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Selective internalization of granule membrane after secretion in mast cells

(membrane sorting/membrane recycling/covalent membrane marker/membrane composition)

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ABSTRACT **[^{3}H]Galactose, covalently bound to cell surface glycoconjugates of rat peritoneal mast cells, was used to study internalization of labeled plasma membrane and granule membrane constituents before or after secretion stimulated by compound 48/80. Internalized label was distinguished quantitatively from label on the cell surface by its inaccessibility to enzymatic removal. Three different situations were compared. (*i*) With label only on the plasma membrane, and in the absence of secretion, incubation at 37°C (but not at 0°C) resulted in a gradual decrease of label on the cell surface until, after ≈2 hr, a steady state was reached with 93% of all cell-bound label remaining on the cell surface. Recycling of internalized label was demonstrated. (*ii*) When cells were labeled on the plasma membrane and then stimulated to secrete, subsequent retrieval of (unlabeled) granule membrane did not affect the rate or extent of simultaneous internalization of labeled plasma membrane. (*iii*) When both plasma membrane and exposed granule membrane were labeled after secretion, subsequent incubation at 37°C (but not at 0°C) resulted in ≈33% of all cell-bound label becoming internalized during 4 hr, indicating additional internalization of label due to retrieval of labeled granule membrane. In all three cases, loss of label into the medium occurred with a half-life of 8–11 hr, showing that no extensive shedding of granule membrane occurred after secretion. The results suggest either that no mixing of labeled membrane constituents occurred between the plasma membrane and granule membrane or that during retrieval of granule membrane, sorting of membrane was taking place at the cell surface.**

Intracellular transport occurring between and by means of the various endomembrane organelles of the eukaryotic cell involves numerous and repeated fusion–fission events between these membranes (cf. ref. 1). In view of the high rate of lateral diffusion in the bilayer membrane, one can expect a rapid mixing of the constituents of the membranes in fusion. Nevertheless, the characteristic composition of each distinctive membrane compartment is maintained. This can be achieved by the cell in operating a mechanism that prevents randomization and/or by "sorting out" the mixed membrane components (cf. ref. 1).

The mast cell provides a cellular system in which fusion between two membrane compartments can be induced under controllable conditions (2–4). Under the influence of biological or artificial stimulants, these cells can be induced to secrete the contents of their numerous secretory granules into the extracellular medium. This process involves extensive fusion of granule membranes with the plasma membrane (5–8). Subsequently, this excess membrane is retrieved from the cell surface by an endocytosis-like process (5, 9–13).

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Two mechanisms by which the mast cell restores the original separation between granule membrane and plasma membrane are conceivable. A randomized part of the enlarged cell membrane can be internalized followed by a selective return of only plasma membrane constituents to the cell surface, or granule membrane inserted into the cell surface can be internalized selectively, leaving plasma membrane constituents in place. The aim of the present study is to determine which of these two mechanisms is operating during retrieval of granule membrane.

MATERIALS AND METHODS

Mast Cells. Peritoneal mast cells were collected by peritoneal lavage of exsanguinated adult male Wistar rats (SAIMR, Rietfontein, South Africa) with phosphate-buffered saline (pH 7.4)/0.9 mM Ca^{2+}/0.5 mM Mg^{2+} using 5 ml per rat three times. The peritoneal cells were pooled by mild centrifugation (200 × *g*; 10 min; 18°C). The mast cells were separated on a preformed Percoll gradient as described (14), using one gradient for cells from 10 rats. The mast cells were washed free from Percoll in two steps, each using 10 ml of conditioned phosphate-buffered saline, consisting of the buffer used for lavage, after removal of cells and filtering through a Millex-GS 0.22-μm filter (Millipore). An average of 0.6 × 10^6 mast cells per rat was obtained, with no detectable (light microscope) contamination by other peritoneal cells. For each experiment, 20 rats were used. The cells were kept strictly at 18°C throughout all procedures, until after secretion when they were cooled on ice.

