Kinetics of Release of Serotonin from Isolated Secretory Granules. II. Ion Exchange Determines the Diffusivity of Serotonin

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ABSTRACT We measured the efflux of 5-hydroxytryptamine (5-HT, serotonin) from an intact secretory granule extracted from the mast cell of the beige mouse. The efflux was measured with amperometry after rupture of the granule membrane was triggered by electroporation. We determined the diffusivity of 5-HT within the secretory granule to be 2.0×10^{-8} cm^2 s^{-1} when the granule is in contact with a physiological saline and found that this diffusivity depends on the valence of the cation in the external electrolyte. There is a fivefold increase in the diffusion coefficient of 5-HT determined in CsCl (150 mM, pH 7.2) at 3.7×10^{-8} cm^2 s^{-1} compared to that determined in histamine dihydrochloride (Hi, 100 mM at pH 4.5) at 0.7×10^{-8} cm^2 s^{-1}. We found that the rate of expansion of the granule matrix observed in physiological medium correlates with the efflux of 5-HT, and that the rate of swelling of the matrix and the efflux depend on the microviscosity within the granule matrix and not the bulk viscosity of the external solution. The low diffusivity of 5-HT (~500-fold less than in the bulk), the observation that the valence of the counterion affects this diffusivity, and the relationship between the volume changes of the matrix and the efflux suggest that 5-HT is released from the granule by ion exchange. We discuss the implications of this result for exocytotic release in mast cells and propose that an ion exchange mechanism could control the rate of release in other secretory systems.

INTRODUCTION

There is increasing evidence that the release of amines from secretory vesicles undergoing exocytosis does not proceed by pure diffusion (Alvarez de Toledo et al., 1993; Chow and von Rüden, 1995; Wightman et al., 1995; Pihel et al., 1996). The recent amperometric measurements of release from single vesicles in mast and chromaffin cells suggest that the rate of release is too slow to be explained by diffusion alone (Alvarez de Toledo et al., 1993; Chow et al., 1992; Wightman et al., 1995) and seems to depend on the properties of the matrix found within the secretory vesicles (Alvarez de Toledo et al., 1993; Wightman et al., 1995). For example, in chromaffin granules the dissociation of catecholamines from the protein matrix was suggested to be one of the factors controlling the rate of release of catecholamines (Schroeder et al., 1996), and there is evidence in mast cells that the rate of release of serotonin (5-HT) during exocytosis is limited by ion exchange between the matrix and the counterions in the external solution (Marszalek et al., 1996). Less is known about what controls the rate of release of other secretory products (e.g., acetylcholine, glutamic acid, peptides, and proteins). Huang et al. (1995) measured the efflux of insulin from β cells during exocytosis with amperometry and found it to be (as measured by the width of the amperometric spike at half of its amplitude, $t_{1/2}^{spike}$) about six times slower than the efflux of 5-HT measured from the same cells (Zhou et al., 1996). The same group measured similar time courses for hormones released by rat melanotrophs (Paras and Kennedy, 1995), but they did not discuss the mechanisms controlling the release of these peptides.

The core of many secretory vesicles contains a charged polymeric matrix. Because electrostatic interactions between the ionic network and cationic/anionic secretory products are often suggested to be responsible for the condensation of products within the vesicles (Uvnäs and Åborg, 1983; Reggio and Palade, 1978; Reggio and Dagorn, 1978; Zanini et al., 1980), it is probable that an electrostatic mechanism like ion exchange influences the exocytotic release of charged secretory products. Other mechanisms like hindered diffusion and reptation (de Gennes, 1971; Doi and Edwards, 1978; Russell et al., 1993) may also contribute to the kinetics of exocytotic release when the secretory products are polymeric.

In the accompanying paper we outline a method for measuring the efflux of 5-HT from isolated intact secretory granules of the mast cell of the beige mouse. This method uses electroporation of the granule membrane to trigger the efflux of 5-HT and amperometry to detect serotonin at a carbon fiber electrode (Marszalek et al., 1997). In this communication we measure the efflux of 5-HT and determine the diffusivity of 5-HT within the granule matrix. We test the hypothesis that the efflux of 5-HT is influenced by the valence of the external counterion; this would be expected if the efflux were controlled by ion exchange and were not a purely diffusional process. Furthermore, we also change the solvent (e.g., viscosity) of the external medium and examine its affect on the efflux of 5-HT. We conclude

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that the efflux of 5-HT is limited by the low diffusivity of 5-HT within the granule matrix and discuss the implications of this result for exocytotic release.

MATERIALS and METHODS

Reagents

Lanthanum chloride (99.99% purity) was obtained from Alfa Aesar (Ward Hill, MA), cesium chloride (purity > 99.99%) and sodium chloride (purity > 99.99%) were obtained from Aldrich Chem. Co. (Milwaukee, WI), and dextran (500,000 mol wt) was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were purchased from Sigma (St. Louis, MO). Two different types of methylcellulose with viscosities of 1) 23 cp at 3% and 2) 1500 cp at 2% were used. All chemicals were used as received.

Experiments were conducted in various solutions at room temperature. The solutions used were 1) an external solution of either Hanks' balanced salt medium or a modified Ringer's medium (Marszalek et al., 1997); 2) 100 mM histamine dihydrochloride, 2–10 mM citric acid, pH 4.5; 3) 155 mM CsCl, 1.5 mM Tris buffer, pH 7.0; 4) 88 mM $LaCl_3$ and 1–10 mM Tris buffer, pH 7.0; 5) 150 mM NaCl with 10 mM HEPES; and 6) solution 1) with various amounts of sucrose, methylcellulose, or dextran added.

Viscosity and osmolality of solutions

The viscosities of the solutions were measured with a Wells-Brookfield cone-and-plate viscosimeter or a falling ball type viscosimeter (Gilmont Instruments, Barrington, IL). Osmolality was measured with a vapor pressure osmometer (model 5100C or 5500; Wescor, Logan, UT). The viscosity of medium containing dextran was adjusted by varying the dextran concentration (e.g., 20%, 30%) in Hanks' medium. The viscosity of medium containing methylcellulose ranged from 4 to 1500 cp. The low-viscosity solutions (up to 23 cp) were obtained by adding different amounts of methylcellulose (type 1)) to Hanks' medium or histamine dihydrochloride solution, and the high-viscosity solutions were made up in a similar way with type 2) methylcellulose.

Experiments with secretory granules

We followed the procedures described in detail in the accompanying paper (Marszalek et al., 1997).

Experiments with cells

Peritoneal mast cells from the beige mouse were isolated as described (Nanavati et al., 1992). During patch-clamp experiments the solution within the pipette contained (in mM) 140 K-glutamate, 7 $MgCl_2$, 3 KOH, 0.2 ATP, 1 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.2), and 0.01 GTPγS, and the extracellular solution was the same as the modified Ringer's medium outlined in the accompanying paper (Marszalek et al., 1997) except that it also contained 25 mM glucose.

Cell membrane admittance was measured with the patch-clamp technique (Hamill et al., 1981) in the whole-cell mode. A detailed description of the experimental procedure is found in Nanavati et al. (1992). Cell membrane capacitance (C_m) and fusion pore conductance (G_p) were calculated from the cell admittance as outlined by Nanavati et al. (1992). 5-HT released during exocytosis was measured by amperometry (Alvarez de Toledo et al., 1993). During patch-clamp experiments the carbon fiber electrode was placed over the cell. The current was amplified by an EPC-7 patch-clamp amplifier and collected at 75 or 104 Hz.

THEORETICAL BACKGROUND

Kinetics of ion exchange

Ion exchange gels carry positive or negative electric surplus charges that are neutralized by ions of opposite sign, the counterions. The counterions are free to move within the matrix of a gel and can be exchanged for a stoichiometrically equivalent amount of counterions from the external electrolyte. The concentration of coions (mobile ions of the same sign as that of the surplus charge of the matrix) is usually very low within an ion exchanger (Donnan exclusion; Helfferich, 1962), and coions do not generally affect the kinetics of ion exchange. A counterion A that is initially present in an ion exchanger can only diffuse into the solution when another counterion B simultaneously migrates into the ion exchanger to compensate the matrix charge (preservation of bulk electroneutrality). Frequently, the exchange of ions generates a swelling pressure within the gel, and because the matrix of a gel is usually elastic, it swells when taking up solvent.

