Atomic Force Microscopy Study of the Secretory Granule Lumen

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ABSTRACT We have used an atomic force microscope to study the mechanical properties of the matrix found in the lumen of secretory granules isolated from mast cells. The matrices were insoluble and had an average height of 474 ± 197 nm. The volume of these matrices increased reversibly about tenfold by decreasing the valency of the bathing external cation (La^{3+} < Ca^{2+} < Na^{+}). The elastic (Young's) modulus was found to decrease by about 100-fold (4.3 MPa in La^{3+} to 37 kPa in Na^{+}) upon a tenfold increase in the matrix volume. A swollen granule matrix had an elastic modulus similar to that of gelatin in water. The elastic modulus was inversely related to the change in the volume of the matrix, following a relationship similar to that predicted for the elasticity of weakly cross-linked polymers. Our results show that the matrix of these secretory granules have the mechanical properties of weak ion exchange resins, lending strong support to an ion exchange mechanism for the storage and release of cationic secretory products.

INTRODUCTION

Secretory products are stored in the lumen of secretory granules and are released upon exocytosis. However, the mechanisms of storage and release are unknown. Uvnäs and Åborg (1989) have proposed that the secretory granule matrix functions as an ion exchanger and that the storage and release of secretory products occur by stoichiometric exchange (ion exchange) with the counterions that neutralize the charged groups that are fixed in the matrix.

Ion exchangers are insoluble cross-linked polymers that contain a high density of fixed charges. Because bulk electroneutrality must be always satisfied, an equal number of counterions must neutralize the fixed charges in the gel. Hence large amounts of charged secretory products could be stored as counterions and then be rapidly mobilized for release by ion exchange (Uvnäs and Åborg, 1989).

The giant mast cell granules from the mutant beige mouse (bg^{j}/bg^{j}) have been shown to contain an insoluble gel matrix. These granules have diameters of up to several micrometers and can be easily studied with a light microscope (Curran and Brodwick, 1991; Fernandez et al., 1991; Brodwick et al., 1992). When the membrane of a secretory granule is removed, the granule matrix is exposed to the bathing cations. An exposed matrix swells in the presence of monovalent cations and shrinks in the presence of divalent cations (Curran and Brodwick, 1991; Fernandez et al., 1991). Furthermore, these granule matrices behave as elastic gels with a bulk modulus (2–40 MPa) comparable to that of gelatin in water (Brodwick et al., 1992). These mechanical properties, together with the observation that mast cell matrices bind and exchange cations stoichiometrically (Uvnäs and Åborg, 1983, 1989), correspond to the typical properties of ion exchange gels (Helfferich, 1962).

Although the data obtained from the giant granules have been informative, most secretory vesicles have diameters smaller than a micrometer and cannot be studied with light microscopes. For example, granules from wild-type mast cells average 624 nm in diameter (Chock and Schmauder-Chock, 1989), and small synaptic vesicles average 50 nm in diameter (Südhof and Jahn, 1991). Because the light microscope resolution is limited by the wavelength of visible light, it cannot be used to study most of secretory granules. High-resolution images of secretory granules can be obtained by electron microscopy (EM). However, EM studies require sample dehydration and fixation; a result of this is the destruction of the functional properties of the matrix. These limitations have prevented a detailed examination of the luminal structure of secretory granules and their possible role as ion exchangers.

The atomic force microscope (AFM) can image samples under conditions that imitate those found in vivo while resolving size changes smaller than 1 nm and forces down to 100 pN with high temporal resolution (~1 ms). For example, the AFM has been used to measure the adhesion forces between single ligand-receptor pairs (Florin et al., 1994) and the height fluctuations of a single enzyme undergoing substrate-product reactions (Radmacher et al., 1994). The remarkable resolution of the AFM and its ability to operate in the presence of physiological saline makes it an ideal choice to image isolated secretory granules (e.g., Parpura et al., 1995).

In this work we have used the AFM to study the mechanical properties of submicrometer-sized secretory granules isolated from rat mast cells. We found that these secretory granules contain an insoluble matrix that reversibly shrinks and swells in response to ion exchange with different cations. Furthermore, we demonstrate that these matrices have an elastic modulus similar to that of gelatin in water and that the elastic modulus is inversely dependent on the swelling of the matrix, following a relationship similar to that pre-

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dicted for the elasticity of weakly cross-linked polymers (Treloar, 1975). Taken together, these observations demonstrate the presence of an ion exchange gel in the lumen of small secretory granules. Our findings extend the observations made in the giant granules of the beige mouse and support the view that the storage and release of cationic secretory products occur by ion exchange. In addition, our studies demonstrate a new approach to study the mechanical properties of intracellular organelles.

MATERIALS AND METHODS

Isolation of secretory granules

Isolation of mast cell secretory granules was based on the modification of several established procedures (Amende and Donlon, 1985; Hamaguchi et al., 1987; Chock and Schmauder-Chock, 1989). Briefly, mast cells were harvested by peritoneal lavage from adult Sprague-Dawley or Long Evans rats. The lavage saline consisted of a CO_2-independent medium (Gibco, Gaithersburg, MD; catalog no. 18045-021) containing 0.175% fatty acid-free bovine serum albumin (ICN Biomedicals, Costa Mesa, CA). The lavage was centrifuged at 100 × *g* for 10 min. The resulting pellet was resuspended in 1 ml of lavage saline and layered over 2 ml of 22.5% metrizamide (dissolved in the lavage saline). After a 20-min 400 × *g* (at interface) centrifugation, the contaminant cells were removed by aspiration. The remaining pellet was resuspended and centrifuged at 100 × *g* for 10 min. The pellet containing pure mast cells was resuspended in 2 ml of lavage saline and then was sonicated to extract the secretory granules, using a sonifier equipped with a microprobe (Branson, Shelton, CT; model 450; six pulses at an output of 2.25 and 25% duty cycle). After vigorous vortexing, the suspension was spun at 100 × *g* for 10 min. The supernatant containing the granules was collected. The pellet containing unbroken mast cells was resuspended and sonicated. This procedure was repeated twice to increase the yield of purified granules. The supernatants were pooled and spun at 100 × *g* for 10 min to remove aggregates. The resulting supernatant was spun at 1000 × *g* for 15 min to pellet the granules. The purified granules were resuspended in lavage saline. This procedure yields a suspension of 85% pure intact granules as determined by inspection with a light microscope equipped with Nomarski optics.

Isolated intact granules were applied to poly-L-lysine (1 mg/ml; M_r 100,000)-coated glass coverslips for 1 h at room temperature. A thin layer of granules adhered firmly to the surface of the coated glass coverslip. To expose the granule matrix, the granules were stripped of their membranes with a mild detergent treatment (0.01% Triton X-100 for 5 min).

AFM Imaging

A Nanoscope E and associated equipment (Digital Instruments, Santa Barbara, CA) were used in this study. All images were obtained at room temperature (22–26°C) by using an E scanner (16.5 μm × 16.5 μm maximum scanning area). The experiments were done using a fluid cell filled with a saline solution. Before imaging, the matrices were condensed by washing them with a solution containing 100 mM histamine dihydrochloride and 1 mM citric acid (pH 3.5; 260–266 mmol/kg; Fernandez et al., 1991). To prevent damage of the matrices, the imaging force was adjusted to 1–2 nN.