Secretion with C48/80. A Falcon Petri dish (60 × 15 mm) was rinsed with 2 ml of conditioned phosphate-buffered saline. The mast cells were resuspended in 2 ml of conditioned phosphate-buffered saline at 18°C and transferred to the Petri dish. The cells were observed by phase-contrast microscopy at a magnification of ×200. After establishing that the cell population consisted of intact nonexocytic cells, the stimulant, compound 48/80 (Sigma), was added to a final concentration of 1 μg/ml during mild agitation by hand for ≈20 sec. Subsequently, the cells were continuously observed for a further 2 min to follow the secretory reaction. This was evident by a change from the sharply contrasted round boundary of nonexocytic cells to a diffuse halo. Simultaneously, secreted granules became visible as a grainy background. Staining with ruthenium red (Sigma) at a concentration of 0.01% selectively stained the exocytic cells (15), leaving the halo-like structure no longer visible (Fig. 1). The advantage of using a Petri dish during stimulation of the cells was that it allowed direct observation of the total population of cells in the sample to be used for further experimentation. For this reason, ruthenium red staining was used only with an aliquot of the cells for additional confirmation of the secretory process. After secretion, the cell suspension was transferred back into a test tube, cooled on ice and washed three times with 5 ml of phosphate-buffered saline (200 × *g*; 5 min).

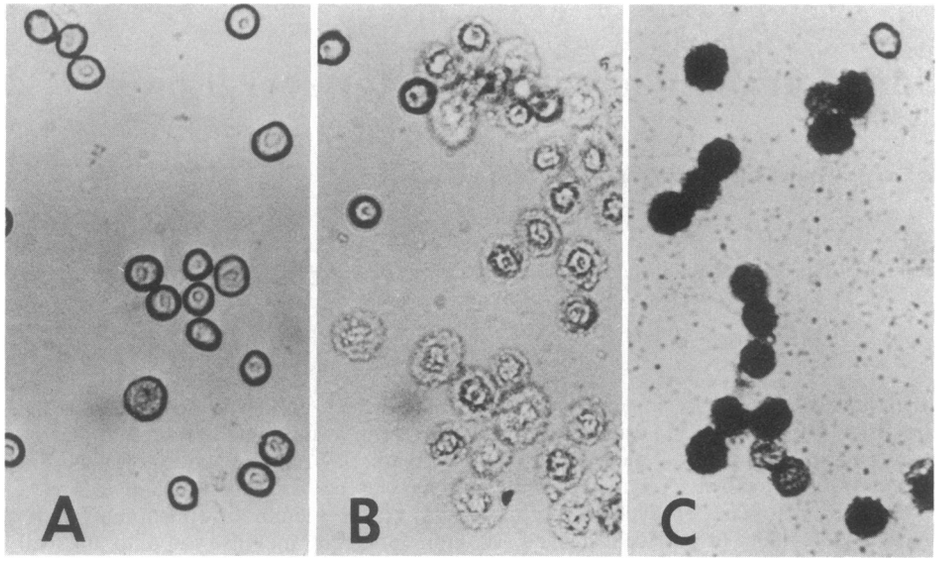

FIG. 1. Light micrographs of unfixed mast cells. (*A*) Nonexocytic cells before addition of C48/80. Cell boundary is characterized by a well-defined contour. (*B*) Exocytic cells after 2 min at 18°C in the presence of 1 μg of C48/80 per ml. Cell boundary is ill defined, resembling a faint halo. Note the few nonexocytic cells as in *A*. (*C*) Exocytic cells as in *B* after staining with ruthenium red at 0.01% for 5 min. The halo-like structure is no longer visible because of heavy staining. Note the grainy background due to secreted granules. Cells used in these micrographs represent a subpopulation of cells as used for one of the experiments represented in Fig. 2.