The core of a secretory granule of the mouse mast cell is a hydrogel that carries a high density of negative charges associated with sulfate and carboxyl groups of heparin sulfate proteoglycan matrix. This matrix attracts positively charged secretory products (e.g., 5-HT, histamine (Hi), and Ca^{2+})). At a native pH of ~6 within the granule (Johnson et al., 1980), the majority of 5-HT (pK_2 = 4.9) is stored in a monovalent form, whereas about half of the histamine (pK_2 = 5.9) is stored in a divalent form. 5-HT, Hi, and Ca^{2+} are the mobile counterions that can diffuse out of the granule matrix. Suspensions of these matrices were shown to display properties of cation exchangers (Uvnäs and Åborg, 1983). A series of images of the matrix extracted from a secretory granule of the mast cell of the beige mouse are shown in Fig. 1 *A*. In the first image a granule devoid of membrane is immersed in sodium saline (r = 3.4 μm). When sodium saline is replaced by an acidic solution of 5-HT (at the pH of 3.9 5-HT is divalent), the granule matrix becomes condensed (r = 2.6 μm) and refractile when viewed with Nomarski optics. When 5-HT is replaced by water, the granule size and refractive index do not change (r = 2.5 μm), at least for the time viewed (15 min). When a high concentration of NaCl was injected into the chamber, the granule matrix expanded (r = 3.4 μm), and an amperometric current of 5-HT was detected at a carbon fiber electrode placed close to the granule (Fig. 1 *B*). Replacement of this solution with 5-HT caused the granule to condense (r = 2.5 μm) again. This simple demonstration shows that the granule matrix of the mast cell of the beige mouse indeed responds to the ionic environment and behaves like a cation exchanger (Uvnäs and Åborg, 1983; Curran and Brodwick, 1991; Fernandez et al., 1991). Moreover, this experiment indicates that, at a single granule level, 5-HT cations can be released rapidly from the matrix only when there is a sufficient number of cations available in the external electrolyte (Fig. 1 *C*).

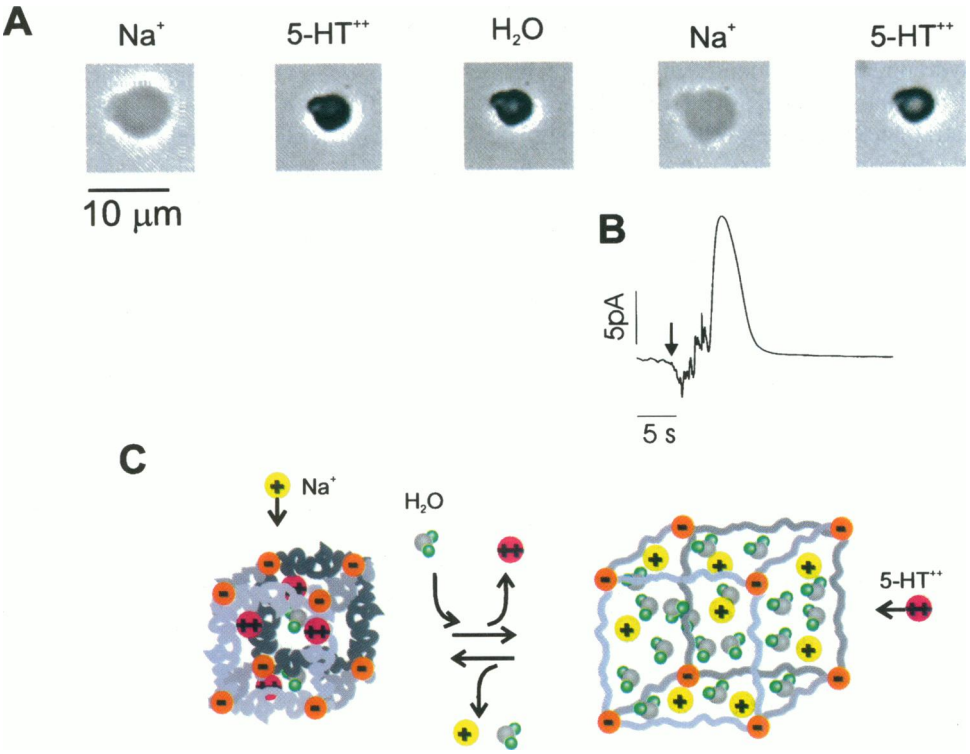

FIGURE 1 Ion exchange between the bathing medium and the polymeric matrix of the secretory granule of the beige mouse mast cell. (*A*) 1) The core of a secretory granule bathed in Ringer's medium, radius $r = 3.4$ μm. 2) Ringer's medium was replaced by 50 mM 5-hydroxytryptamine hydrochloride solution (5-HT · HCl; pH 3.9), $r = 2.6$ μm. 3) 5-HT solution was replaced by water (18 Ω cm), $r = 2.5$ μm. 4) Fifteen microliters of concentrated NaCl solution was injected into the chamber (final concentration ≈375 mM NaCl), $r = 3.4$ μm. 5) This medium was then replaced by 50 mM 5-HT · HCl (pH 3.9), $r = 2.5$ μm. (*B*) An oxidation current of 5-HT that was released from a granule upon ion exchange with Na^+. This current was generated at a carbon fiber electrode placed close to the granule. The injection of NaCl into the chamber is marked by an arrow. The initial part of the signal in *B* (large noise) is an artifact related to the injection procedure itself. (*C*) Schematic showing the exchange of Na^+ with 5-HT^{2+}; both cations bind to the fixed negative charges of the granular matrix.

Ion exchange is an electrodiffusion process and its rate depends upon the mobilities of all exchanging ions; the influx and efflux of all ions are coupled and equal (in equivalents). This is in contrast to pure diffusion, where the fluxes are not coupled and concentration gradients by themselves determine mass transport (cf. Fick's first equation; Crank, 1956). The fluxes of ions during ion exchange can be described by the Nernst-Planck electrodiffusion equation (Bockris and Reddy, 1977; Helfferich, 1962). The rate of ion exchange is found by solving this equation under several initial boundary conditions (Helfferich, 1962; Appendix 1). When ion exchange is limited by the diffusivity of counterions within the secretory granule ("particle" diffusion), the time it takes to exchange half of the 5-HT molecules ($t_{1/2}$) is

$$t_{1/2} = 0.03 \frac{r^2}{D_{5\text{-HT}}} \qquad (1)$$

where r is the radius of a granule, and $D_{5\text{-HT}}$ is the effective diffusion coefficient of 5-HT within the matrix that approximates the interdiffusion of all exchanging counterions (e.g., interdiffusion between 5-HT^+ and Na^+; Appendix 1).

When ion exchange is limited by the diffusivity of counterions within the "unstirred layer" at the interface between the secretory granule and the surrounding medium ("film" diffusion; Helfferich, 1962; Appendix 1), the time it takes to exchange half of the 5-HT molecules is

$$t_{1/2} = 0.23 \frac{r\delta C_{5\text{-HT}}}{D_{5\text{-HT}} C_B} \qquad (2)$$

where δ is the thickness of the film, $C_{5\text{-HT}}$ is the concentration of 5-HT within the matrix, C_B is bulk concentration of external counterion B, and $D_{5\text{-HT}}$ is the diffusion coefficient of 5-HT in the film.

If the rate of ion exchange is limited by film diffusion (Eq. 2), $t_{1/2}$ for exchange should be proportional to the radius of the exchanger and inversely proportional to the concentration of the external counterion ($t \propto r/C_B$). If particle diffusion limits the rate of ion exchange (Eq. 1), $t_{1/2}$ should be proportional to the square of the radius and should not depend on the concentration of the external counterion ($t \propto r^2$).

Kinetics of swelling

In the example illustrated in Fig. 1, two monovalent sodium cations replace one divalent 5-HT cation. This exchange

decreases the chemical potential of water within the granule matrix, and water is absorbed by the gel. In addition to free water, the water hydrating dissolved ions is also taken up by the gel. The total amount of water affects diffusivities of ions within the matrix and therefore swelling can affect the efflux of 5-HT. We describe the kinetics of swelling of the granule matrix with Tanaka and Fillmore's model (Tanaka and Fillmore, 1979; Tanaka et al., 1973). The swelling of a gel is treated as a diffusional process; the polymeric matrix of a gel diffuses as it adsorbs fluid from the surrounding medium. For a spherical matrix with an initial radius of r_o, the rate of expansion of the matrix is given by (Tanaka and Fillmore, 1979)

$$r(t) = r_f - (r_f - r_o)\frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-tn^2}{\tau}\right) \quad (3)$$

where τ is the characteristic time of swelling and is given by $\tau = r_f^2/(D_{gel}\pi^2)$, where r_f is the radius of the gel at its fully swollen state and D_{gel} is the diffusion coefficient of the matrix itself. $D_{gel} \cong K/f$, where K is the bulk modulus of the matrix and f is the frictional constant (Tanaka et al., 1973) between the matrix and the fluid.

