molecules (Rothman 1985).

There are a number of examples where cells have the ability to secrete molecules through mechanisms other than classic granule exocytosis. For instance, vesicular transport of mediators has been suggested for basophils (Dvorak et al. 1980), as well as in cutaneous T cell-dependent delayed-type hypersensitivity reactions in mice (Kops et al. 1984; Van Loveren et al. 1984) in the absence of overt degranulation. Ichikawa et al. (1977) showed that serotonin, but not histamine, is released from normal and neoplastic mast cells treated with N-(2-ethylhexyl)-3-hydroxybutyramide and catecholamine. Secretion of granule-stored substances without degranulation has also been observed by Woodbury et al. (1984) in the case of protease release by mucosal mast cells in *N. brasilienis*-infected rats and in the secretion of low and high molecular weight enkephalin (Vindrola et al. 1988).

Mast-cell release of serotonin without histamine is induced by a variety of drugs which are tricyclic in structure (Theoharides et al. 1985) and some of these have also been shown to inhibit histamine release from human basophils (Lichtenstein and Gillespie 1975). The mechanism by which these agents permit serotonin release while inhibiting that of histamine is not known. A recent study using the calcium indicator Quinn 2 showed that the tricyclic antidepressant amitriptyline causes an immediate decrease in cytoplasmic calcium ions (Theoharides and Kotkow 1986), which could lead to inhibition of granule exocytosis, while still permitting vesicular transport or transmembrane release of serotonin. Finally, selective sorting and transport of secretory and membrane proteins has also been shown to involve distinct compartments in the secretory pathway (Moore and Kelly 1985) and "individually recruitable" secretory pools depending on the maturity of the molecule to be secreted (Morrissey and Cohn 1979).

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References

Benyon R, Robinson C, Church MK (1989) Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli. Br J Pharmacol 97:892–904

Berlin G, Enerbäck L (1986) Non-differential inhibition of histamine and serotonin release from mast cells by amitriptyline. Agents Actions 18:89–91

Carlsson SA, Ritzen M (1969) Mast cells and 5-HT. Intracellular release of 5-hydroxytryptamine (5-HT) from storage granules during anaphylaxis or treatment with compound 48/80. Acta Physiol Scand 77:449–464

Carraway RE, Cochrane DE, Granier C, Kitabgi P, Leemman E, Singer EA (1984) Parallel secretion of endogenous 5-hydroxytryptamine and histamine from mast cells stimulated by vasoactive peptides and compound 48/80. Br J Pharmacol 81:227–229

Cochrane DE, Douglas WW (1974) Calcium-induced extrusion of secretory granules (exocytosis) in mast cells exposed to 48/80 or the ionophores A_{23187} and X_{537A}. Proc Nat Acad Sci USA 71:408–412

Csaba G (1971) Mechanism and function of mast cell granules. Acad Sci Hung 22:155–168

Dvorak AM, Hammond ME, Morgan E, Orenstein NS, Galli SJ, Dvorak HF (1980) Evidence for a vesicular transport mechanism in guinea pig basophilic leukocytes. Lab Invest 42:263–276

Gustaffson B, Enerbäck L (1980) A cytofluorometric analysis of polymer-induced mast cell secretion. Exp Cell Biol 48:15–30

Hammel I, Lagunoff D, Bauza M, Chi E (1983) Periodic, multimodal distribution of granule volumes in mast cells. Cell Tissue Res 228:51–59

Ichikawa A, Kanedo H, Mon V, Tomita K (1975) Release of serotonin from mast cells induced by N-(2)-ethylhexyl-3-hydroxybutramide and catecholamine. Biochem Pharmacol 26:197–202

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27:137A

Kashgarian M, Taylor CR, Binder HJ, Hayslett JP (1980) Amplification of cell membrane surface in potassium adaptation. Lab Invest 42:581–588