Labeling of the Cell Surface. Galactosyltransferase was used to bind radioactive galactose to terminal *N*-acetylglucosamine (GlnNAc) moieties on the cell surface. The labeling was done as described (16) with the following modifications. Because not enough labeling sites were available on the mast-cell surface, the cells were first treated with a mixture of neuraminidase and β-galactosidase (see ref. 16; this is also the reason why selective labeling of the plasma membrane against granule membrane using ^3H vs. ^{14}C was not feasible): 2–5 × 10^6 cells were resuspended in 1 ml of phosphate-buffered saline to which the enzyme mixture (a gift from Rudolf Weil, Sandoz, Vienna) was added at 0.25 unit per ml for 10 min. After three washings in 5 ml of phosphate-buffered saline, labeling was started by resuspending the cells in 250 μl of the final incubation mixture for 5 min, after which a further 250 μl was added for another 5 min. The cells were then washed in 5 ml of Hepes saline (10 mM Hepes, pH 7.4/140 mM NaCl/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$) followed by two washing steps in conditioned phosphate-buffered saline. The above procedure was done at 18°C and on ice for nonexocytic cells and exocytic cells, respectively. A 10-fold higher concentration of UDP[6-^3H]Gal was used in the incubation mixture than that described previously (16). The labeling intensity was between 5000 and 15,000 dpm of ^3H per 10^6 cells.

Removal of Label from the Cell Surface. The procedure was as described (16). Cells were fixed in 2.5% glutaraldehyde for 30 min at 20°C, washed, and treated with β-galactosidase at 0.25 unit per ml overnight at 20°C. The fraction of label released from the cell surface was determined as the quotient of the radioactivity in the total sample and in the supernatant after centrifugation for 10 min at 500 × *g*. ^3H was measured after conversion to ^3H_2O in a sample oxidizer (Packard, model 306).

Culture Conditions. Labeled mast cells were resuspended in medium 199 (10 mM Hepes, pH 7.2/10% inactivated fetal calf serum) at ≈0.2 × 10^6 cells per ml and incubated at 37°C under mild agitation. Samples of 0.5 ml were taken at the indicated times and added to 0.16 ml of a 10% glutaraldehyde solution in small test tubes (500 × 6 mm) and mixed in a Vortex immediately after addition. Siliconized glassware was used for all procedures.

Isolation of Total Membrane. Washed cells were resuspended in 10 mM Tris·HCl, pH 7.8/0.1 mM phenylmethylsulfonyl fluoride/1 mM EDTA and stored at −20°C until further use. After thawing, the remaining cells were disrupted in a Dounce homogenizer. The total membrane was collected after centrifugation at 100,000 × *g* for 1 hr at 4°C.

Isolation and Labeling of Secreted Granular Material. The procedure is based on a method described (17). Unlabeled mast cells were stimulated to secrete in Hepes saline, cooled on ice, and separated from secreted material by centrifugation (1000 × *g*; 15 min). Secreted granular material was collected by centrifugation at 3000 × *g* for 20 min, washed once in Hepes saline, treated with β-galactosidase followed by labeling, all as described for whole cells except that centrifugation was at 3000 × *g* for 20 min. The pellet of labeled granular material was prepared for electrophoresis as described.

Polyacrylamide Gel Electrophoresis. Membrane proteins were dissociated by heating at 90°C for 3 min in 1% $NaDodSO_4$/0.1 M 2-mercaptoethanol. Electrophoresis was carried out in gradient slab gels (250 × 180 × 1.4 mm; 10%–13% polyacrylamide/0.1% $NaDodSO_4$) with a discontinuous buffer system (18) at 5 mA for 48 hr at room temperature. Molecular weight standards used were a standard mixture (SDS-6H, Sigma). Bands were stained with Coomassie brilliant blue. Gels were dried and lanes were cut into 2.5-mm slices, which were converted to ^3H_2O and $^{14}CO_2$ in a sample oxidizer before determining the radioactivity. Where radioactivity profiles were compared for different membrane samples, these were labeled with ^3H vs. ^{14}C and run in the same lane to eliminate all errors arising from inhomogeneities in the gel or from slice cutting.