Force-distance curves

For the acquisition of force-distance curves we controlled the positioning of the AFM tip by means of a custom-built high-voltage amplifier connected to the *x*, *y*, and *z* axis inputs of the piezoelectric positioner of the Nanoscope E. To gain access to the piezoelectric positioner we used a signal access module (Digital Instruments). From the signal access module we also obtained a readout of the displacement of the AFM tip, which was sampled at 2–5 kHz and filtered at the Nyquist frequency. The data acquisition and the voltage control of the *z* axis of the piezoelectric positioner were done with an AT-MIO-16X interface (National Instruments, Austin, TX). The *x* and *y* axes were controlled with an AT-AO-6 interface (National Instruments). Both interfaces were installed in a P5-100 personal computer (Gateway 2000; Sioux City, SD) and driven by custom-written software (LabView for Windows 3.1; National Instruments).

Elasticity measurements

The elastic (Young's) modulus was estimated from measurements of the elastic displacement of a granule matrix pressed on by an AFM tip. The force-displacement relationship was well represented by a theory formulated by Hertz (Hertz, 1882; Johnson et al., 1971) describing the elastic displacement of a spherical object (the granule matrix) pressed on by another spherical object (the AFM tip), that is,

$$F_{tip} = \frac{4}{3\pi} \times \frac{1}{k_{matrix} + k_{tip}} \times \sqrt{\frac{R_{matrix}R_{tip}}{R_{matrix} + R_{tip}}} \times \Delta z^{1.5}, \quad (1)$$

where k_{matrix} and k_{tip} are elastic constants of the material of each sphere defined by

$$k_{matrix} = \frac{1 - \nu^2_{matrix}}{\pi E_{matrix}} \text{ and } k_{tip} = \frac{1 - \nu^2_{tip}}{\pi E_{tip}},$$

where E is Young's modulus and ν is the Poisson ratio of each material. Because the AFM tip has an elastic modulus of 150 GPa (Weisenhorn et al., 1993), $k_{matrix} \gg k_{tip}$, then Eq. 1 can be simplified to

$$F_{tip} = \frac{4}{3} \times \frac{E_{matrix}}{1 - \nu^2_{matrix}} \times \sqrt{\frac{R_{matrix}R_{tip}}{R_{matrix} + R_{tip}}} \times \Delta z^{1.5}, \quad (2)$$

where Δz is the elastic displacement of the granule matrix and R is the radius of each sphere. The Poisson ratio for the granule matrix was chosen to be 0.5. The granule matrix radius was calculated from its height, assuming that the height of a matrix represents the diameter of a spherical object. The tip radius was determined by imaging gold grains (10 nm; Vesenka et al., 1993) and was found to be 44 ± 12 nm (mean ± SD). The force applied by the AFM tip to the matrices was estimated from the displacement of the AFM tip and the spring constant of the AFM cantilever. The spring constant of the cantilevers (193 μm long and 36 μm wide, silicon nitride with integral tips; Digital Instruments) was found to be 53 ± 8 mN/m, as measured from their unloaded resonant frequency in air (Cleveland et al., 1993).

Saline solutions

In addition to the histamine dihydrochloride solution (see AFM imaging), we used three different isosmotic solutions containing cations bearing various valencies: a sodium chloride solution contained 135 mM NaCl and 10 mM Trizma (pH 7.4; 271–275 mmol/kg), a calcium chloride solution contained 110 mM $CaCl_2$ and 10 mM Trizma (pH 7.4; 276–278 mmol/kg), and a lanthanum chloride solution contained 80 mM $LaCl_3$ and 10 mM Trizma (pH 7.4; 265–267 mmol/kg). All solutions were filtered through a 0.2 μm filter. The different solutions were exchanged slowly using a hand-held syringe connected with flexible tubing to the AFM chamber. To keep the AFM tip in the same scanning position, the tip was not withdrawn during perfusion. In the case of contact AFM imaging we reduced the scan area to zero to prevent damage of the sample during the solution exchange. After the exchange was completed we readjusted the imaging force and restored the scan area.

RESULTS AND DISCUSSION

Imaging the granule matrix

We used the AFM to localize isolated secretory granules from mast cells and to determine whether these granules contain insoluble matrices. Isolated mast cell granules were applied to the surface of a poly-L-lysine-coated glass coverslip and allowed to adhere. Attached granules were stripped of their membranes (see Materials and Methods) and imaged in solution using a conventional contact AFM. After removal of their membranes, the granules appeared as insoluble matrices (Fig. 1, *inset*).

The heights of the isolated matrices were compared with the size distribution of intact granules obtained from EM studies (Chock and Schmauder-Chock, 1989). In our experiments the matrices were condensed in a histamine dihydrochloride solution that imitates the soluble intraluminar content of an intact granule. Under these conditions the heights of the isolated matrices were found to be normally distributed ($r = 0.985$) with an average of 474 ± 197 nm (mean ± SD, $n = 630$; Fig. 1, *bars*). However, EM studies of intact mast cell granules have reported a mean diameter of 624 nm (Chock and Schmauder-Chock, 1989; Fig. 1, *open circles*). This difference in size between intact granules and isolated matrices (624 nm versus 474 nm) cannot be accounted for by the removal of the granule membrane (~5 nm).

The size difference could be due to the softness of the matrix. Because a force must be applied to the sample (about 1–2 nN) to obtain an image in the contact mode, the sample deforms. This can be seen in the inset of Fig. 1, where the matrices do not appear to be spherical and deform in the scanning direction. Similarly, the height of a soft matrix will be underestimated as the AFM tip compresses it. An accurate estimate of this error can be made only if the elastic modulus of the matrices is known. In spite of these inaccuracies, imaging confirmed the presence of an insoluble and elastic matrix in the lumen of mast cell granules. Additionally, the images provided the x,y coordinates that we used to place the AFM tip on top of a single granule matrix to examine their mechanical properties.

Swelling of the granule matrix

Swelling is a revealing mechanical property of ion exchange gels. The degree of swelling of an ion exchange gel depends on the degree of cross-linking of a polymer and the nature of the counterion. In weakly cross-linked gels, such as those found in the lumen of beige mast cell granules, the valency of the counterion is the most important factor in swelling (Curran and Brodwick, 1991; Fernandez et al., 1991; Helfferich, 1962). The ability of a gel to take up free water depends on the number of counterions in the gel. Because a gel has a fixed number of charges, the requirement for electroneutrality predicts that replacement of a monovalent counterion by a divalent counterion cuts the number of counterions in the gel by half. Consequently, the number of water molecules also decreases and the gel shrinks. Thus it is evident that the degree of swelling decreases with the increase in the valency of the counterion.

