Reversible condensation of mast cell secretory products *in vitro*

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ABSTRACT We have investigated the mechanisms responsible for the condensation and decondensation of secretory products that occur in mast cell secretion. We show here that the hydrated matrix of an exocytosed secretory granule can be recondensed to its original volume by exposure to acidic solutions containing histamine at concentrations that mimic those found in vivo. Recondensation by acidic histamine began in the range of 1–10 mM with a dose response curve that was accurately predicted by a Hill type equation with four highly cooperative binding sites and a half maximum concentration of $[H_i^{++}] = 3.9$ mM. Recondensation by histamine showed a sigmoidal dependency on pH (critical range pH 5.5–6.5) and was fully reversible. These experiments suggest that histamine, possibly by binding to anionic sites in the protein-heparin complex of the granule matrix, triggers a change in the polymeric structures of the granule matrix from an extended coil to a collapsed globular state. This may be a useful model for understanding the condensation of secretory products into dense core granules and their subsequent decondensation upon exocytosis.

INTRODUCTION

A fundamental event in regulated secretion is the packaging of neurotransmitters and other secretory products by their condensation inside secretory granules (Kelly, 1985; Griffiths and Simons, 1986). Secretory products selected in the Golgi for the regulated secretory pathway reach the condensing vacuole in a dilute form. The condensing vacuole, an immature form of secretory granule, undergoes a progressive acidification and a large condensation of the enclosed products until they acquire the density of mature secretory granules. The mechanisms by which proteins and other secretory products can be condensed by up to several hundredfold in the condensing vacuole remains unknown.

In mast cells, histamine and a protein-heparin complex are stored at high concentrations and are densely packed inside secretory granules (Lindahl et al., 1986). The protein-heparin complex that forms the matrix of the secretory granule is synthesized first as a nonsulfated proteoglycan molecule with ten or more polysaccharide chains linked to the peptide core, forming long brushes with 100–200 disaccharides units each. The long sugar polymers are then heterogeneously sulfated, creating complex patterns with a high density of negative charge, which is characteristic of heparin glycosaminoglycans. The resulting proteoglycan molecule is highly charged and hydrophilic, able to attract large amounts of water and form a hydrated gel that occupies a very large volume for its mass. Similarly, proteins like the chromogranin/secretogranin family form the matrix of various neuronal and neuroendocrine secretory granules and are also highly sulfated and thus have an abundance of negative charges. These examples suggest that a common structural characteristic of the proteins and proteoglycans that form the matrix of dense core granules is that they are highly charged and consequently can form hydrated gels where the proteins and glycosaminoglycans tend to exist in a coil configuration. Mechanisms by which these proteins can be condensed in their passage from the *trans* Golgi to the mature secretory vesicles have been proposed. For example, it has been shown recently that secretogranin II, a member of the chromogranin/secretogranin family, aggregates in vitro when exposed to a medium containing calcium at acidic pH (Gerdes et al., 1989). The suggestion was made that calcium and pH act by neutralizing the excess negative charge of secretogranin II, reducing electrostatic repulsion and causing the observed aggregation. Similarly, it has been proposed that calcium is the shielding cation that permits condensation of the highly charged mucins inside the secretory granules of goblet cells (Verdugo, 1990).

In the case of mast cell granules, it is likely that the charged and hydrated coils of the heparin proteoglycan must be neutralized to reduce electrostatic repulsion and allow a dehydrated, globular form to be packed into the secretory granules. The isolated and membrane-free

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matrix of mast cell granules binds inorganic cations as well as biogenic amines (Uvnas and Aborg, 1977). It has been proposed that organic and inorganic cations compete for the same binding sites in the granule matrix and that the protein-heparin matrix has the properties of a cation exchanger (Uvnas and Aborg, 1977). However, a relationship between cation binding and matrix condensation has not been noted before, possibly because typical mast cell granules are submicron in size making it difficult to measure their size with the optical microscope. In contrast, mast cells of the pigment mutant beige mouse (bg^j/bg^j) have large secretory granules which are easily observed under the light microscope. Stimulation of beige mouse mast cells with various secretagogues causes a massive degranulation. Upon exocytosis the granule matrix, made of a protein-heparin complex, swells several-fold and remains visible near the cell for at least 24 h. The exocytosed granule matrices are ideal structures with which to study the mechanisms of condensation and decondensation of secretory products in a physiological setting. Preliminary reports have suggested that histamine and divalent ions are capable of causing changes in the volume of the hydrated matrix of exocytosed beige granules (Curran and Brodwick, 1985; Villalon et al., 1990). In this paper we expand these observations and attempt to define the ionic conditions that are necessary to cause the reversible condensation of exocytosed mast cell granules and probe its possible mechanisms.