RESULTS

The labeling system used in this study provided a biochemical method for measuring internalization and recycling of labeled membrane components. (*i*) Internalized label is no longer accessible to enzymatic release and can therefore be distinguished quantitatively from label remaining on the cell surface. (*ii*) Previously internalized label becomes accessible to enzymatic release when it is recycled back to the cell surface.

First, nonexocytic mast cells were labeled on the cell surface to study internalization of labeled plasma membrane constituents under conditions when the cell is not retrieving granule membrane. As shown in Fig. 2, incubation at 37°C resulted in a small fraction of label gradually becoming inaccessible to enzymatic release until a steady state had been reached after ≈120 min. This process did not occur when the cells were kept on ice. When cells were disrupted by sonication, the label again became accessible to enzymatic release from the remaining membrane fraction to the same extent as for whole cells immediately after labeling, in the absence of membrane flow (≈90% after 10 hr at 20°C).

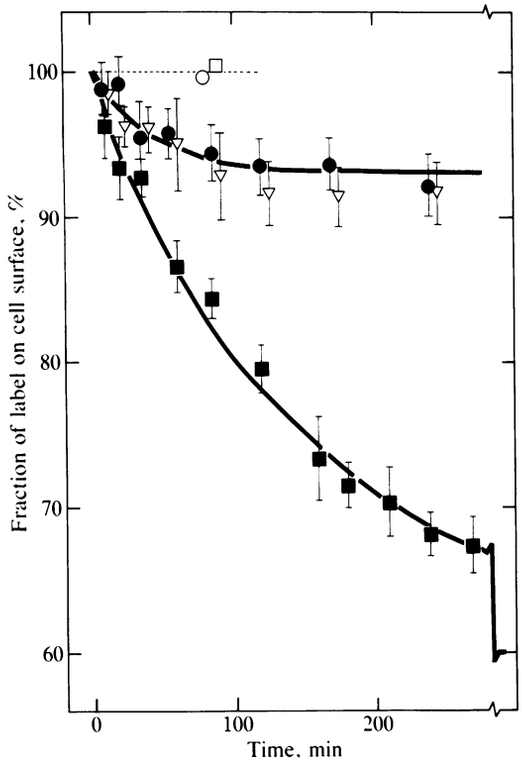

FIG. 2. Redistribution of labeled membrane glycoconjugates between cell-surface and intracellular membranes in the presence and absence of retrieval of granule membrane. Labeled cells were incubated in medium at 37°C, and aliquots were fixed in glutaraldehyde at the indicated times. Fraction of label remaining on cell surface was determined by enzymatic removal. ●, Cells labeled on plasma membrane without subsequent secretion; ○, same as ● but cells were kept on ice; ▽, cells labeled on plasma membrane followed by secretion; ■, cells labeled on cell surface after secretion; □, same as ■ but cells were kept on ice. Points represent mean of 3–5 independent experiments with the error bars indicating the average variation (mean standard error; the SEM is ≈50% smaller.) For values at $t = 0$, cells were fixed prior to warming; the fraction of label being released from these samples by overnight enzyme treatment varied between 85% and 95% for different experiments (variation for a single experiment was within 3%, corresponding to the average experimental accuracy) and was normalized to represent 100% of label on the cell surface (cf. refs. 19 and 20). All other points within a single experiment were normalized accordingly. Normalization was done before averaging data from different experiments. The following evaluation can be made. Independent of membrane retrieval, plasma membrane (PM) is shuttling between the cell surface and an intracellular membrane pool (IM). Label initially introduced into PM will redistribute between PM and IM as follows: $x = \rho + (1 - \rho)\cdot e^{-\lambda t}$, with ρ = PM/(PM + IM), $\lambda = [t_0\cdot(1 - \rho)]^{-1}$ and t_0 = PM/k, where x is the fraction of label in PM, k (membrane area/time) is the rate constant of membrane flow in both directions, and t_0 indicates the time taken to shuttle membrane equivalent to PM (cf. ref. 19). Fitting the data (●) to Eq. 1 results in $\rho = 0.93$ or IM/PM = 0.075, and $t_0 \simeq 11$ hr. Retrieval of granule membrane can be described by first-order kinetics (half-life, 2 hr) as indicated (■).