We tested whether granule matrices, isolated from normal mast cells, behave like weakly cross-linked ion exchangers. Initially we condensed granule matrices in a histamine dihydrochloride solution and imaged them using contact AFM (Fig. 2 *A*). After perfusion with a sodium

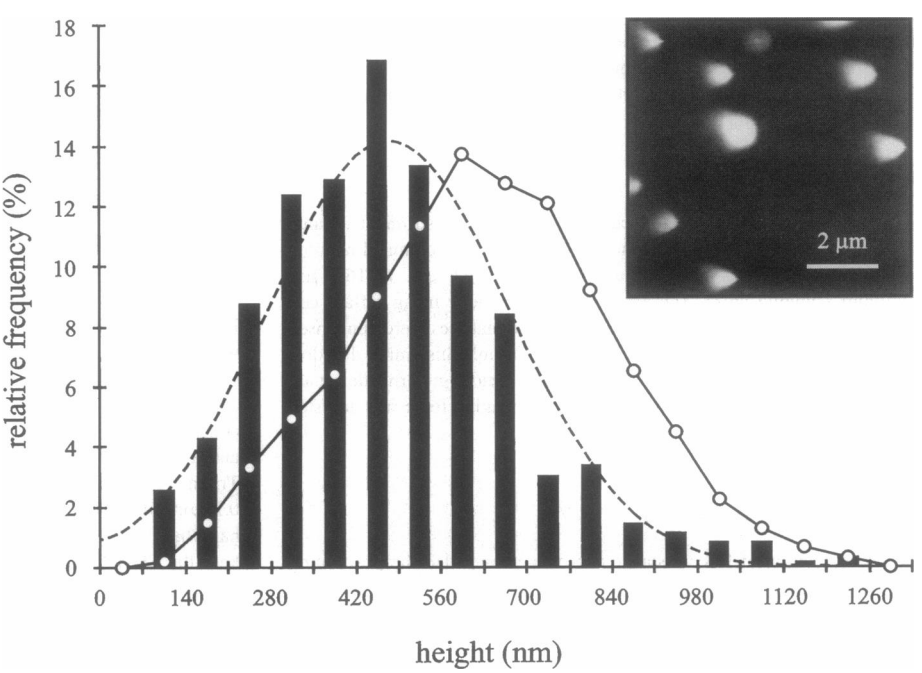

FIGURE 1 Height distribution of condensed granule matrices measured with the atomic force microscope (*solid bars*). This distribution is similar to the diameter distribution of mast cell granules measured with electron microscopy (*circles*; adapted from figure 2 of Chock and Schmauder-Chock, 1989). The dashed lines indicate a fitted Gaussian distribution to the height data. The inset shows a typical contact mode AFM image of isolated granule matrices. The matrices were kept condensed in a histamine solution.

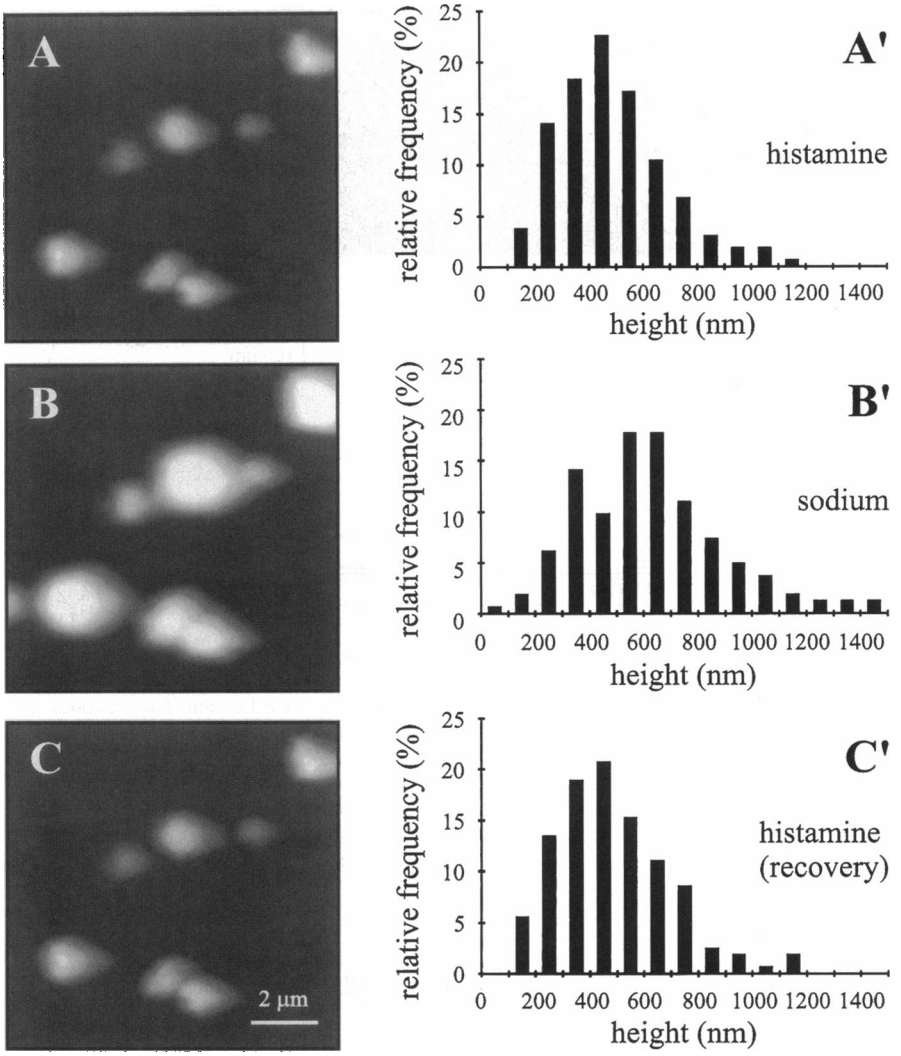

FIGURE 2 Isolated secretory granule matrices swell reversibly in response to ion exchange. (*A*) Contact AFM image of isolated granule matrices condensed in a histamine-containing solution. (*B*) Contact AFM image of the same granule matrices as in *A*, after exchange of the histamine-containing solution by a sodium-containing solution. It is apparent from the images that the granule matrices swell. (*C*) The swelling was reversible because the granule matrices recover their original size after perfusion with a histamine-containing solution. The insets on the right (*A'–C'*) show height distributions (n = 164) measured under the three conditions utilized in *A*, *B*, and *C*. In all cases the secretory granule matrices were imaged by the same force (1.5 nN).

chloride solution, the matrices increased their height (from 488 ± 205 nm in histamine to 622 ± 267 nm in sodium; n = 164; Fig. 2 *B*). The increase of the matrix height was reversible because the granule matrices returned to their original height after perfusion with a histamine-containing solution (488 ± 209 nm; Fig. 2 *C*). The increase in the granule matrix height due to ion exchange was 29% on average, as measured by contact AFM imaging. This value is smaller than the amount of swelling measured in the giant granules of the beige mouse mast cell (>50%; Fernandez et al., 1991). This discrepancy may be the result of changes in the elasticity of the granule matrix during swelling. Because the contact AFM requires relatively large tip forces (1–2 nN), a decrease in the elastic modulus of the granule matrix during swelling will cause a larger deformation of the matrix for the same applied force, underreporting the height of the swollen matrix. Hence we sought to develop an alternative method for measuring the height of a secretory granule matrix that requires forces substantially smaller than those used in contact AFM imaging. This method is demonstrated in Fig. 3 and makes use of force-distance curves acquired on top of an isolated granule matrix.