METHODS

Mast cells were obtained from the peritoneal cavity of adult beige mice (bg^j/bg^j) (Jackson Laboratories, Bar Harbor, ME) after a procedure described elsewhere (Breckenridge and Almers, 1987). They were equilibrated in a standard extracellular saline defined as containing 150 mM NaCl, 10 mM Hepes, 3 mM KOH, 0.9 mM NaOH, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 12 mM Glucose, 310 mmol/kg, pH 7.3, at room temperature. Test solutions consisted of the test salt at the given concentration (see figure legends) buffered to the indicated pH with either citric acid or Hepes depending on the pH. Secretion was stimulated by 10 μg/ml of compound 48/80 (Sigma Chemical Co., St. Louis, MO). Swelling of secretory granules was imaged by videomicroscopy at ~3,500 magnification with a Zeiss (IM35) inverted microscope equipped with Nomarski optics. Swelling was monitored using a video camera (model IKC30M; Toshiba, Japan), and recorded at 30 frames/s on a video recorder (BV-1000; Mitsubishi, Japan). The diameter of the granules was measured by single frame video image analysis. Digital image analysis was implemented using a frame grabber (DT 2851; Data Translation, Marlboro, MA) installed on a microcomputer (AT-286; Beltron, Feasterville, PA), operated by the Image-Pro software package (Media Cybernetics, Silver Spring, MD). Repeated measurements of granule diameter performed in single images rendered a measuring error <2%. Volumetric expansion was calculated assuming a spherical shape for the secretory granules and expressed as a percent of their final post-exocytotic volume in standard extracellular saline (pH 7.3). All solutions were replaced by bath perfusion using a flow of ~1 ml/s using a total exchange volume of 5 ml. The glass well containing the cells had a volume of 300 μl.

RESULTS

An isolated beige mouse mast cell, with its typically large histamine-containing granules, is shown before stimulation in Fig. 1*A*. Degranulation began 20–30 s after stimulation with compound 48/80, and was complete within the first minute of exposure to this secretagogue. Upon release, the granule matrices expanded three to four times (3.3 ± 0.3 [mean ± SEM, n = 10]) their intracellular volume (Fig. 1 *B*). Decondensation of the granular matrix upon exocytosis reaches a maximum in <1 s as previously reported (Breckenridge and Almers, 1987). After exocytosis, the expanded polymer matrices of the

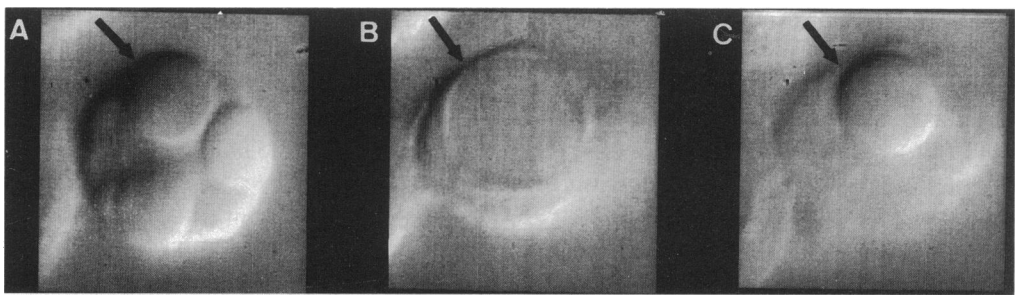

FIGURE 1 Changes in the volume of individual beige mast cell granules upon histamine-induced recondensation. *A–C* show a sequence of photographs of a granule during an expansion/recondensation cycle (see arrow). An isolated mast cell containing several large intracellular secretory granules before stimulation is shown in *A*. Upon stimulation with 10 μg/ml of compound 48/80, the cell rapidly degranulates. The granules are exocytosed and expand to about five times their original intracellular volume in ~600 ms (*B*). Equilibrating the cell in a solution containing 50 mM histamine at pH 3.0 (50 mM histamine, 5 mM citric acid) produced a rapid recondensation of the exocytosed granules to approximately their original volume (*C*).