These and the following results can therefore be interpreted in terms of a redistribution of label between the cell surface and intracellular membranes due to internalization and recycling (see below; Fig. 3) of membrane. Previously, such an interpretation was corroborated by direct morphometric measurements using this label as an autoradiographic membrane marker (20–22).

Second, nonexocytic mast cells were labeled on their plasma membrane and were then stimulated to secrete. Under these conditions, between 60% and 80% of all cells secreted, as judged by the criteria indicated in Fig. 1. These

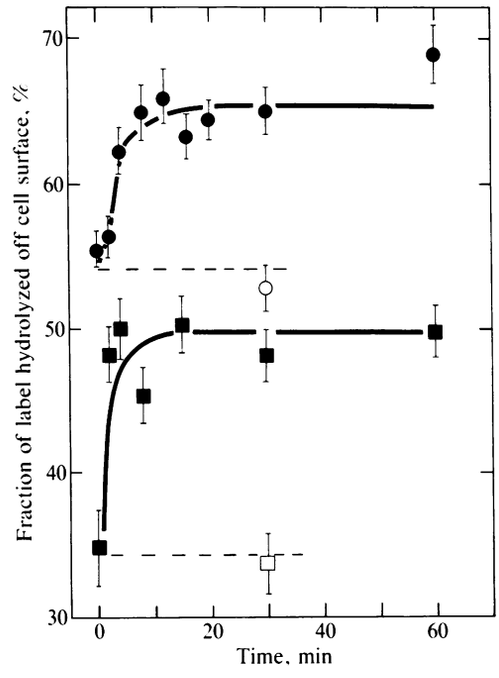

FIG. 3. Recycling of previously internalized labeled membrane glycoconjugates. Labeled cells were incubated in medium at 37°C for 180 min as described in Fig. 2. Cells were rapidly cooled on ice, washed once in phosphate-buffered saline, and treated with a mixture of neuraminidase and β-galactosidase at 0.5 unit/ml for 30 min on ice. This treatment removed ≈80% of all label on the cell surface. Cells were washed three times in phosphate-buffered saline and resuspended in medium for further incubation. Samples were taken as described in Fig. 2 to determine the fraction of label becoming accessible to enzymatic release as it was recycled back to the cell surface. ●, Cells labeled on plasma membrane without subsequent secretion; ○, same as ●, but cells were kept on ice; ■, cells labeled on cell surface after secretion; □, same as ■, but cells were kept on ice. Points are from a single experiment with error bars (SEM) indicating experimental accuracy. Curves were drawn by eye.

cells were then used to study internalization of labeled plasma membrane constituents during retrieval of (nonlabeled) granule membrane. As shown in Fig. 2, internalization of label occurred at the same rate and to the same extent as in the absence of membrane retrieval.

Third, mast cells were first stimulated to secrete followed by labeling of cell-surface components. As described below (see Fig. 5), these cells carried labeled membrane constituents in addition to those previously labeled on the plasma membrane and that presumably belonged to granule membranes. As shown in Fig. 2, label became internalized to a significantly larger extent, in agreement with the idea of retrieval of (labeled) granule membrane (cf. *Discussion* for quantitative considerations). When cells were kept on ice, no internalization of label was observed (Fig. 2).