We collected force-distance curves on top of a single matrix (Fig. 3 *A*, position 2) in both histamine (Fig. 3 *D*, Hist) and sodium (Fig. 3 *E*, Na^+) solutions. Force-distance curves on the glass surface (Fig. 3 *A*, position 1) in the vicinity of a matrix were acquired to determine the baseline height (Fig. 3 *C*). The height measurement was obtained from the intercept of a force-distance curve and a horizontal line (Fig. 3 *C–E*). This intercept typically occurred at a force of about 100 pN, which is 10- to 20-fold less that the forces required for contact AFM imaging. Every force-distance curve has a linear region where the cantilever deflection is constant because the tip is off the surface (right side of force-distance curves in Fig. 3 *C–E*). Using linear regression and the first 200 sampling points from the linear region of the force-distance curves, we established a line that fits the linear region of the force-distance curve. The line intercepts the sloped region of the force-distance curve. This intercept represents the height of a sample. To compensate

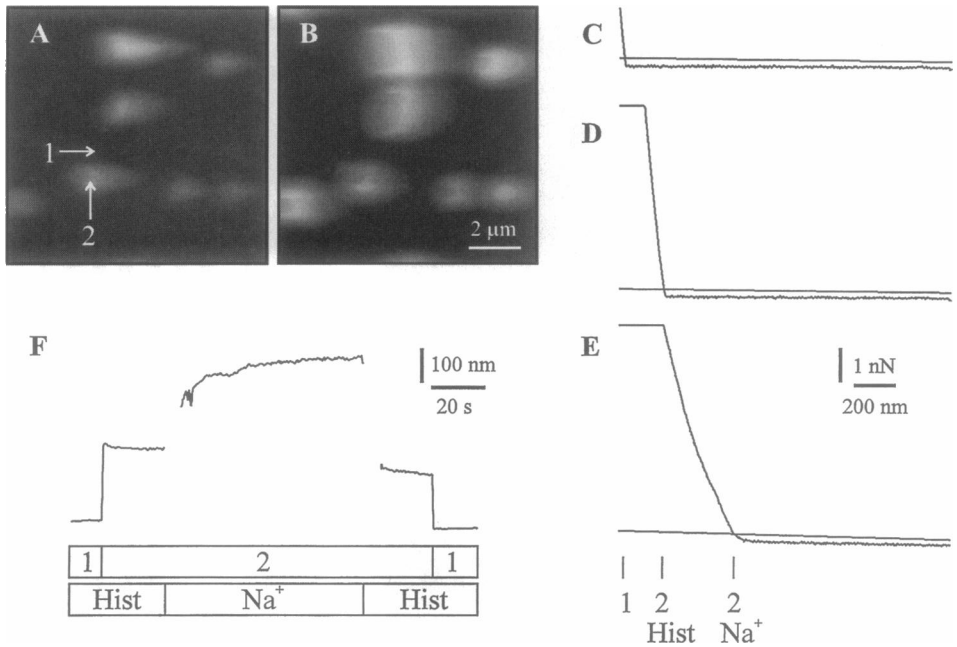

FIGURE 3 Isolated secretory granule matrices reversibly change their volume in response to ion exchange. (*A*) Isolated granule matrices, condensed in a solution containing histamine, were imaged in contact AFM to find their location. Two positions are noted: 1, a contact point on the glass surface; 2, a contact point at the top of a granule matrix. (*B*) Replacement of histamine by sodium causes swelling of the matrices. (*C–E*) Force-versus-distance curves obtained on the glass surface at position 1 (*C*); at position 2 in histamine (*D*) and at position 2 in sodium (*E*). The height of the contact point is measured as the intercept of the force-distance curve with a horizontal line. The contact point on the glass surface marks the baseline height (*C*). The height of the condensed matrix is obtained at contact point 2 (*D*). The height of the matrix swollen in sodium is measured in *E*. A continuous sampling of the height, using the technique shown in *C* and *D*, demonstrates reversible swelling of the matrix upon ion exchange (*F*). The labels under the trace indicate the position of the contact point (1 or 2) and the main cation of the bathing solution (*F*). The gaps in the trace correspond to artifacts generated by the perfusion of the AFM chamber.

for the noise in the system, an offset was applied to the line, so that we can reliably measure the intercept.

Changing the bath electrolyte from histamine dihydrochloride to sodium chloride caused the matrix to swell, as monitored by the change in height (from 390 ± 103 nm in histamine to 698 ± 170 nm in sodium; 83 ± 29% increase; $n = 15$; Fig. 3 *D–F*). The matrix returned to its original size after perfusion with a histamine-containing solution (Fig. 3 *F*). To account for any changes in height due to a perfusion artifact, we have done identical experiments on a glass surface. Changing the electrolyte from histamine dihydrochloride to sodium chloride caused an insignificant change in the height of the glass surface (−5 ± 5 nm; $n = 6$). The swelling of the granule matrices, in response to the replacement of histamine by sodium, is similar to that measured in the giant granules of the beige mouse using a light microscope (Curran and Brodwick, 1991; Fernandez et al., 1991).

In an intact granule the gel matrix is surrounded with a lipid membrane. The intact granular membrane acts as an ionic barrier. Upon exocytosis the granular matrix is exposed to the extracellular space through an open fusion pore. Opening of a fusion pore allows extracellular cations (e.g., sodium) to penetrate the gel and exchange with the stored granular cations (e.g., histamine and serotonin). This ion exchange causes swelling of the matrix (Curran and Brodwick, 1991; Fernandez et al., 1991). In contrast, isolated granules with an intact membrane do not change size in response to external ionic replacements because ion exchange is prevented by the granule membrane. This can be seen in Fig. 4, where it is shown that replacing the bathing histamine dihydrochloride solution with a sodium chloride solution has no effect on the size of the intact granule. However, exposure of the granule to a detergent (0.01% Triton X-100) broke down the granule membrane and exposed the gel matrix to the bathing saline. Under these conditions the gel matrix was observed to swell reversibly in response to ion exchange, as previously shown in Fig. 3.

As predicted from the physical chemistry of weakly cross-linked ion exchangers (Helfferich, 1962), the matrix of mast cell granules changed its volume after the replacement of the bathing cations. Furthermore, when the divalent histamine stored in an intact granule was replaced by sodium ions, rapid swelling of the granule matrix was observed, similar to the swelling of individual granules observed in vivo (Fernandez et al., 1984).

Elasticity of the granule matrix

The mechanical properties of a material depend on its molecular structure. If secretory products are stored in the lumen of a secretory granule as a salt, then the lumen should

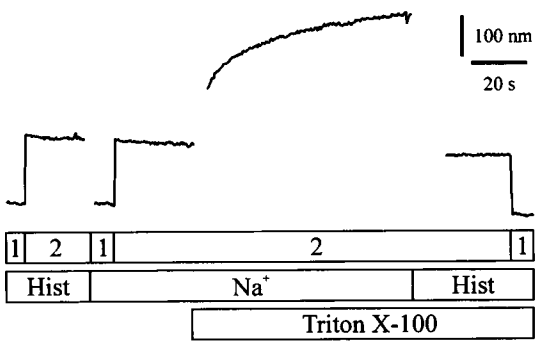

FIGURE 4 An intact granule membrane prevents ion exchange between the granule matrix and the bathing cations, entrapping histamine and serotonin inside a condensed matrix. The removal of the granule membrane with a detergent exposes the granule matrix to the bathing solution containing sodium ions. Ion exchange between the stored amines and sodium causes swelling of the matrix, as observed in vivo. Swelling is reversible because the addition of histamine recondenses the matrix to a height similar to that of an intact granule. The labels under the trace indicate the position of the contact point (1, on the glass surface; 2, on top of a granule), the main cation of the bathing solution, and the presence of a detergent in the bathing solution. The gaps in the trace correspond to artifacts generated by the perfusion of the AFM chamber.

have an elastic modulus similar to that of wet crystals (e.g., $E \approx 10^9$ Pa; Morozov and Morozova, 1993). In contrast, if secretory products are stored bound to a gel matrix, the lumen should have an elastic modulus similar to that of hydrated gelatin (e.g., $E \approx 10^5$ Pa; Radmacher et al., 1995).