We fit the data (Verdugo, 1993) with only the first exponential term of Eq. 3, namely,

$$r(t) = r_f - (r_f - r_o)\exp(-t/\tau) \quad (4)$$

RESULTS AND DISCUSSION

Particle versus film diffusion control

As discussed in the accompanying paper (Marszalek et al., 1997), the rising phase of the amperometric spike recorded from the electroporated granule reflects the time required for complete rupture of the granule membrane. Membrane rupture is initiated by electroporation, driven by the elasticity of the membrane, and augmented by swelling of the granule matrix. This process is initially anisotropic because poration of the membrane occurs with the highest probability at the poles of the granule (see Figure 3 in Marszalek et al., 1997). At this stage the initial efflux of 5-HT is also anisotropic, because it is limited by the position and small size of these early pores. As rupture proceeds (pores expand), the efflux of 5-HT increases. When rupture is complete, 5-HT can diffuse in all directions and therefore the efflux becomes radial. We assumed that the efflux was radial from the onset when we use the model (Eqs. 1 and 2) to describe ion exchange of 5-HT within the secretory granule (Appendix 1). This is a reasonable approximation, because the majority of molecules (65–85%) are released during the exponential phase of the spike (see Figures 6–8 in Marszalek et al., 1997).

To establish if particle (Eq. 1) or film (Eq. 2) diffusion controls the rate of ion exchange, we examined the dependence of the half-time of the integral of the amperometric spike $t_{1/2}^{int}$ on the size of the granule and on the concentration of the counterions in the external saline (Fig. 2 *A*; Ringer's medium, *open symbols*; histamine solution, *filled symbols*). In this figure, r represents the radius of the granule at its fully swollen state r_f, and we plot it instead of the initial radius of the granule (r_0) because the majority of molecules exchange after the granular matrix is fully swollen (see Figure 8 in Marszalek et al., 1997). Although Fig. 2 *A* shows clearly that $t_{1/2}^{int}$ increases with r_f in both Ringer's medium

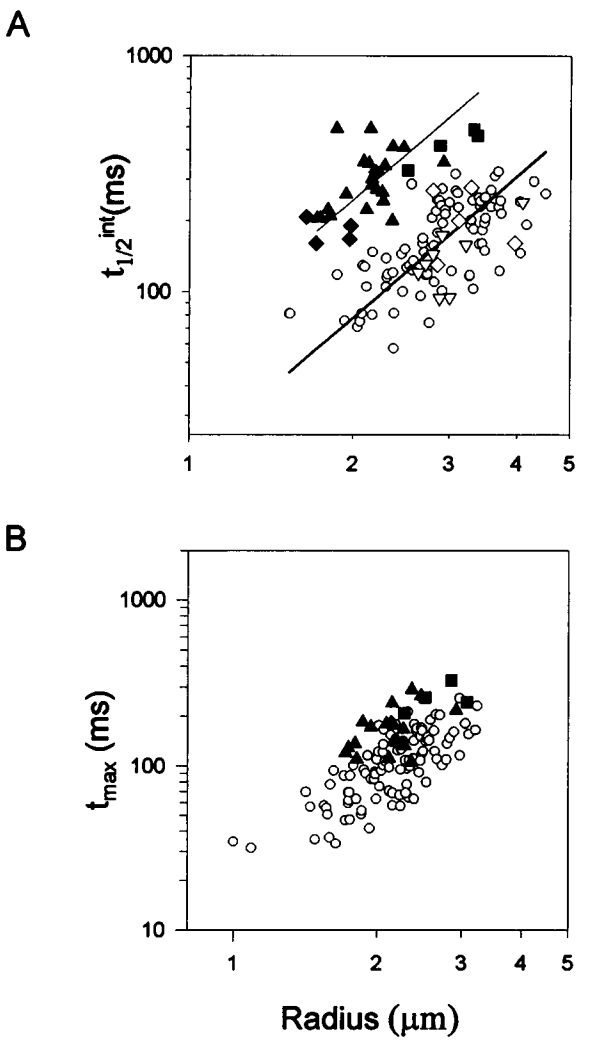

FIGURE 2 A comparison of the half-time $t_{1/2}^{int}$ of the integral of the amperometric spike of 5-HT obtained from granules that were immersed in histamine dihydrochloride (▲, ◆, ■), modified Ringer's medium (○, ◇), and 150 mM NaCl solution (▽, pH 7.2). ▲, ■, ○, ▽, Data obtained when the granule membrane was electroporated; ◆, ◇, data obtained when the granule membrane was dissolved in Triton X-100 (final concentration ~0.005%). The solid lines are fits of $t_{1/2}^{int} = Ar^2$ to data where A is found to be 61 (*upper line*, histamine), and A = 19 (*lower line*, Ringer's). The data obtained in Triton X-100 were not included in the fits. (*B*) A comparison of the rising phase of the amperometric spike recorded in Ringer's (○) and in histamine dihydrochloride (100 mM, ▲) and 1.5 mM (■). t_{max} normalized by r_0^2 is 37 for histamine-containing solution and 21 for Ringer's medium. All data shown were obtained by electroporating the granule membrane.

and histamine solution, we cannot determine unequivocally which process is rate limiting just from the relationship between $t_{1/2}^{int}$ and r_f because the range of r is small (~1.5–4.5 μm), the scatter in the data is relatively large, and fitting a function ($t_{1/2} = Ar^x$; cf. Eqs. 1 and 2) with two unknown parameters (A, x) becomes ambiguous. We therefore measured the efflux of 5-HT at various concentrations of counterion in the external solution because $t_{1/2}$ is proportional to C^{-1} when film diffusion is limiting, but is independent of the external concentration if particle diffusion is limiting (cf. Eqs. 1 and 2 and Appendix 1). We found no significant difference between the efflux of 5-HT measured at 1.5 mM and 100 mM histamine dihydrochloride (*filled squares* and *filled triangles* in Fig. 2 *A*), although Eq. 2 predicts that $t_{1/2}^{int}$ should increase by ~70-fold at 1.5 mM histamine (*filled squares*). A similar result was observed in sodium saline (15 mM versus 150 mM NaCl, data not shown), and this suggests that the efflux of 5-HT is independent of concentration within the 1–100 mM range. We conclude that the efflux is controlled by particle diffusion within the granule matrix and not by film diffusion in the "unstirred" layer.

Comparison of the efflux of 5-HT from granules immersed in physiological medium and histamine dihydrochloride solution

When granules were porated in Ringer's medium, the average $t_{1/2}^{int}$ of the amperometric spike was 173 ms (n = 88; Fig. 2 *A*, *open circles*). We observed similar results when the granule membrane was electroporated in 150 mM NaCl at pH 7.2 (Fig. 2 *A*, *open triangles*). The average $t_{1/2}^{int}$ obtained from the spikes is 144 ms, and the points are within the scatter of the data obtained in Ringer's medium and suggest that the rates of ion exchange in Ringer's medium and NaCl are not significantly different. As expected (Marszalek et al., 1997), similar results were obtained when the granule membrane was not electroporated, but dissolved by the addition of surfactant (Fig. 2 *A*, *open diamonds*).

When granules were porated in 100 mM histamine solution, the average $t_{1/2}^{int}$ was 323 ms (n = 21; Fig. 2 *A*, *solid triangles*). These experiments were conducted at pH 4.5 rather than pH 7.2, because at pH 4.5 histamine is divalent. In contrast to physiological saline, there was no swelling of the granule matrix in histamine solution, showing that release of 5-HT can occur without significant volume changes. This lack of swelling is expected at this concentration of histamine dihydrochloride (Fernandez et al., 1991), because, like 5-HT (Fig. 1), divalent histamine condenses the matrix of the secretory granule. The rising phase of the amperometric spike is ~30% shorter in Ringer's medium than in histamine dihydrochloride (Fig. 2 *B*). This supports the conjecture that the rupture of the membrane is accelerated by swelling of the granule matrix (Marszalek et al., 1997).