granules remain visible. They often detach from the cell, but sometimes they remain attached, thereby facilitating their observation (Fig. 1 *B*). Upon exocytosis, the bathing saline has direct access to the granule matrix either through the expanded fusion pore that connects the lumen of the secretory granule with the extracellular space, or directly when the granule matrix has been expelled clear of the cell through the open fusion pore. An exocytosed granule matrix can be recondensed to within 5% of its original intracellular volume by replacing the extracellular saline with a solution containing 50 mM histamine at pH 3 (Figs. 1 *C* and 4). In contrast, granules isolated by mild sonication of beige mast cells have an intact granule membrane, retain their condensed form and are unaffected by exposure to either acidic histamine or standard extracellular saline (Monck et al., 1991). Recondensation does not depend on the association of the granule matrix and the cell or the cell membrane, as identical results were obtained in exocytosed, membrane-free granules detached from the cell surface. Detached granules recondensed to 28.6% ± 3.0% (mean ± SEM, $n = 5$) of their expanded post-exocytotic volume, whereas attached granules recondensed to 29.9% ± 2.3% (mean ± SEM, n = 4) of their post-exocytotic volume in standard saline. Thus histamine-induced condensation results solely from a specific interaction between histamine and the heparin polymer matrix. Exposure of the exocytosed granular content to histamine concentrations and pH that mimic those found inside the granule in vivo (150 mM histamine, at pH 5.4) (Alter and Schwartz, 1989; Johnson et al., 1980), also resulted in recondensation of the granule matrix (e.g., see Fig. 3). Recondensation always occurred as fast as we were able to exchange the bathing saline ($< 1\,s$).

Recondensation of the granule matrix was reversible. Replacement of the histamine-containing solution by standard extracellular saline restored the matrix to its hydrated size. Expansion and recondensation cycles could be repeated many times, without significant changes.

At constant pH (3.0) histamine became effective in recondensing the granule matrix within a narrow range (1–10 mM). Fig. 2 shows the dose response that relates matrix condensation, expressed as a percentage of its volume in standard saline, %, with the histamine concentration, $[H_i]$. The solid curve is given by the function $\% = 95.5\% - D_{max}[H_i]^n/([H_i]^n + K_d)$ with $n = 3.6$, $K_d = [3.88\ mM]^{3.6}$ and $D_{max} = 68\%$, corresponding to the maximum attainable change at a saturating concentration of histamine. Fig. 2 (*inset*) shows a linear regression of the data represented as a Hill plot. The fit (solid line in inset) returned the values used for the constants n and K_d. The excellent agreement between the Hill equation

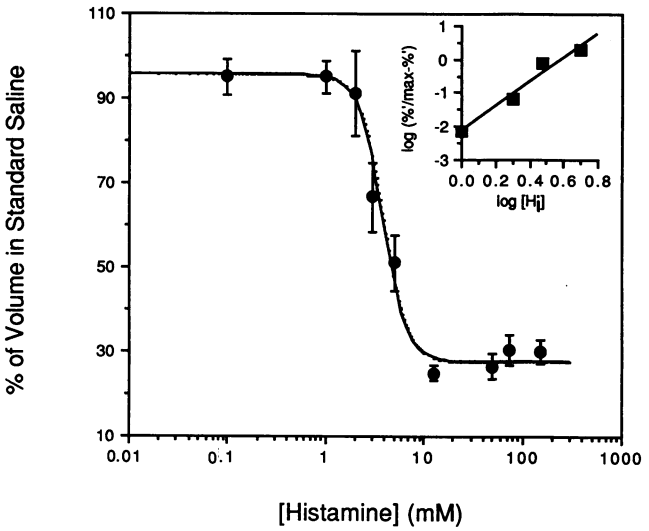

FIGURE 2 Effect of increasing histamine concentration on the condensation of exocytosed secretory granules of mast cells at pH 3.0. The solid trace is a plot of the Hill equation with $n = 3.6$. (*Inset*) the constant n was obtained from a Hill plot of the data (see text) where $\%' = 95.5$-data and max = 68%. All test solutions contained the indicated concentration of histamine-dihydrochloride, 5 mM citric acid, and were adjusted to pH 3.0. Changes in volume are expressed as a percent of the volume in standard saline (mean ± SEM). Each data point is the average of eight experiments.

with $n = 3.6$ and the data (Fig. 2) is suggestive that a high degree of cooperativity is required among the divalent histamine binding sites of the granule matrix to bring about condensation (see Discussion).