To examine whether granule matrices found in the lumen of secretory granules are hard objects or have the elasticity of gels, we measured the elastic modulus of isolated granule matrices using the AFM. The elastic modulus was determined by measuring the elastic displacement of the granule matrix by the AFM tip for a given applied force. The AFM tip cannot measurably deform the hard surface of glass. Hence, upon touching the glass surface, the AFM cantilever will be deflected linearly by a z axis displacement of the sample (Fig. 5 *A*). However, when the AFM tip is in contact with the granule matrix, the same z axis displacement of the sample results in a smaller cantilever deflection. The elastic displacement of the granule matrix by the AFM tip is calculated as the difference between the displacement of the cantilever when the AFM tip touches glass and when it touches the matrix (Fig. 5 *A*). The loading force applied by the tip to cause the elastic displacement of the sample is calculated as the product of the cantilever deflection multiplied by its spring constant.

The loading force versus elastic displacement relationship (Fig. 5 *B*) was well described by a model derived by Hertz (1882) for the elastic displacement of a spherical body (the granule matrix) caused by a spherical object (the AFM tip) (see Eq. 2 and Materials and Methods). A fit of Eq. 2 to the data (Fig. 5 *B*, *solid lines*) gives an estimate of the elastic (Young's) modulus of the sample. Individual granule matrices swollen in a sodium-containing solution had an elastic modulus of 46 kPa (median; range 4–114 kPa; $n = 11$; Fig. 5 *B*). Granule matrices condensed in a histamine-containing solution were about 39 times stiffer, showing an elastic modulus of 1.96 MPa (median; range 175 kPa to 5.16 MPa; $n = 11$). The elastic modulus of the granule matrices measured here is at least two orders of magnitude smaller than that expected for a wet crystal (Morozov and Morozova, 1993). However, it falls in the range of the elastic modulus of gelatin under various degrees of swelling (Radmacher et al., 1995).

The elastic modulus of cross-linked polymers is known to depend on the degree of swelling in a given solvent. Because rubber is one of the most commonly studied cross-linked polymers, it is useful to briefly consider its elastic properties (Treloar, 1975). There are three known parameters that influence the elastic modulus of rubber: temperature, degree of cross-linking, and swelling caused by the sorption of a solvent. Assuming that the temperature and the degree of cross-linking remain constant, the relationship between the degree of swelling and the elastic modulus is given by

$$E \cong a \times (V_f/V_i)^{-5/3}, \quad (3)$$

where E is elastic modulus, V_f/V_i is the swelling relative to the initial volume (V_i), and a is a constant. Thus the elastic modulus of a rubber-like polymer decreases with swelling.

We examined whether granule matrices have the properties of a rubber-like cross-linked polymer. To vary the degree of swelling, we exposed isolated granule matrices to electrolytes containing cations of increasing valency. As previously shown for the giant granules of the beige mouse (Curran and Brodwick, 1991; Fernandez et al., 1991), we found that the degree of swelling of the granule matrix decreases with increased valency ($Na^+ > Ca^{2+} > La^{3+}$) of the external cation (Fig. 6 *A*). We estimated the swelling of the granule matrix in a given solution (V_f) relative to the size of a matrix condensed in a histamine-containing solution (V_i) (Fig. 6 *B*). The estimates were obtained assuming that the height of a matrix represents the diameter of a spherical object. We measured the elastic modulus of the isolated granule matrices under various degrees of swelling by fitting the Hertz model to force-elastic displacement curves, as was done in Fig. 5 *B*. We found that the elastic modulus increased with the valency of the external cation ($Na^+ < Ca^{2+} < La^{3+}$; Fig. 6, *C* and *D*). Fig. 7 shows that the elastic modulus is inversely related to the degree of swelling. Furthermore, the data are well described by a power law similar to Eq. 3 (Fig. 7, *solid line*), as predicted for a rubber-like cross-linked polymer.

Taken together, these data indicate that the lumen of the secretory granules found in mast cells contains a cross-linked, rubber-like polymer that has an elasticity similar to that of gelatin.

Scatter in the elasticity measurements

For any given ionic condition we have observed large scatter in the measurement of elasticity of granule matrices

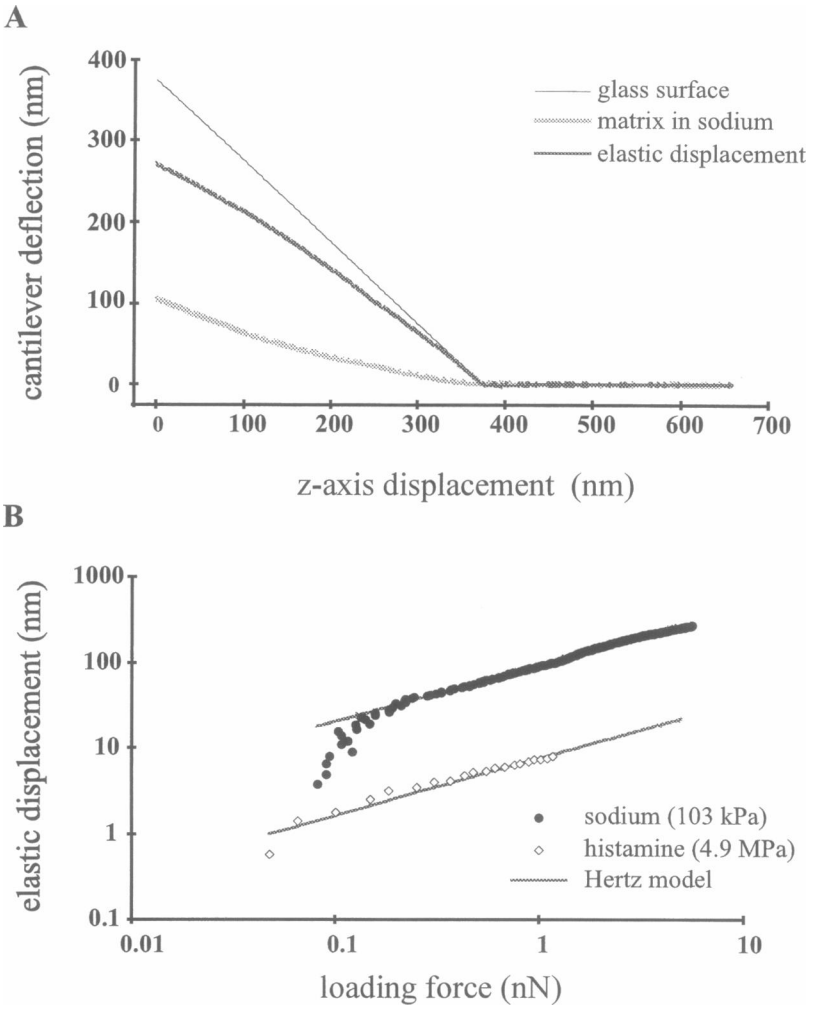

FIGURE 5 Estimate of the elastic modulus of a granule matrix from its elastic displacement by an AFM tip. (*A*) Force-distance curves were taken on the glass surface and on top of a swollen granule matrix bathed in a sodium-containing solution. Because of the elastic displacement of the granule matrix by the AFM tip, the same z axis displacement results in a smaller cantilever deflection when the tip is in contact with the matrix than with the glass surface. The elastic displacement of the matrix by the AFM tip is calculated as the difference between these two force-distance curves. (*B*) Plots of elastic displacement versus loading force obtained from matrices bathed in solutions containing sodium (●) or histamine (◇). The loading force is calculated as the product of the cantilever deflection multiplied by its spring constant. The force-elastic displacement relationship is best described by the Hertz model (*solid lines*) for the elastic displacement of a spherical elastic body (granule matrix) by a spherical object (the AFM tip). The Young's modulus was estimated to be 103 kPa for a swollen matrix (in sodium) and 4.9 MPa for a condensed matrix (in histamine).