Kraeuter Kops SK, Van Loveren H, Rosenstein RW, Ptak W, Askenase PW (1984) Mast cell activation and vascular alterations in immediate hypersensitivity-like reactions induced by a T cell derived antigen binding factor. Lab Invest 50:421–434

Lagunoff D (1972) Vital staining of mast cells wtih ruthenium red. J Histochem Cytochem 20:938–944

Lagunoff D (1973) Membrane fusion during mast cell secretion. J Cell Biol 57:252–259

Lawson D, Raff MC, Gomperts B, Fewtrell C, Gilula NB (1977) Molecular events during membrane fusion: A study of exocytosis in rat peritoneal mast cells. J Cell Biol 72:242–259

Lichentstein LM, Gillespie E (1975) The effects of the H_1 and H_2 antihistamines on "allergic" histamine release and its inhibition by histamine. J Pharmacol Exp Ther 192:441–450

Moore HH, Kelly RB (1985) Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. J Cell Biol 101:1773–1781

Morrissey JJ, Cohn DV (1979) Regulation of secretion of parathyroid hormone and secretory protein I from separate intracellular pools by calcium, dibutyryl cyclic-AMP and isoproterenol. J Cell Biol 82:93–102

Padawer J (1970) The reaction of rat mast cells to polylysine. J Cell Biol 47:352–372

Richardson KL, Jarret L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313–323

Röhlich P, Anderson P, Uvnäs B (1971) Electron microscope observations on compound 48/80-induced degranulation in rat mast cells. J Cell Biol 51:465–483

Rothman SS (1985) Nonvesicular transport. In: Ho JJL (ed) Protein Secretion: A critical analysis of the vesicular model. John Wiley and Sons, New York, pp 205–223

Rudnick G (1987) Platelet responses and metabolism. In: Holmsen H (ed) Receptors and metabolism, vol. 2. CRC Press, Boca Raton, FL, pp 119–133

Tamir H, Theoharides TC, Gershon MD, Askenase PW (1982)

Serotonin storage pools in basophil leukemia and mast cells: Characterization of two types of serotonin binding protein and radio-autographic analysis of the intracellular distribution of ^{3}H-serotonin. J Cell Biol 93:638–647

Theoharides TC, Douglas WW (1978) Somatostatin induces histamine secretion from rat peritoneal mast cells. Endocrinology 102:1637–1640

Theoharides TC, Kotkow KJ (1986) Calcium ion levels during differential mast cell secretion. FASEB 45:387

Theoharides TC, Bondy PK, Tsakalos ND, Askenase PW (1980) Differential release of serotonin and histamine from mast cells. Nature 297:229–231

Theoharides TC, Kraeuter-Kops S, Bondy PK, Askenase PW (1985) Differential release of serotonin without comparable histamine under diverse conditions in the rat mast cell. Biochem Pharmacol 34:1289–1398

Van Loveren H, Kraeuter-Kops SK, Askenase PW (1984) Different mechanisms of release of vasoactive amines by mast cells occur in T-cell dependent compared to IgE-dependent cutaneous hypersensitivity response. Eur J Immunol 14:40–47

Vindrola O, Ase A, Finkeilman S, Nahmod VE (1988) Differential release of enkephalin and enkephalin-containing peptides from perfused cat adrenal glands. J Neurochem 50:424–430

Viveros OH, Diliberto EJ, Daniels AJ (1983) Biochemical and functional evidence for the cosecretion of multiple messengers from single and multiple compartments. Fed Proc 42:2923–2928

Vliagoftis H, Dimitriadou V, Theoharides TC (1990) Progesterone triggers selective mast cell secretion of 5-hydroxytryptamine, Int Arch Allergy Appl Immunol (in press)

Weibel ER (1973) Stereological techniques for electron microscopy morphometry. In: Hayat MA (ed) Principles and techniques of electron microscopy: Biological applications, vol 3, pp 237–296

Woodbury RG, Miller HRP, Huntely JF, Newlands GFJ, Palliser AC, Wakelin D (1984) Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. Nature 312:450–452