Interdiffusion of 5-HT and the counterions within the granule matrix

We fitted the data obtained in histamine (pH 4.5) and Ringer's medium (pH 7.2) to $t_{1/2}^{int} = Ar_f^2$ (Eq. 1, particle diffusion), and the best fits are $t_{1/2}^{int} = 61r_f^2$ (ρ = 0.62) and $t_{1/2}^{int} = 19.4r_f^2$ (ρ = 0.49), respectively. We used Eq. 1 and calculated the effective diffusion coefficient ($D_{5\text{-HT}}$) of 5-HT within the granular matrix to be 4.9×10^{-9} cm^2 s^{-1} for granules in contact with histamine dihydrochloride and 1.5×10^{-8} cm^2 s^{-1} for granules in contact with Ringer's medium. After the diffusion coefficients were corrected for the rising phase of the spikes and the initial anisotropy in the efflux (Appendix 2), the $D_{5\text{-HT}}$ of 5-HT in histamine and Ringer's medium are 0.7×10^{-8} cm^2 s^{-1} and 2.1×10^{-8} cm^2 s^{-1}, respectively (Table 1). The low diffusivity of 5-HT within the granular matrix is ~500 times slower than the diffusivity in the bulk (~10^{-5}), but is typical for diffusion of charged molecules within an ion exchanger (Helfferich, 1962). The diffusivity measured in histamine dihydrochloride is ~3.0 times smaller than that obtained when the external solution was Ringer's medium. This clearly suggests that the external counterion affects the efflux of 5-HT and that this efflux must be coupled to the influx of the external counterion histamine (Appendix 1). We conclude that the interdiffusion of 5-HT$^+$ and histamine (Hi^{++}) within the granular matrix is slower than interdiffusion of 5-HT$^+$ and Na$^+$. This is consistent with observations reported for ion exchangers; the interdiffusion coefficient is greater with exchanging ions of lower valencies (Helfferich, 1962). The difference between the interdiffusion coefficients of 5-HT/Na and 5-HT/Hi is not the result of the lower pH of the histamine solution (cf. 4.5 versus 7.2), because the time course of exchange of 5-HT by sodium at pH 4.5 was similar to the time course measured at pH 7.2 (data not shown). We conclude that protons, at least in this pH range, do not significantly affect the kinetics of 5-HT exchange in the mast cell secretory granules.

TABLE 1 Comparison of the diffusion coefficient ($D_{5\text{-HT}}$) determined from the efflux from the secretory granules of mast cells of the beige mouse when they were immersed in different electrolytes

	% released*	t_{max}/r^2 (ms/μm^2)	$D_{5\text{-HT}}$ (10^{-8} cm^2 s^{-1})#§
CsCl	28	14 (5)	3.7 (0.2)
NaCl	27	20 (6)	3.1 (0.1)
Ringer's	25	21 (7)	2.1 (0.1)
$Hi(HCl)_2$	23	37 (10)	0.7 (0.2)
$LaCl_3$	19	43 (25)	0.6 (0.3)

*Percentage of molecules released during the rising phase of the spike.
#Corrected for rising phase (see Appendix 2).
§At physiological temperatures (37°C), the viscosity of the solution decreases and the diffusivity of 5-HT should be ~45% greater.
Standard deviations are shown in brackets.
Statistical significance of difference in the diffusion coefficient were tested with the Mann-Whitney test for nonparametric data. We found statistical significance ($p < 0.0004$) between Ringer's/CsCl, Ringer's/$Hi(HCl)_2$, CsCl/$Hi(HCl)_2$ and NaCl/$Hi(HCl)_2$.

The low diffusion coefficient of 5-HT within the granule matrix clearly indicates that there are significant intermolecular interactions (e.g., 5-HT$^+$-matrix, Na^+-matrix, Hi^{++}-matrix) that slow down the efflux of 5-HT. This low diffusivity of 5-HT is consistent with ^1H NMR studies of histamine interactions within the granule matrix of the rat mast cell (Rabenstein et al., 1987). They found that exchange between bound and free histamine (intragranular) was fast, but the exchange between intragranular histamine and the extragranular histamine was slow and suggested that electrostatic interactions between the positively charged histamine and negatively charged granule matrix slow down this exchange. It is likely that similar interactions should affect the diffusivity of 5-HT within the matrix. Because the physical properties (size and functional groups) of Hi are closer to 5-HT than to Na^+, exchange of 5-HT for histamine should be closer to isotopic exchange (Appendix 1) than the exchange of 5-HT for Na^+. The D for the 5-HT/Hi pair of 0.7×10^{-8} cm^2 s^{-1} should therefore be close to the self-diffusion coefficient of 5-HT, $D^{self}_{5\text{-HT}}$. The self-diffusion coefficient for sodium within the granule matrix D^{self}_{Na} must be significantly higher than $D^{self}_{5\text{-HT}}$ and D^{self}_{Hi} to account for the threefold increase in the effective diffusion coefficient of the 5-HT/Na^+ pair (nonisotopic exchange) over the 5-HT/Hi pair (quasi-isotopic exchange). Using numerical solutions of the equation describing the kinetics of nonisotopic ion exchange provided by Helfferich and Plesset (1958), we can estimate D^{self}_{Na} to be at least 10-fold higher than $D^{self}_{5\text{-HT}}$.

Efflux of 5-HT depends on the valence of a counterion

To further investigate the influence of external counterions on the efflux of 5-HT, we conducted a similar series of experiments on CsCl and $LaCl_3$. We chose cesium not only to verify if the efflux of 5-HT is faster when the counterion is monovalent as opposed to divalent, but also because its diffusivity in water is 1.5 times faster than the diffusivity of sodium. This is a consequence of the lower hydration of the cesium ion. We chose lanthanum, because it is a trivalent cation and was shown to induce substantial contraction of the granule matrix (Curran and Brodwick, 1991).

A typical amperometric spike of 5-HT obtained when granules were electroporated in 155 mM CsCl is shown in Fig. 3 *A*. The spikes are similar to those obtained in Ringer's medium and exhibit a noninstantaneous but fast-rising phase (Fig. 3 *A*). A plot of the $t^{int}_{1/2}$ of the amperometric spike versus the radius of the granule at its fully swollen state is outlined in Fig. 3 *B* (*solid circles*). The data were fitted to the equation $t_{1/2} = Ar^2$, where A was found to be 13 (n = 31, ρ = 0.72). We calculate an effective diffusion coefficient with Eq. 1 and find it to be 3.7×10^{-8} cm^2 s^{-1} after