(Fig. 6 *D*). In our experiments we used the elastic displacement of a gel matrix by the AFM tip to calculate the elastic modulus of the gel (Figs. 5 *B* and 6, *C* and *D*). Because this calculation (Eq. 2) takes in account the radii of both the AFM tip and the granule matrix, the elastic modulus should be independent of the matrix size. Surprisingly, we observed that the elastic modulus depended upon the radius of the matrix (Fig. 8). This observation suggests that the elastic modulus is not constant. The reason for this is unknown, but there could be some biological and biochemical differences among granule matrices that can explain this radial dependency. For example, the gel matrix that forms the core of mast cell granules is made of cross-linked proteoglycans. In a cross-linked gel, the elastic modulus is directly related to the molecular weight of the polymer chains and to the degree of cross-linking. The elastic modulus is given by the expression

$$E \approx N \times k \times T = \rho \times R \times T/M_w, \qquad (4)$$

where E is the elastic modulus; N is the number of chains per unit volume, which is determined by the degree of cross-linking; k is the Boltzman constant; T is temperature; ρ is density; R is the gas constant; and M_w is the average molecular weight of the polymer chains (Treloar, 1975). It is possible that secretory granules vary in the degree of cross-linking of their gel matrices, changing N. Likewise, the average molecular weight of the proteoglycans may vary, changing M_w.

It has been demonstrated that the molecular weight of the proteoglycan (heparin), from the mast cell granule matrices, varies from about 200,000 to 2,000,000 Da (from figure 2 of Yurt et al., 1976). Because the elastic modulus is directly related to the molecular weight (Eq. 4), this variability in molecular weight, by up to 10-fold, may explain some of the scatter. Furthermore, Marszalek et al. (manuscript in preparation) measured the diffusion coefficient (D_{gel}) for a single isolated granule matrix of the beige mouse mast cell and found it to range from 1.1×10^{-7} cm^2/s to 2.6×10^{-7} cm^2/s. This diffusion coefficient is related to its elastic modulus (E) by $E \approx D_{gel}/a$, where a is a constant (Tanaka and Fillmore, 1979). Based on the scatter obtained from the diffusion coefficient measurements, we would expect about a threefold scatter in the elastic modulus for granules of similar size. Indeed, we found that the elasticity measure-

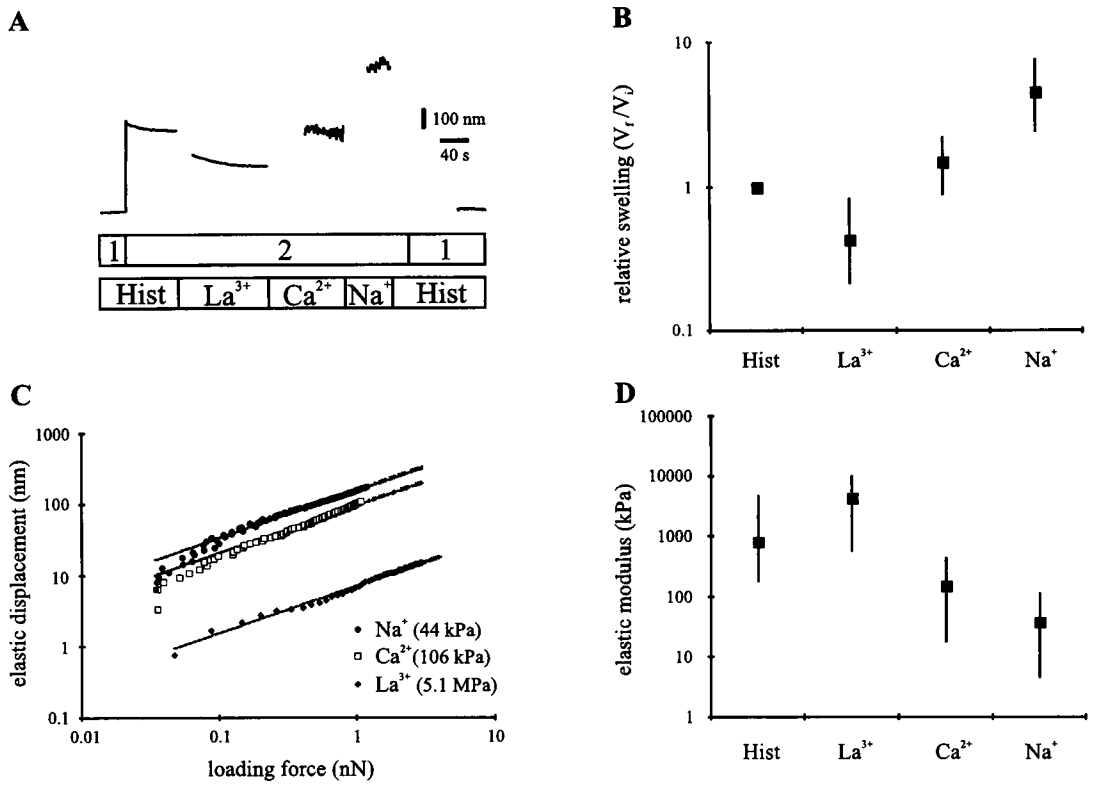

FIGURE 6 The swelling of a granule matrix decreases with an increase in the valency of the bathing cations. (*A*) Continuous sampling of the height of an isolated granule matrix under different ionic conditions. As before, the labels under the figure describe the position of the AFM tip during the recordings (top label: 1, on the glass surface; 2, on top of a matrix) and the cation of the bathing saline (bottom label). The gaps in the trace are due to perfusion artifacts. (*B*) The relative swelling of a matrix (expressed as a ratio of the final volume in any given electrolyte and the initial volume in histamine) decreased with an increase in the valency of the bathing cation. The points indicate medians (n = 6), and the vertical lines indicate the range. (*C*) The elastic modulus of a granule matrix estimated by fitting the Hertz model (*solid lines*) to force-elastic displacement curves obtained for a matrix bathed with saline solutions containing sodium (●), calcium (□), or lanthanum (◆). (*D*) The elastic modulus of a granule matrix strongly depends on the valency of the bathing cation. The values of the elastic modulus shown in the figure were calculated by fitting the Hertz model to the data obtained from granule matrices with different radii, in the range of loading forces from 0.8 to 1.2 nN. The points indicate medians (n = 6), and the vertical lines indicate the range.