Recondensation of the exocytosed granule matrices showed a strong pH dependence. At a constant histamine concentration (150 mM) recondensation occurred only at acidic pH (Fig. 3). Condensation of the granule matrix is likely to be caused by the divalent species of histamine (H_i^{++}) because at neutral pH, histamine behaves as a monovalent cation, whereas at more acidic pH (3–4) it becomes divalent ($pK_1 = 10$; $pK_2 = 5.9$) (Uvnas, et al., 1970). In support of this hypothesis is the observation that the half-maximal effect of pH occurs at pH = 6.2, which is very close to pK_2. Low pH could affect the dissociation of sulfate and carboxylic groups of the heparin polyion, thereby favoring condensation. Nonetheless, the finding that Ca^{++} can partially condense the matrix at both neutral (data not shown) and acidic pH (see below), suggests that the pH dependence of histamine-induced condensation is primarily due to the effect of pH on the cationic charge of histamine.

As shown in Figs. 2 and 3, recondensation was not driven simply by osmotic water loss. The data show that the condensation induced by a high pH, high osmolality solution (i.e., 150 mM histamine at pH 9.3; 550 mmol/

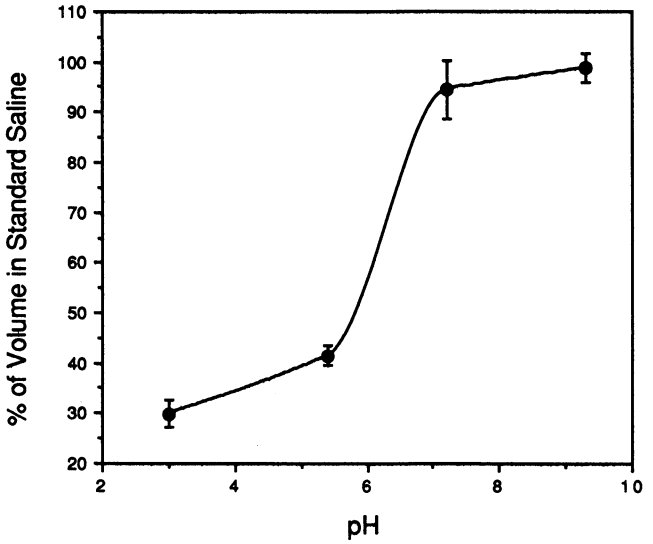

FIGURE 3 Influence of pH on histamine-induced condensation of exocytosed granules from mast cells. Changes in volume are expressed as a percent of the expanded exocytosed volume (mean ± SEM). Each data point is the average of 10 observations. The concentration of histamine in this experiment was 150 mM. Histamine solutions were buffered with 10 mM citric acid at pH 3.0 (348 mmol/kg), and 5.4 (375 mmol/kg); or with 10 mM Hepes at pH 7.25 (500 mmol/kg), and 9.3 (550 mmol/kg).