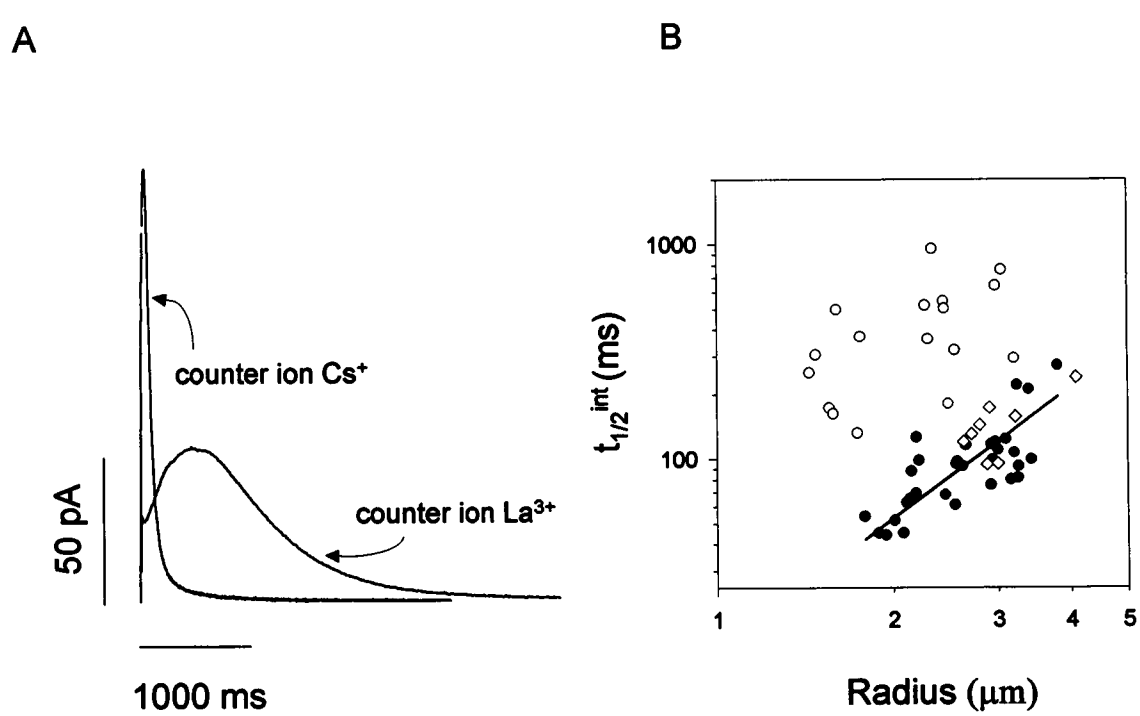

FIGURE 3 (*A*) Amperometric spikes obtained when granules where bathed in CsCl and $LaCl_3$ solutions. The Q, $t^{int}_{1/2}$, $t^{spike}_{1/2}$, t_{max}, $r_{initial}$, and r_{final} for CsCl are 18 pC, 80 ms, 87 ms, 52 ms, 2.3 μm, and 3.2 μm, and for $LaCl_3$ are 68 pC, 763 ms, 1187 ms, 472 ms, 3.1 μm, and 2.9 μm. (*B*) Comparison of the half-time $t^{int}_{1/2}$ of the integral of the amperometric spike of 5-HT obtained from granules that were immersed in CsCl (*solid circles*), NaCl (*open diamonds*), and $LaCl_3$ (*open circles*). The lower solid line is a fit of $t^{int}_{1/2} = Ar^2$ to the CsCl data, where A is found to be 13. The lanthanum solution ($LaCl_3$, 88 mM) was buffered with 1–10 mM Tris buffer (pH 7.0). The cesium solution (CsCl, 155 mM) was buffered with 1 mM Tris buffer (pH 7.0).

correcting for the rising phase (Table 1, Appendix 2). As predicted, this is faster (5.3 times) than the $D_{5\text{-HT}}$ measured for the granules immersed in histamine-containing solution. This result once again supports our conjecture that the efflux of 5-HT is coupled to the influx of external counterions; Cs^+ accelerates electrodiffusion of 5-HT^+ within the matrix, whereas Hi^{++} slows it down. The diffusivity of 5-HT^+ is also ~1.8 times faster than that measured for granules immersed in Ringer's medium, but is only slightly faster than the diffusivity measured in NaCl (Fig. 3 *B*, Table 1). The reason that the diffusivity of 5-HT is slower when the external solution is Ringer's medium as compared to a solution containing Cs is that the Ringer's medium also contains divalent counterions (2 mM $CaCl_2$ and 1 mM $MgCl_2$). This result suggests that the valence of the counterion affects the interdiffusion more than the size and/or degree of hydration of the ions. This conclusion supports the conjecture that the matrix of the granule of the mast cell is a weakly cross-linked gel (Parpura and Fernandez, 1996).

We repeated the experiments in 88 mM $LaCl_3$ and observed that the granular matrix contracted (up to 30% of its initial volume) as the efflux of 5-HT was recorded at the carbon fiber electrode (Fig. 3). Two different types of spikes were recorded. The first was similar to that observed in Na, Cs, and Hi, except that the rising phase and exponential decay were longer (see *spike* in Fig. 3 *A*). The decaying phase of the amperometric spike in the second group (~30% of the cases) exhibited a more complex time course and was not exponential. The reasons for the anomalous time course are unknown. It is possible that La^{3+} interacts with the granular membrane and stabilizes the pores induced by the electric field. Therefore, we measured the $t_{1/2}^{int}$ from the amperometric spike of the first group only (see Fig. 3 *B*; *open circles*). Because there is significant scatter in the data, we calculated the diffusivity of 5-HT from each data point with Eq. 1 and averaged the values ($n = 17$). After correcting for the rising phase, we found $D_{5\text{-HT}}$ to be 0.6×10^{-8} cm^2 s^{-1} (Table 1 and Appendix 2). This diffusivity is fivefold slower than that measured in Cs-containing solutions, but is not significantly different from that measured in Hi (Table 1). The observation that the granule matrix contracts upon ion exchange does suggest, however, that La^{3+} interacts with the polymeric chains of the heparin sulfate proteoglycan matrix. The contraction occurs because one La^{3+} ion replaces three 5-HT^+ cations, which increases the chemical potential of water within the granule (Fig. 1 *C*).

In experiments conducted in all electrolytes except lanthanum chloride, $t_{1/2}^{int}$ varied by two- to threefold for granules of the same size (Figs. 2 *A* and 3 *B*). Possible explanations for this scatter are 1) large variation in the shape of granules (giant granules of the mast cell of the beige mouse are formed from smaller granules and often appear to be nonhomogenous; Figures 5 and 6 *C* in Marszalek et al., 1997); 2) nonuniform concentration of 5-HT within a granule; or 3) variability in the physical properties of the granular matrix (e.g., elastic modulus, porosity, charge distribution). These are not unreasonable conjectures and would affect the diffusivity of serotonin within the matrix. It is likely that they account for the relatively poor fit of the $t_{1/2}^{int}$ to the granule radius (Figs. 2 *A* and 3 *B*). We observe bigger scatter (up to fivefold) in lanthanum chloride solution (Fig. 3 *B*), and the reasons for this increased variation in $t_{1/2}^{int}$ are not entirely clear. Other studies also show that secretory vesicles display variability. Parpura and Fernandez (1996) recently found that there was sixfold difference in the elastic moduli of the granule matrix of rat mast cells granules, and Wightman et al. (1995) reported that there was significant variation in the concentration of catecholamines within the secretory vesicles of chromaffin cells.

Microviscosity within the granule matrix affects the kinetics of swelling and the rate of exchange of 5-HT

Kinetics of swelling of the matrix

Because swelling of the granule matrix accompanies exchange of secretory products in physiological medium (Curran and Brodwick, 1991; Fernandez et al., 1991), it is important to investigate further the conditions that may affect its kinetics. It is also useful to compare the time course of swelling with the rate of release of 5-HT.

Outlined in Fig. 4 *A* is the time course of swelling measured when the membranes of two granules were electroporated in Hanks' medium. The effective radius of the granules increased by ~0.7 μm (the area increased by ~1.6–2-fold). It took slightly longer for the bigger granule to reach its maximum swollen state. We fitted the data to Eq. 4, ignoring the load that the membrane exerts on the expanding matrix, and found the diffusivities of the matrices (D_{gel}) to be 1.4×10^{-7} (*open diamonds*) and 1.2×10^{-7} cm^2 s^{-1} (*solid diamonds*). The average value ($n = 7$) for the diffusivity of the gel in physiological medium is 1.4×10^{-7} ($\pm 0.7 \times 10^{-7}$) cm^2 s^{-1}. This diffusivity is on the same order of magnitude as reported for other gels (Tanaka and Fillmore, 1979).

The rate of ion exchange depends upon the diffusivities of counterions within the granule matrix. The diffusivity of the matrix (D_{gel}) determines the kinetics of swelling. Whereas diffusivity of an ion depends upon its interaction with a charged matrix and the solution, the diffusivity of the matrix depends upon its elastic properties and the friction between the matrix and the solution (Tanaka and Fillmore, 1979). Both diffusivities are affected by the viscosity of the solution. In the case of ions this is well expressed in the Stokes-Einstein equation that describes the dependence of the diffusivity of a small molecule (e.g., ion) of radius a on the viscosity (η) of a solution ($D = kT/6\pi a\eta$, where k is the Boltzmann constant and T is the absolute temperature; Bockris and Reddy, 1977). In the next series of experiments we examine the effect of increasing η on the kinetics of swelling of the matrix and on the rate of exchange of 5-HT in different solvents.