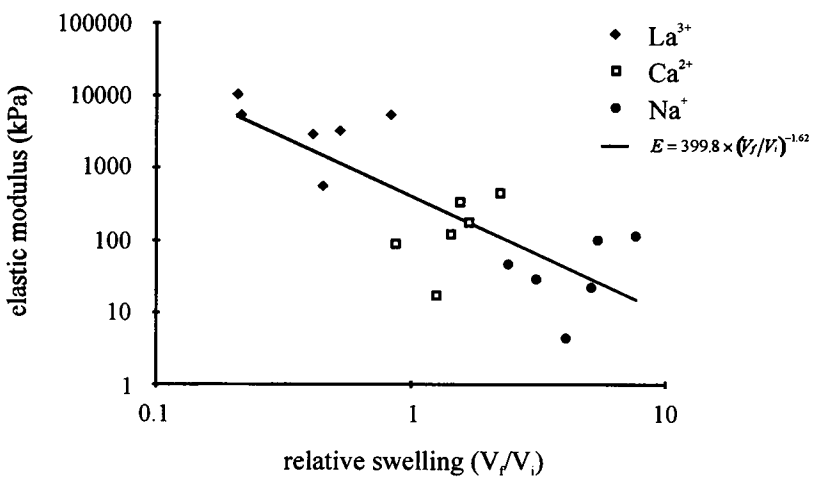

FIGURE 7 The relationship between the elastic modulus and swelling was described by a power relationship given by $E = a \times (V_f/V_i)^b$ (*solid line*) (a = 399.8 kPa; b = −1.62; r = −0.781).

ments exhibit a similar scatter and the elastic modulus of the matrix varies up to sixfold for granules of the same size (e.g., for granule matrices with radii of 211 and 225 nm, respectively, elastic moduli of 560 kPa and 3.25 MPa were calculated when the matrices were immersed in a lanthanum-containing solution). This observation may indicate that the variability in the measurements of the elastic modulus originates from differences in the biochemical composition among granule matrices. It is possible that elasticity measurements of granule matrices, such as those demon-

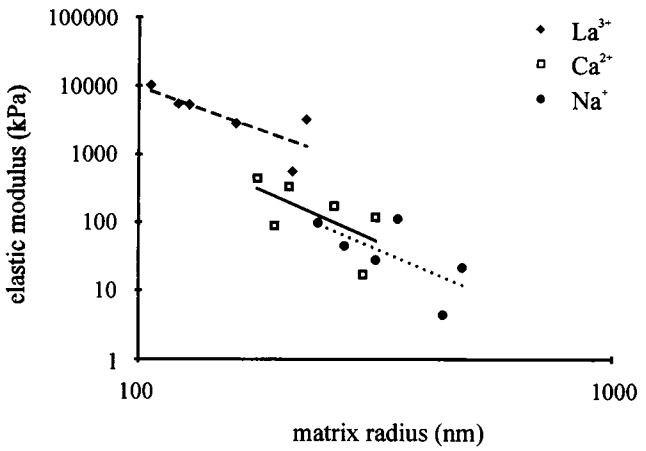

FIGURE 8 Elastic modulus of granule matrices as a function of their radius, measured in saline solutions containing La^{3+}, Ca^{2+}, or Na^{+}. The elastic modulus of the granule matrices depends on their radii. The solid lines are fits of the equation $E = a \times r^{b}$ (solid lines) (a = 1.06 TPa, 2.29 TPa, and 760 GPa for La^{3+}, Ca^{2+}, and Na^{+}, respectively; b = −2.52, −3.05, and −2.91 for La^{3+}, Ca^{2+}, and Na^{+}, respectively). The radial dependency could be the result of a variability in a molecular weight of a proteoglycan (heparin) that composes the granule matrix.

strated in this work, could be used to determine the molecular parameters (e.g., molecular weight and degree of cross-linking) of the underlying proteoglycans.

Contact AFM imaging of secretory granule matrices

Although our initial intention was to use the standard contact AFM mode of imaging for our studies, it became apparent that standard AFM imaging is of limited use when studying small elastic samples like secretory granule matrices. When used to make a measurement, the AFM tip applies a force to the sample. Soft samples such as gelatin deform in response to the applied force. Hence a spherical gel will appear deformed in the scanning direction and will have a reduced height (Fig. 1, *inset*, Fig. 2, and Fig. 3, *A* and *B*). This problem is evident in Fig. 1, where the distribution of heights of the granule matrices is smaller than that of the intact granules observed with EM techniques. Only a small part of this difference may be the result of the removal of the granule membrane. The difference may also be caused by the preparation of the samples for EM or real size differences between the granule samples included in these studies. However, it is likely that the height distribution measured with contact AFM imaging is an underestimate due to compression of the gel matrices. We found that matrices condensed with a histamine dihydrochloride solution were deformed in the z direction by 12 nm at 1 nN and by 21 nm at 2 nN of force, a typical force range used for contact AFM imaging. As the first approximation we can simply add these values to the height distribution (Fig. 1) to correct for the compression of the sample. After this correction the average height of the granule matrices was increased from 474 ± 197 nm to 490 ± 196 nm (the histogram of Fig. 1 includes matrices imaged with different applied forces). However, the corrected mean height is still smaller than the mean diameter of 624 nm measured by EM.

The compression of an elastic sample by the AFM tip depends upon the elastic modulus of the sample; it decreases as the elastic modulus increases. This is evident in Fig. 2, where the average height of granule matrix in a histamine-containing solution increased by 29% on average after perfusion with a sodium-containing solution when imaged by contact AFM. This swelling is substantially smaller than expected (>50%; Fernandez et al., 1991). The discrepancy can be explained by an ~40-fold decrease in the elastic modulus when histamine is replaced by sodium, indicating that the softer granular matrix in sodium can be deformed by an AFM tip more than the same matrix in histamine. For example, at an imaging force of 1.5 nN (the force that was used to acquire images shown in Fig. 2), the AFM tip deforms granule matrices by 16 and 201 nm in histamine- and sodium-containing solutions, respectively. From these observations it is desirable to minimize forces when measuring the height of soft secretory granule matrices. In this paper we used the force-distance curve technique because it requires 10- to 20-fold lower forces than contact AFM imaging to measure the height of the secretory granule matrices. These forces (~100 pN) decrease the compression of the granule matrix. For example, for a force of 100 pN a granule matrix condensed in histamine deforms by only 3 nm (median, n = 11), whereas a matrix swollen in sodium deforms by 30 nm (median, n = 11). Furthermore, when the force-distance curve technique is used to measure height, we observed that upon ion exchange of histamine by sodium, granules swelled by 83% (Fig. 3 *C–F*). These measurements are in good agreement with previously reported values (Fernandez et al., 1991). Although the force-distance curve technique is more accurate, it is far more laborious than contact AFM imaging, and therefore a combination of two techniques is optimal for studying the mechanical properties of secretoy granule matrices.

In addition to compression of the matrix by the AFM tip, another explanation for the nonspherical and elongated shape of the granule matrices originates from the tip shape and the time delay of the feedback loop. Scanning with the pyramidal tip results in a square-like shape of the granule matrix (e.g., Fig. 1, *inset*), whereas the time delay of the feedback loop results in "shadows" within the images in the scanning direction (e.g., the scanning direction is from left to right in Fig. 3, hence the shadows appear on the right of the granule matrix).