It could be shown that recycling of label took place when nonexocytic cells were labeled as well as when cells were labeled after secretion. Labeled cells were incubated for 180 min, as before (Fig. 2), cooled on ice, and treated with β-galactosidase to remove label from the cell surface. After resuspension at 37°C (but not at 0°C), previously internalized label reappeared at the cell surface (Fig. 3). Because of technical reasons (20), no quantitative assessment could be made concerning the degree of recycling. As the measurement of recycling was based on the accessibility of label to release by β-galactosidase and neuraminidase, recycled label remained unmodified as far as the specificities of these enzymes are concerned (cf. refs. 20 and 22).

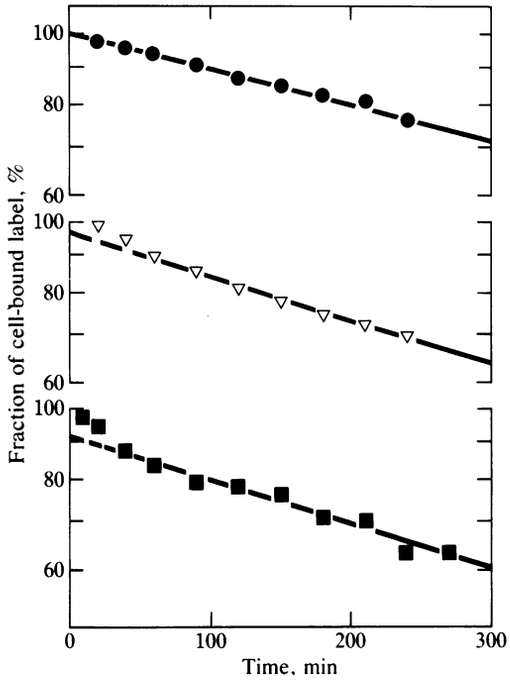

FIG. 4. Fraction of label remaining cell bound. Labeled cells were incubated as described in Fig. 2. At indicated times, samples were taken to distinguish cell-bound from released label by differential centrifugation at 200 × *g* for 10 min at 4°C. Total label remained constant. ●, Cells labeled on plasma membrane without subsequent secretion (half-life, 10.5 hr); ▽, cells labeled on plasma membrane followed by secretion (half-life, 8.5 hr); ■, cells labeled on cell surface after secretion (half-life, 8.5 hr).