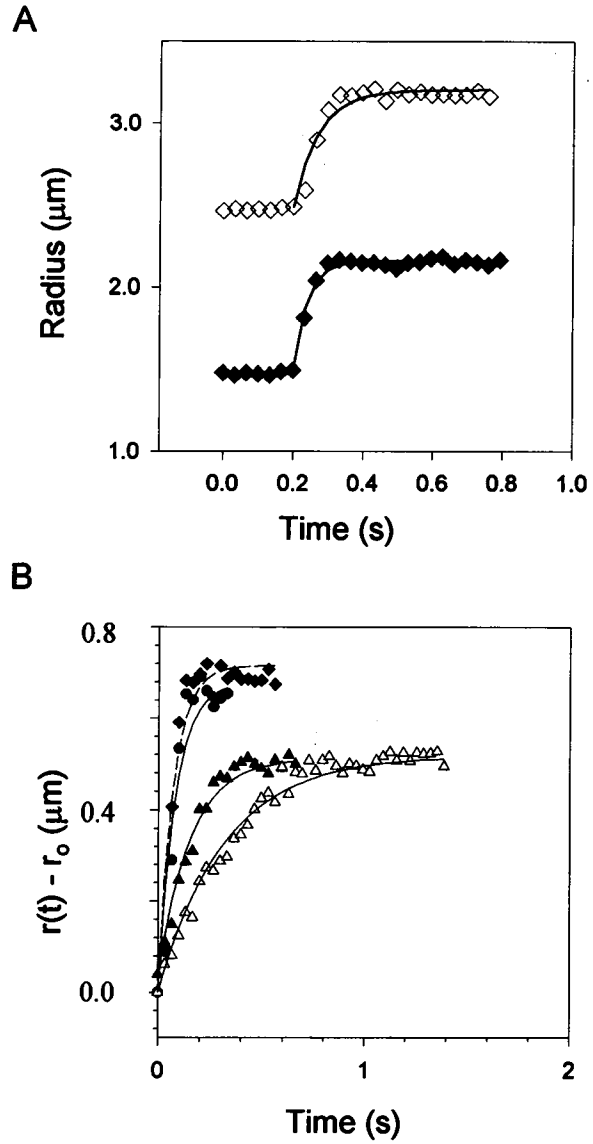

FIGURE 4 Kinetics of swelling of the granule matrix in different media. (*A*) The granules were immersed in Hanks' medium. The different symbols represent the results obtained from two different secretory granules of the mast cell of the beige mouse. The solid lines represent a fit of Eq. 4 to the data, where D_{gel} was found to be 1.2×10^{-7} cm^2 s^{-1} (◇) and 1.4×10^{-7} cm^2 s^{-1} (◆). (*B*) Kinetics of swelling of the granular matrix depends upon the viscosity and the type of solvent. Secretory granules of the beige mouse mast cell were immersed in Hanks' medium (◆, viscosity η = 1 cp); 3% methyl cellulose dissolved in Hanks' medium (●, η = 23 cp); Hanks' medium with 20% dextran (▲, η = 70 cp); and Hanks' medium with 30% dextran (△, $\eta \approx$ 200 cp). The dashed lines represent a fit of Eq. 4 to the Hanks' medium data, and the solid lines represent a fit of Eq. 4 to the rest of the data, where r_o is the initial radius of the granule. The osmolality of all solutions is 280 mmol kg^{-1}.

In Fig. 4 *B* we show examples of the time course of swelling of granules that were electroporated in sodium saline (*solid diamonds*), saline with 3% methyl cellulose (*filled circles*), saline with 20% dextran (*filled triangles*), and saline with 30% dextran dissolved (*open triangles*). The osmolality of the solutions is the same (~280 mOsm). The kinetics of swelling in saline solution with methylcellulose added was slightly reduced ($D_{gel} = 0.8 \times 10^{-7}$ cm^2 s^{-1}; η = 23 cp) compared to saline alone ($D_{gel} = 1.4 \times 10^{-7}$ cm^2 s^{-1}), but was found to be independent of the bulk viscosity of the medium up to ~1500 cp. We observed a decrease in the rate of swelling of the matrix in the dextran-containing saline (D_{gel} dropped to 0.5×10^{-7} cm^2 s^{-1}) only when the viscosity of the medium reached 70 cp. D_{gel} dropped further to 0.2×10^{-7} cm^2 s^{-1} at η = 200 cp. These experiments show that the addition of large polymeric molecules to saline does not significantly affect the kinetics of swelling of the matrix. Although the molecules increase the bulk viscosity of the medium, they do not interact strongly with the matrix and do not significantly modify its viscoelastic properties. In contrast, sucrose, when added to Hanks' medium, dramatically affected the rate of swelling of the matrix. We found that the D_{gel} of the polymeric matrix decreases linearly (ρ = 0.97) with decreasing fluidity ($1/\eta$) of the sucrose saline medium (Fig. 5).

Exchange of 5-HT in viscous media

To assess the effect that the viscosity of the solution has on the rate of ion exchange, we measured the efflux of 5-HT in 100 mM Hi at pH 4.5 supplemented with different solutes (e.g., sucrose, methylcellulose). Consistent with the swelling experiments in Hanks' medium, we found that the efflux of 5-HT was not affected when methylcellulose (η = 23–1500 cp) was dissolved in histamine dihydrochloride. The $t_{1/2}^{int}$ is the same as that measured for the controls (Figs. 6 *A* and 7 *A*), and the granular matrix did not swell and remained refractile under Normaski optics. This was not the case when the efflux was measured in 100 mM histamine dihydrochloride with 0.8–1.8 M sucrose dissolved. Intact gran-

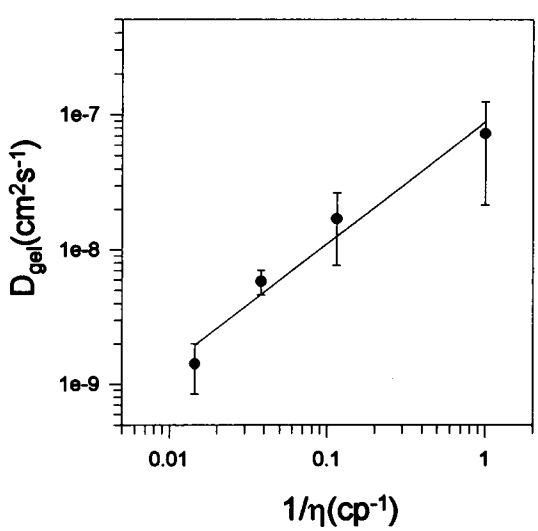

FIGURE 5 The matrix diffusivity D_{gel} increases linearly with the fluidity of the external solution ($1/\eta$). The viscosity was increased by adding sucrose to Hanks' medium. The solid line is a linear regression of the relationship (ρ = 0.98).

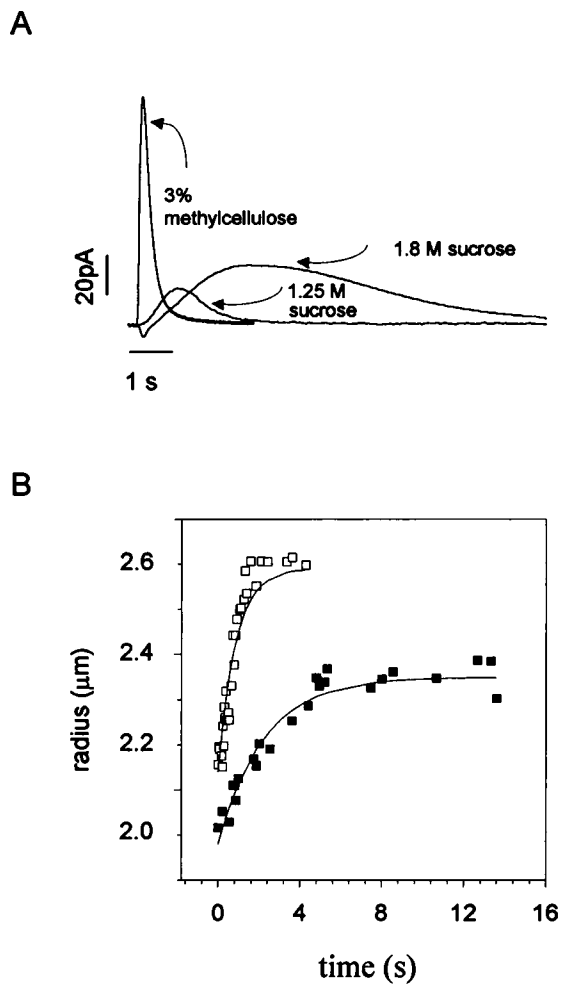

FIGURE 6 The efflux of 5-HT (*A*) and the kinetics of swelling (*B*) are affected by the microviscosity of the medium within the matrix. (*A*) Amperometric spikes obtained when granules where bathed in 100 mM histamine dihydrochloride with 3% methyl cellulose (Q, $t_{1/2}^{int}$, $t_{1/2}^{spike}$, and r_o are 36.2 pC, 241 ms, 264 ms, 2.6 μm), 1.25 M sucrose (η = 4.5 cp; Q, $t_{1/2}^{int}$, $t_{1/2}^{spike}$, t_{max}, r_o, and r_f are 25 pC, 1205 ms, 1180 ms, 895 ms, 1.9 μm, and 2.4 μm), and 1.8 M sucrose (η = 12 cp; Q, $t_{1/2}^{int}$, $t_{1/2}^{spike}$, t_{max}, r_o, and r_f are 139 pC, 3630 ms, 5005 ms, 2296 ms, 2.02 μm, and 2.35 μm) dissolved in the solutions. (*B*) Kinetics of swelling of the granule matrix in 100 mM histamine dihydrochloride (pH 4.5) with 1.25 M sucrose (□) and 1.8 M sucrose dissolved (■). The solid lines represent a fit of the data to Eq. 4, where D_{gel} was found to be 8.05×10^{-9} cm^2 s^{-1} (1.25 M sucrose) and 2.5×10^{-9} cm^2 s^{-1} (1.8 M sucrose).