The mast cell matrix as an ion-exchange gel

Mast cell secretory granules are known to contain heparin proteoglycans (e.g., Metcalfe et al., 1981) and biogenic amines such as histamine and serotonin. The heparin proteoglycans are highly sulfated, resulting in an abundance of

negative charges, causing these proteoglycans to behave as ion exchangers, as observed by Uvnäs and Åborg (1989). Our results show that the amine-proteoglycan complex forms a cross-linked soft gel with ion exchange properties. A simplified view of the hypothetical architecture of a secretory granule is shown in Fig. 9. We speculate that the gel is assembled from miniature units, represented by the cross-linked cubes shown in Fig. 9 *A*. In this model every unit contains a certain number of fixed negative charges. Because bulk electroneutrality must be satisfied at all times, the fixed negative charges are always screened by counterions (Fig. 9 *B*). In native intact granules the counterions are acidic histamine ($Hist^{2+}$) and serotonin ($5\text{-}HT^{2+}$). In our experiments the endogenous counterions were replaced by the cations contained in our test solutions (e.g., Na^+ and Ca^{2+}). Because the negative charges of the cross-linked gel are fixed, counterion replacement can only be done by ion exchange. Displacement of a divalent counterion (e.g., Ca^{2+}) by ion exchange with a monovalent counterion (e.g., Na^+) causes the gel to swell. The swelling is the result of doubling the number of counterions per cross-linked unit, as required by electroneutrality. The increase in the number of counterions inside the gel causes an increase in osmotic pressure. As a result of increased osmotic pressure, water molecules move into the gel, causing swelling (Fig. 9 *B*). This simple description of ion exchange gels is valid only

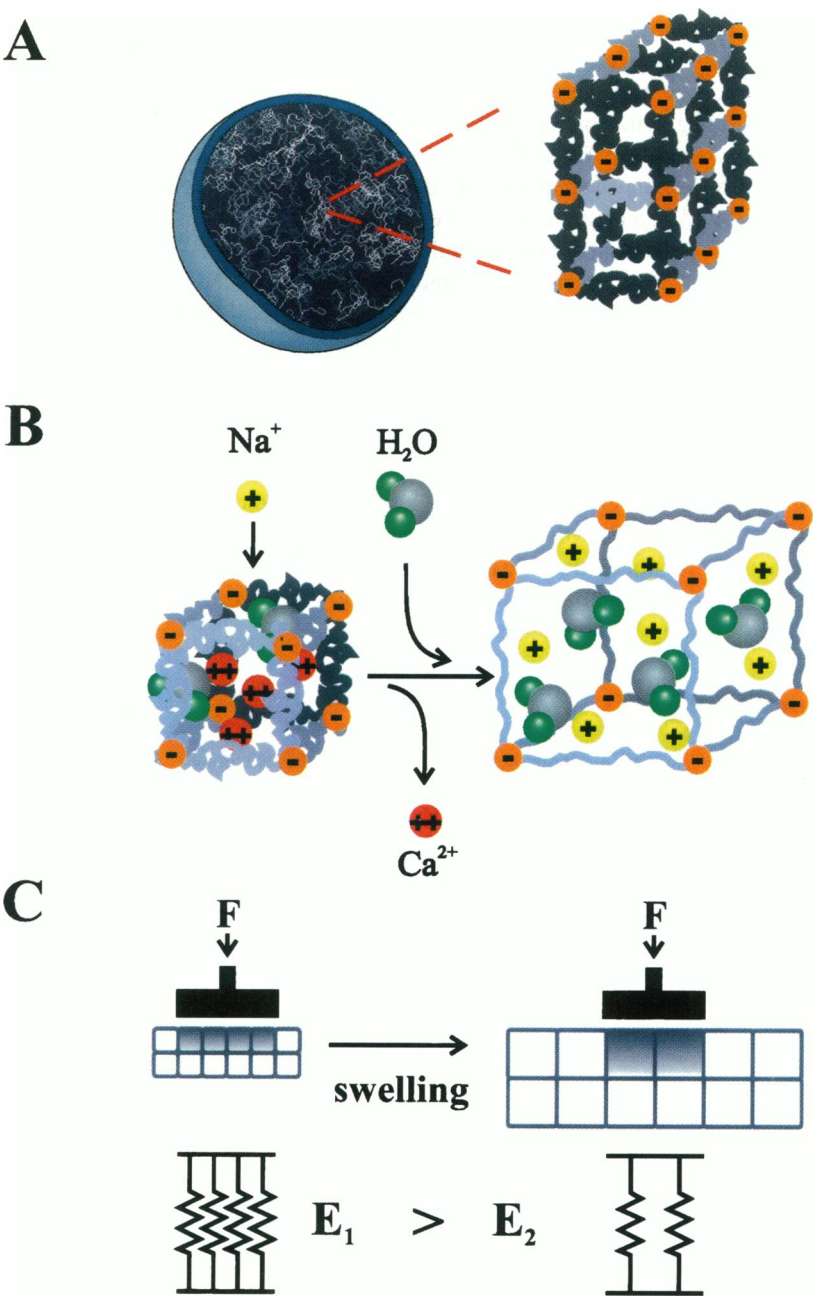

FIGURE 9 A hypothetical representation of the structure of a secretory granule. (*A*) A mast cell secretory granule consists of a lipid membrane coating a condensed heparin proteoglycan gel matrix. The matrix can be envisioned as an assembly of units (cubes) formed by cross-linked polymers. (*B*) A unit contains a fixed number of negative charges (eight). Electroneutrality requires four divalent cations (e.g., calcium) to screen these charges. Upon ion exchange with a monovalent cation (e.g., sodium), the number of cations in the gel doubles, increasing its osmotic pressure and causing it to swell. (*C*) The elastic displacement of the granule matrix by the AFM tip is resisted by the elastic properties of the cross-linked polymers. Each unit of the matrix is represented by a spring. The density of the polymers (springs) is reduced with swelling of the matrix, reducing the elastic modulus.

for gels that are weakly cross-linked (Helfferich, 1962) and describes the swelling of the secretory granule matrices (Figs. 3, 4, and 6 *A*).

As a consequence of the swelling of the gel matrix, the density of the gel decreases. This explains the large reductions in the elastic modulus that are observed when the granule matrices are allowed to swell. Fig. 9 *C* explains this mechanism. It is helpful to consider each miniature cross-linked polymer unit as a spring. By applying force to the AFM tip we can deform the granule matrix. The amount of elastic displacement depends on the number of springs that are opposing the action of the tip (Fig. 9 *C*). When the density of the gel decreases during swelling, fewer equivalent springs oppose the AFM tip action, revealing a reduced elastic modulus.

In conclusion, we have demonstrated the use of atomic force microscopy to study the mechanical properties of the lumen of a submicroscopic secretory granule. Using these techniques, we have shown that mast cell granules contain an insoluble gel matrix that has the mechanical properties of an ion exchange gel. These findings extend previous studies done with the giant secretory granules of the beige mouse mast cell and demonstrate that ion exchange gels may be a common feature of secretory granules.

Like mast cells, secretory granules from many cells, including clear synaptic vesicles, contain charged proteoglycans, suggesting that ion-exchange mechanisms controlling the storage and the release of secretory products may be widespread. For example, the neurotransmitter transporter SV2 (a ubiquitous synaptic vesicle protein) is a keratan sulfate proteoglycan (Feany et al., 1992; Scranton et al., 1993). The glycosyl domains from a few SV2 molecules may fill the vesicle volume and form an ion exchange matrix that regulates the release of neurotransmitters. The AFM techniques demonstrated in our study may prove useful in testing this hypothesis.

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