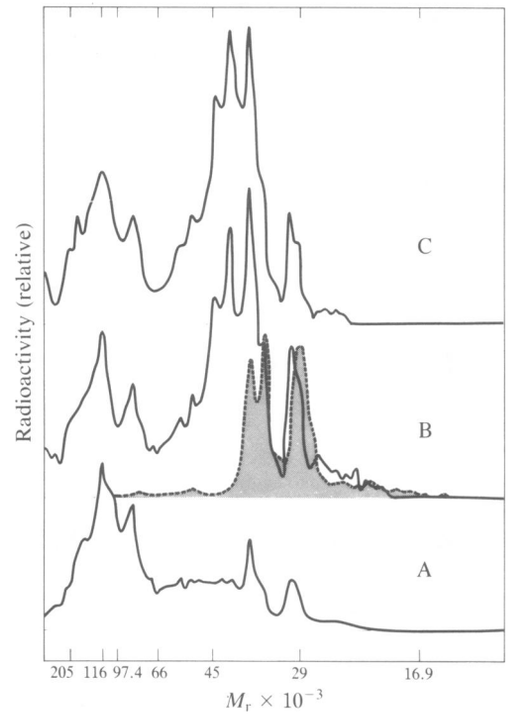

FIG. 5. Composition of labeled membrane glycoconjugates on $NaDodSO_4$/polyacrylamide gels. (*A*) Total membrane of cells labeled with [^{3}H]galactose on plasma membrane in the absence of secretion. (*B*) Total membrane of cells labeled with [^{14}C]galactose on cell surface after secretion; area covered by the profile in the M_r range <66,000, except for shaded area, is interpreted as representing label on granule membrane; shaded area indicates profile for secreted granular material. (*C*) Total membrane of cells labeled with [^{3}H]galactose on cell surface after secretion, followed by 4 hr of incubation at 37°C, resulting in ≈65% of the original label remaining cell bound (see Fig. 4). For direct comparison, ^{14}C- and ^{3}H-labeled membrane samples were run in the same lane on the gel. After drying, the lane was cut and radioactivity was determined as described. To obtain an estimate for relative contribution to total label by plasma membrane (55%), granule membrane (28%), and cell-bound secretory product (17%), values on the ordinate were normalized to yield approximately the same area under each profile in the M_r range >66,000. Peak heights of the shaded profile were arbitrarily adjusted, without changing their relative heights, to the corresponding peak heights of profile *B*. Depicted in this way, the shaded area indicates the maximum contribution of this label (17% of total).

The possibility of post-secretive membrane shedding was investigated by measuring the release of label into the medium. Label was lost from the cells with a half-life of ≈8.5 hr (Fig. 4). However, practically the same rate was observed when only the plasma membrane had been labeled in exocytic (8.5 hr) and nonexocytic cells (10.5 hr).

The composition of labeled membrane components was determined by measuring the radioactivity profiles for total membrane fractions after separation on $NaDodSO_4$/polyacrylamide gels. The profile obtained for labeled plasma membrane constituents from nonexocytic cells is shown in Fig. 5*A* in comparison with the profile obtained when the cell surface was labeled after secretory insertion of granule membrane (Fig. 5*B*). It can be seen that, after secretion, additional molecular species became accessible to labeling (M_r, <66,000). To determine whether these molecular species were actually constituents of the newly exposed granule membrane or merely belonged to secretory material remaining attached to the surface of exocytic cells, secreted granular material was isolated and labeled selectively followed by electrophoresis. The shaded area in Fig. 5*B* shows the labeling profile of secreted granular material. Qualitatively, it can be seen that only part of the additional labeled species could belong to secretory material rather than to granule membrane. Quantitatively, when exocytic cells labeled after secretion were disrupted, <15% of the label could be collected in the fraction consisting of granular material (cf. *Materials and Methods* and ref. 17). The labeling pattern of exocytic cells after 4 hr of membrane flow [i.e., when ≈35% of the label had been lost from the cells (Fig. 4)] is not significantly different from that obtained directly after labeling, without prior loss of label (Fig. 5*C* vs. Fig. 5*B*). This indicated that the loss of label was not selective for only certain labeled species.

DISCUSSION

For simplicity, when describing the results, inaccessibility of label to enzymatic release was equated to internalization of label. In previous studies, using ameba (21, 22) and a macrophage cell line (20), this assumption could be corroborated by electron microscopic autoradiography. Until such data become available also for the mast-cell system, the justification of the assumption has to rely on reasons given in the results section (namely, label remaining accessible to enzymatic release in cells kept at 0°C or becoming accessible when cells are disrupted; recycling of label occurring at 37°C but not at 0°C).

The most critical aspect of the present study, both from a technical point of view as well as for interpretation of the data (Fig. 2), was to ensure that after treatment with glycosidases and subsequent labeling, the cells reacted properly when stimulated to secrete. In this respect, the use of conditioned buffer and keeping all manipulations to a minimum, in both time and intensity, were significant factors. Two of seven experiments had to be discontinued, because only 10%–20% of the cells reacted to stimulation according

to the criteria indicated in Fig. 1. In the other five cases, on which the data in Fig. 2 are based, 60%–80% of the cells secreted (40%–50% histamine release; not shown) compared to >95% when cells were stimulated before enzyme treatment and labeling. The labeling procedure resulted in <5% damaged or exocytic cells, as judged by susceptibility to ruthenium red staining (15). In no instance were cells treated with concentrations of C48/80 >1 μg/ml, in order to avoid nonselective histamine release (23). Cells were kept strictly at 18°C until after secretion, because prior cooling also resulted in a lower degree of secretion (cf. ref. 23). The time, at 18°C, between applying the label until secretion and cooling on ice (≈30 min) did not result in any detectable internalization of label.