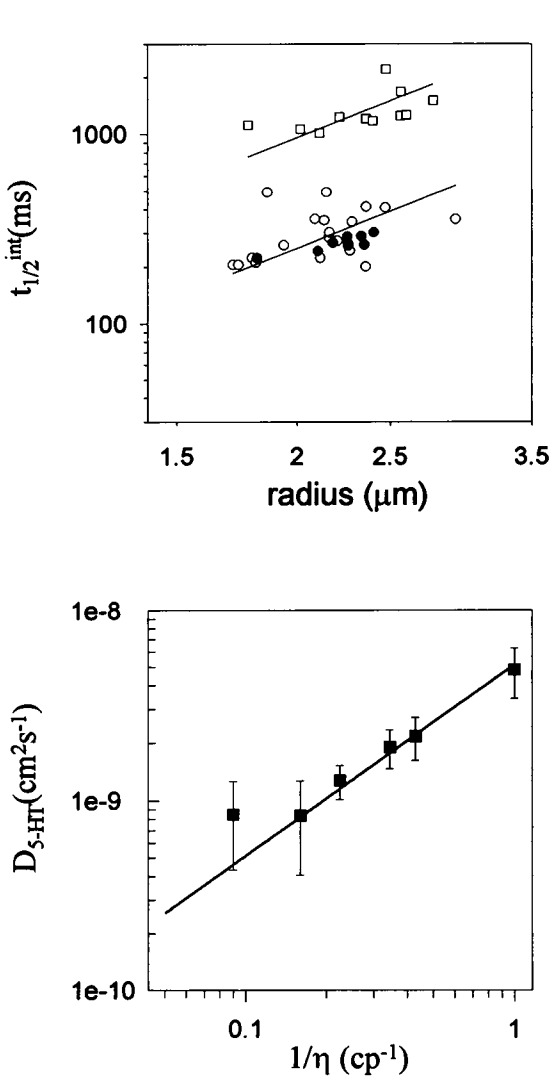

FIGURE 7 (*A*) Comparison of the half-time $t_{1/2}^{int}$ of the integral of the amperometric spike of 5-HT obtained from granules that were immersed in 100 mM histamine dihydrochloride (○), 3% methyl cellulose dissolved in histamine dihydrochloride (●), 1.25 M sucrose dissolved in histamine dihydrochloride (□), η = 4.5 cp. The upper solid line is a fit of $t_{1/2}^{int} = Ar^2$ to the data, where A is found to be 239. The lower solid line is described in Fig. 2 *A*. (*B*) The diffusivity of 5-HT within the secretory granule is proportional to the fluidity ($1/\eta$) of the sucrose solution. The granules were bathed in 100 mM histamine dihydrochloride (pH 4.5), and the viscosity was adjusted by adding sucrose to give a final concentration of 0.8–1.8 M sucrose. $D_{5\text{-}HT}$ was calculated from Eq. 1. The points are the average of 5–23 measurements. The solid line is calculated with $D_{5\text{-}HT}(\eta) = D_{5\text{-}HT}(\eta = 1\text{ cp})/\eta$, where $D_{5\text{-}HT}(\eta = 1\text{ cp})$ is the measured diffusivity of 5-HT within the granule immersed in the external solution (η = 1 cp).

ules are osmotically active (Brodwick et al., 1992) and shrink in sucrose media 10–50% (change in area) relative to their size in the standard solution (280 mOsm). When the granule membrane was porated, the matrix expanded to about ±10% of the initial size measured in the standard solution, and the granule matrix appeared less refractile under Nomarski optics. Consistent with the swelling experiments in Hanks' medium, the rate of swelling (Fig. 6 *B*) decreased as the viscosity of the sucrose solution increased from 2 to 12 cp. This is also reflected in the diffusivity of the 5-HT, which was observed to decrease as the concentration of sucrose increased (cf. amperometric spikes obtained from granules electroporated in histamine dihydrochloride with 1.25 or 1.8 M sucrose dissolved; Fig. 6 *A*). In Fig. 7 *A* we compare the $t_{1/2}^{int}$ data obtained in histamine dihydrochloride (*open circles*) with that obtained in the same solution with 1.25 M sucrose dissolved (*open squares*). We fitted the data to $t_{1/2}^{int} = Ar^2$ and found A to be 239, which is four times more than the control. There is an approximately fivefold increase in the rising phase with ~33% (cf. 23% in standard medium) of the molecules

released during this phase. This suggests that membrane rupture, and therefore the efflux of 5-HT, is more anisotropic in sucrose-containing solutions as opposed to histamine dihydrochloride, Ringer's medium, or CsCl solution. Given this reservation, we use Eq. 1 to estimate the diffusivity of 5-HT within the matrix that is in contact with 1.25 M sucrose solution and found it to be 1.3×10^{-9} cm^2 s^{-1}. We estimated the $D_{5\text{-HT}}$ at the various concentrations of sucrose and found that the effective diffusivity of 5-HT within the matrix decreases as the bulk viscosity of the external electrolyte increases. The reduction of the diffusivity of 5-HT in the external solution is also proportional to the bulk viscosity, but has minimal influence on the time course of the amperometric spike, because the detector is close to the granule and the molecules take an insignificant time to reach the detector after they leave the granule. There is a linear relationship (Fig. 7 *B*) between $D_{5\text{-HT}}$ and $1/\eta$ showing some signs of deviation at the highest viscosity. This plot does show that the viscosity and diffusivity of ions within the polymeric matrix are correlated in a fashion similar to that of the Stokes-Einstein equation derived for the bulk.

Sucrose strongly affects the rate of ion exchange and the kinetics of swelling of the matrix because it is a small uncharged molecule that is able to penetrate the granule matrix quickly, increasing the viscosity of the fluid phase within the gel. This decreases the diffusivity of all of the ions within the matrix and the diffusivity of the matrix itself (by increasing the friction coefficient between the polymer molecules and the solutes and water of the matrix). Methylcellulose is a highly branched molecule that cannot penetrate the granular matrix and does not affect the rate of ion exchange and the kinetics of swelling. Because we observed some reduction in the rate of swelling in the presence of dextran, it is likely that some molecules are able to penetrate the polymeric network of the granule (Fig. 4 *B*), and this observation supports the hypothesis that the granule matrix is a weakly cross-linked gel. Although we cannot exclude the possibility that the effects of sucrose on the kinetics of swelling and ion exchange could result from increased osmotic pressure, the results of the experiments conducted in dextran solutions, at constant osmotic pressure, suggest that viscosity of the medium is the critical parameter. In contrast to sucrose, large molecules affect the bulk viscosity of the external solution but have minimal influence on the microviscosity experienced by an ion within and outside the granule matrix (Cussler 1984; Evans et al. 1981). The results obtained in viscous media further support our hypothesis that the rate of release of 5-HT is primarily determined by the interactions of 5-HT within the granule matrix rather than in bulk.

Biological implications of the low diffusivity of 5-HT in mast cell granules

We determined the diffusivity of 5-HT within the matrix of the secretory granule of the mast cell of the beige mouse to be $\sim 2 \times 10^{-8}$ cm^2 s^{-1} when the matrix is in contact with physiological medium. The diffusivity of 5-HT should be similar in the granule of an intact mast cell, and it is not correct to assume that it is the same as the bulk diffusivity of 5-HT ($\sim 10^{-5}$ cm^2 s^{-1}). We also showed that this diffusivity is affected by the nature of the counterion (valence) within the granule matrix and found a similar trend when we measured exocytotic release of 5-HT from beige mouse mast cells. The $t_{1/2}$ of the amperometric spike was about two to three times longer when cells were bathed in histamine dihydrochloride solution compared to Ringer's medium (data not shown). This supports the conjecture that release of 5-HT during exocytosis is not a purely diffusional efflux, but that it is electrically coupled to the influx of external ions. This is in agreement with recent measurements in rat mast cells and bovine chromaffin cells (Pihel et al., 1996). They found that the amplitude of 5-HT current increased in medium containing Cs^+ ions compared to the control and was significantly decreased in medium containing divalent Zn^{2+}.