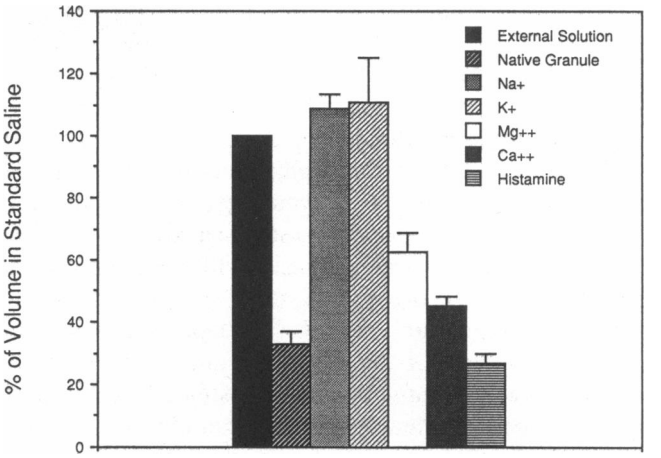

FIGURE 4 The effect of solutions containing 50 mM of either NaCl, KCl, $MgCl_2$, $CaCl_2$, or histamine-dihydrochloride (102 mmol/kg, 98 mmol/kg, 132 mmol/kg, 136 mmol/kg, and 125 mmol/kg, respectively) on the condensation of exocytosed mast cell secretory granules. All solutions contained 5 mM citric acid as a buffer and were adjusted to pH 3.0 with KOH. Each column represents changes in volume (measured 30–60 s after changing solutions) expressed as a percentage of the expanded volume of exocytosed granules in extracellular saline as described in the legend to Fig. 1. The volume of the pre-exocytosed native granules is shown in column 2 for comparison. Each data point (mean ± SEM) is the average of seven experiments.

kg) is negligible compared with that induced by a low pH, low osmolality solution (i.e., 10 mM histamine at pH 3.0; 48 mmol/kg). Thus the effect is, at least in this instance, in the wrong direction to be explained by osmotic water loss. Furthermore, granular matrices recondensed in 150 mM histamine at acidic pH remained condensed even after being exposed to 100-fold dilution of the bathing solution. However, they swelled readily upon exposure to the standard saline containing sodium ions.

It is well known that the matrix of mast cell secretory granules is made of heparin proteoglycans. Due to the presence of ester sulphate groups, heparin is a highly polyanionic polymer and binds a variety of monovalent and divalent cations. Cation binding is unselective and appears to occur by ionic exchange in a concentration and pH-dependent fashion (Uvnas et al., 1970; Uvnas and Aborg, 1977). In the present studies we compared the effectiveness of divalent cations (histamine, Ca^{++}, and Mg^{++}) and monovalent cations (Na^+ and K^+) on their capacity to recondense the exocytosed granules at low pH. While monovalent cations (Na^+ and K^+) were ineffective, Ca^{++} and Mg^{++} readily recondensed the granules, although not as effectively as histamine (Fig. 4). As the figure shows, the valency of the condensing cation is not the only factor determining their effectiveness. In spite of Ca^{++}, Mg^{++}, and H_i^{++} (at pH 3.0) having the same valency, H_i^{++} is more effective.

DISCUSSION

It has been long suspected that the polymeric structures found in the secretory granules may be important for the condensation, and perhaps the sorting of secretory products (Kelly, 1985). Condensed polyanionic polymer networks, and one or more cations or polycations are ubiquitously present in secretory granules (De Camilli and Jahn, 1990). Furthermore, condensation and subsequent decondensation of these polymeric structures is a general feature in the storage and release of secretory products during regulated secretion.

Depending upon the nature of the interconnections among polymer chains, and the charge density of the polymer network, the swelling properties of hydrogels can vary widely. For instance, in gels containing a cross-linked polymer network, swelling is limited and reversible. Conversely, in gels having a tangled polymer matrix, swelling is not limited, and can bring the gel to complete dispersion (Edwards, 1986). In gels made of neutral polymers, swelling is driven by simple osmotic forces, and governed by the diffusion of the polymer network (Tanaka et al., 1980), whereas in gels contain-

ing a polyionic matrix, swelling is driven by the fixed charges of the polyions, and is governed by a Donnan equilibrium (Tam and Verdugo, 1981). Moreover, depending upon the composition of the solvent, polyionic gels can undergo discrete changes of their swelling properties that have the typical features of a critical phenomenon known as polymer gel phase transition. The gel can be in either of two states: a condensed phase or an expanded hydrated phase. While condensed, the polymer network occupies a small fraction of the expanded volume, and behaves independently of the osmotic pressure of the solvent (Tanaka et al., 1980). Discontinuous volume changes in isolated mucin granules induced by immersing the granules in acetone-water or glycerol-water mixtures, led to the proposition that a phase transition mechanism might explain both the condensation of the polymer matrix in the granule, and its decondensation during exocytosis (Verdugo, 1984, 1990). Those experiments, however, failed to establish conditions under which a reversible polymer gel phase transition may occur in vivo. The most striking feature of the present findings is that histamine at concentrations similar to those believed to exist inside the granule in vivo (i.e., 150 mM histamine, at pH 5.4) (Alter and Schwartz, 1989; Johnson et al., 1980) can readily recondense the matrix of exocytosed granules (Fig. 3). Furthermore, it is remarkable that the reversible collapse of the granule matrix can be induced by simple ionic solution substitutions and does not depend on enzymatic activity or sources of metabolic energy. As shown, the matrix of mast cell secretory granules exhibit two extreme configurations: condensed and decondensed. The transition from one to the other was reversible, could be induced by acidic histamine, and its dependence on the concentration of histamine was steep, with a Hill coefficient of 3.6, suggestive of a highly cooperative phenomena. These features closely resemble the effect of salts on polyionic hydrogels (Ohmine and Tanaka, 1982). Thus, it is possible that the reversible collapse of the granule matrix can be explained as a coil-globule phase transition of the polymeric structures that form the matrix.