In the present case, label was lost into the medium at a higher rate than in the case of ameba and macrophages (half-life, 8–11 hr vs. 50 hr and 17 hr, respectively; see refs. 22 and 20). This loss of label could not be ascribed to post-secretive shedding of granule membrane because it was also observed for nonexocytic and exocytic cells where only the plasma membrane had been labeled (Fig. 4). Furthermore, loss of label affected all labeled membrane species to the same extent (Fig. 5). The fact that 60% of the label released into the medium was not precipitable at 100,000 × *g* during 60 min indicated that some loss of label could be due to the action of endogenous glycosidases, which are known to be present in secretory granules of mast cells (table 1 in ref. 4). However, a turnover time characterized by a half-life of 10 hr can still be explained by the normal turnover found for membrane proteins (cf. ref. 24).

The labeling profile for exocytic cells (Fig. 5*B*; M_r, <66,000) strongly suggests that additional molecular species, characteristic of the granule membrane, became inserted into the cell surface during secretion. It was shown that the bulk of the new label could not be ascribed to labeling of secretory granular material remaining bound to the cell surface. On the other hand, the present data do not exclude the possibility that soluble secretory product was readsorbed to the cell surface where it became labeled. However, the extensive washing steps during treatment with β-galactosidase and labeling do not favor this possibility, at least not in terms of quantitative considerations. The appearance on the cell surface of additional molecular species, characteristic of secretory granule membrane, has previously been reported for other cells (ref. 25 and refs. therein).

The results presented in Fig. 2 can be interpreted as indicating selective internalization of granule membrane. Such an interpretation is based on the assumption that label on the plasma membrane and on granule membrane behaves in a way that is representative for the bulk of the respective membrane. The data in Fig. 2 conform to the following model of membrane flow (cf. legend to Fig. 2). (*i*) Independent of whether retrieval of granule membrane is taking place, plasma membrane is internalized and recycled between the cell surface and an intracellular membrane pool 7.5% the size of the plasma membrane. Membrane flow in both directions between these two compartments occurs at a rate of one plasma membrane equivalent every 11 hr. This rate of membrane flow is much slower than that found for pinocytosing macrophages (≈0.5 hr) (20, 26). Endocytosis in mast cells has previously been demonstrated (12). (*ii*) Retrieval of granule membrane can be described by first-order kinetics with a half-life of 120 min. This rate is much slower than the rate observed previously by morphometric means for the sealing of exocytotic cavities during the first 30 min (≈85% sealing) but agrees well with the slower sealing rate observed between 30 and 90 min, occurring with a half-life of ≈100 min (13). The present data, extending to only 4 hr after secretion, give no information concerning the long-term recovery of granule membrane (10). However, approximate extrapolation to steady-state conditions (40% label internalized; the steady-state value depends on the ratio of label on plasma membrane to that on granule membrane) indicates that after 4 hr the recovery process has been completed to ≈80%. This estimate, as well as the result of Németh and Röhlich (13), disagrees with a previous report that a large exocytic cavity remains "in free communication with the extracellular space" even after 48 hr (10). According to the present results, such communication must at least exclude the access of β-galactosidase to the membranes in the cavity.

The difference observed for the internalization of label between cells labeled either before or after secretion relates directly to the problem of "control of membrane specificity" (27). Examples of granule membrane components that did not get randomized after insertion at the cell surface have been reported for the parotid gland (28) and for cultured adrenal chromaffin cells (29). The present data are in agreement with the concept that the characteristic constituents of granule membrane do not mix with labeled constituents on the plasma membrane after fusion of these membranes; if membrane mixing should take place, then "membrane sorting" occurs at the cell surface during retrieval of granule membrane.

I thank Dr. Tom Haylett for helping me to collect the peritoneal cells and for taking the photographs depicted in Fig. 1.

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