We have found that the granule matrix can undergo multiple cycles of condensation-decondensation without losing its original shape. This is the case for spherical granules as well as for the oddly shaped granules that are frequently observed in beige mouse mast cells. These results suggest that the granule matrix is a stable, possibly homogeneously, cross-linked polymer network. The stable links between individual proteoglycans could occur between their protein backbones and/or their heparin glycosaminoglycans. A simple way of representing the granule matrix is to assume that it is made of small unitary cells. Each individual cell could be made of a collapsible polymeric structure. For the granule matrix to collapse into the condensed globular form, each individual cell would have to collapse. If this hypothesis is correct, the data of Fig. 2 would imply that to cause the collapse of the granule matrix, divalent histamine molecules must occupy a minimum of four highly cooperative binding sites in each unitary cell. The unitary cell can be either single glycosaminoglycans, single proteoglycan molecules, or more complex structures. This model predicts that single heparin proteoglycans should be able to collapse and expand under conditions similar to those demonstrated here. Furthermore, it is possible that the individual glycosaminoglycan chains (i.e., heparin) are capable of collapse, caused by the binding of divalent histamine molecules. These predictions can be tested by measuring the hydrodynamic radius of heparin molecules in a dilute solution by using light-scattering techniques (Izumi et al., 1979).

Studies using NMR spectroscopy indicate that there is rapid exchange between free and bound histamine inside the heparin matrix of mast cell granules, while the exchange with free histamine outside the matrix is slow (Rabenstein et al., 1987). These observations are consistent with the idea of Rabenstein et al. (1987), that electrostatic and also steric constraints, may hinder the mobility and virtually entrap histamine inside the condensed heparin matrix. Thus, it is likely that histamine-induced condensation leads to its own sequestration inside the heparin network. The granule matrix can be readily decondensed by saline solutions containing Na^+ in concentrations equal or lower than the extracellular fluid and at neutral pH. Because condensation results from the cooperative effect of at least four H_i^{++} binding sites (Fig. 2), the replacement of a single histamine by a sodium ion may be sufficient to decondense an individual polymeric unit. Thus, it seems likely that upon exocytosis, the replacement of the bound histamine (H_i^{++}) by extracellular sodium, perhaps through an ion-exchange mechanism (Uvnas and Aborg, 1977), triggers the explosive decondensation of the heparin matrix, releasing histamine from its polymer trap.

Sulfated proteoglycans are found in a wide variety of secretory granules including mast cells. Because of their molecular composition and their unusually high negative charge, they have been proposed to have a role in sorting and condensation of secretory products (Kelly, 1985; Palade, 1975; Hashimoto et al., 1987; Griffiths and Simons, 1986). It is tempting to speculate that condensation of the granule matrix by histamine, as shown here, may reveal part of the sorting mechanism operating in mast cells. If, for instance, the inward transporter of histamine that normally functions in the granular membrane (Lagunoff and Bauza, 1982) begins to function in the Golgi before the formation of the condensing

vacuole, the resultant inflow of histamine could make operational the condensing-sorting mechanism proposed by Palade (1975), in this case, for sorting the heparin matrix of the mast cell granule. Furthermore, a significant event during secretory granule formation is the progressive acidification of the condensing vacuole (Orci et al., 1987) and its subsequent condensation into a mature secretory granule. It is likely that the ability of the granule matrix to condense in vitro, as shown here, demonstrates the mechanism by which secretory granules condense in vivo.

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