Our measurement of the diffusivity of 5-HT within the granule and the earlier measurements of exocytotic release suggest that in addition to the geometry of the exocytotic fusion pore, the diffusivity of 5-HT within the granular matrix affects the rate of exocytotic release. Indeed, the low diffusivity of charged secretory products might explain the very slow release during the "foot" phase recorded from mast cells (Alvarez de Toledo et al., 1993; Marszalek et al., 1996) and chromaffin cells (Chow et al., 1992; Schroeder et al., 1996). To further examine this hypothesis, we simultaneously measured the cell admittance and release of 5-HT during the foot phase of exocytotic release in the beige mouse mast cell (Fig. 8). We calculated the conductance (G_p) of the fusion pore from the real (Re) and imaginary (Im) components of the change in cell admittance that occurs upon fusion of the vesicle with the plasma membrane. We determined the total charge detected by integrating the spike and foot signals (Fig. 8 *A*, *bottom left* and *upper right traces*) and estimated the concentration by assuming that the vesicle is a sphere and that the oxidation of 5-HT requires four electrons (Bruns and Jahn, 1995; Wrona and Dryhurst, 1987). We plot the flux, I_{foot}, during the foot phase against the conductance of the pore and fit these data, assuming that the flux of 5-HT is limited by 1) the size of the pore alone or 2) the size of the pore and diffusion of 5-HT within the matrix. For 1) we calculate the flux (Fig. 8 *B*, *dashed lines*) with $[(\pi r_p^2 DC)/(l + \pi r_p/2)]nF$ (Hille, 1994), where n is 4, F is Faraday's constant, l is the length of the pore (4 nm), D is the diffusion coefficient (assumed to be the same as the bulk; 1×10^{-5} cm^2 s^{-1}), and r_p is the radius of the pore calculated from the conductance with $G_p = [(\rho/\pi r_p^2)(l + \pi r_p/2)]^{-1}$, where ρ is the resistivity of the solution ($\sim 100\ \Omega$ cm). At 3 nS the flux determined in this way is 10 (*open circles*) and 25 (*solid circles*) times more than that measured experimentally. For 2) (Fig. 8 *B*, *solid lines*) we fit the data to $\phi = 2\pi r_p DCnF$, where r_p is also calculated from the G_p. We found (four different experi-

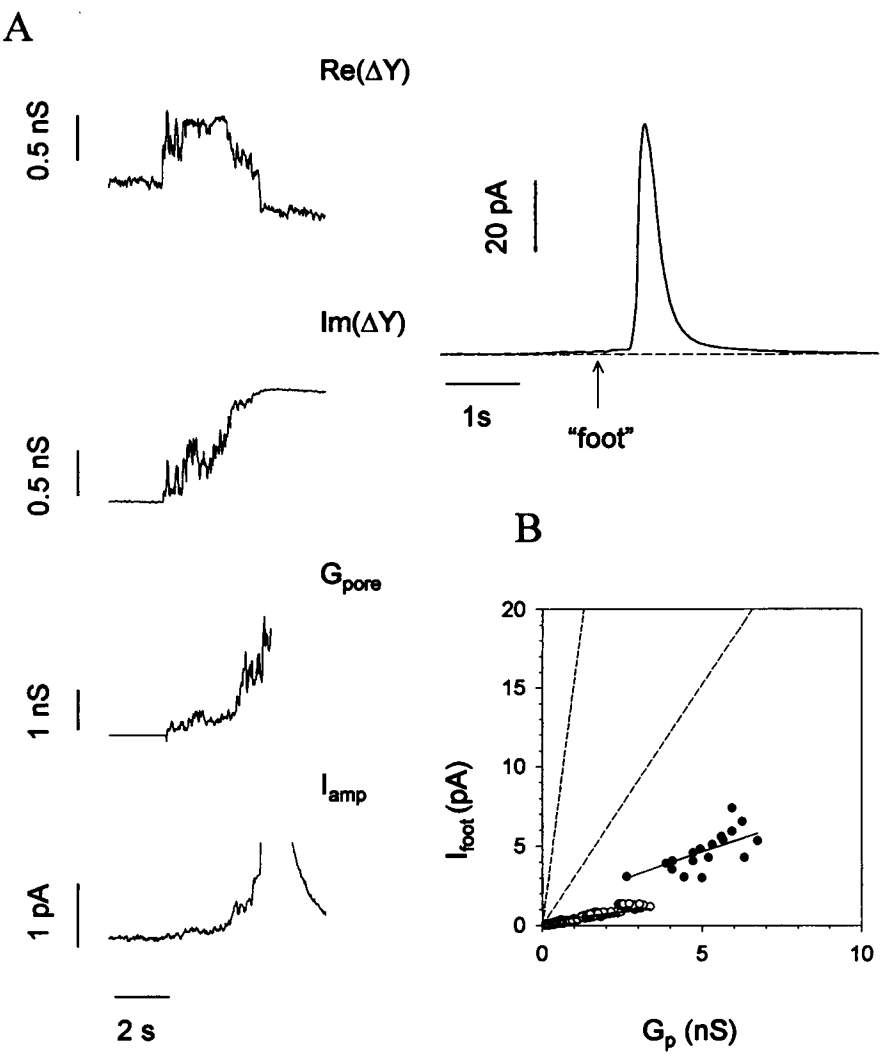

FIGURE 8 Simultaneous recording of cell admittance and serotonin release during exocytosis in the beige mouse mast cell. (*A*) The fusion pore conductance (G_p) was calculated from the real (Re) and Imaginary (Im) components of the change in cell admittance with $2\pi f C_g(\mathrm{Im}(\Delta Y))/\mathrm{Re}(\Delta Y))$, where f is the frequency (833 Hz) of the stimulus voltage and C_g is the capacitance of the secretory granule that fused with the plasma membrane. C_g was calculated from a change in the Im(ΔY) of the admittance and found to be 207fF. The surface area and then the volume V_g of the secretory granule were calculated from C_g, assuming a specific membrane capacitance of 0.57 μF/cm^2 (Zimmerberg et al., 1987). A small amperometric current of 5-HT, I_{foot} (*bottom left trace*), precedes the main amperometric spike (*top right trace*). The $t_{1/2}^{spike}$ is 200 ms, and the total charge detected from the spike and foot, Q_{tot}, is 27 pC. The concentration of 5-HT ($C = Q_{tot}/nFV_g$) was estimated to be 3.37×10^{-6} mol/cm^3. (*B*) Plot of the amperometric current of 5-HT measured during the foot (I_{foot}) versus G_p. The open circles represent the data outlined in *A*; the solid circles are data obtained from Figure 3 of Alvarez de Toledo et al. (1993), where the concentration of 5-HT in the granule was 1.84×10^{-5} mol/cm^3. The solid lines represent fits of the data to model 2). The dashed lines were calculated according to model 1). See text for details.

ments) the diffusivity of 5-HT to be 2.9 ($\pm$0.9) $\times 10^{-7}$ cm^2 s^{-1}. This is ~30 times less than the bulk diffusivity. Therefore we conclude that not only the size of the fusion pore but also the diffusivity of 5-HT (~10^{-7} cm^2 s^{-1}) limits release.

It is interesting that the diffusivity of 5-HT calculated from an exocytotic event (Fig. 8) is ~10 times higher than that estimated from the measurements on isolated granules (Fig. 2 *A*). This discrepancy may result in part from underestimating the concentration of 5-HT within the granule in the intact cell. Alternatively, the difference in $D_{5\text{-HT}}$ could reflect different mechanisms of release between intact cells and electroporated granules. In both cases, $D_{5\text{-HT}}$ was calculated assuming that the efflux of serotonin occurred by diffusional exchange. Although we have shown that the release of 5-HT from an isolated granule occurs by ion exchange with external counterions, this may not be the case in an intact cell. For example, it has been proposed that neurotransmitters are released by ion exchange driven by ionic currents crossing the vesicular membrane, traversing the ion exchange gel and passing through the fusion pore (Rahamimoff and Fernandez, 1997). Hence, release may be accelerated by this mechanism in vivo. It is possible that during mast cell exocytosis 5-HT is displaced by intracellular counterions (e.g., K^+) that cross the granule membrane through ion channels and/or ion pumps. If some of the cell energy is utilized to deliver the intracellular counterions for exchange with 5-HT, the apparent diffusivity of 5-HT will be faster than the interdiffusion of 5-HT and extracellular counterions.

Mechanisms of release in other secretory systems

Most small transmitters and secretory products (e.g., amines, acetylcholine, glutamate) are charged molecules. They and a variety of peptides (e.g., insulin, vasopressin, endorphin, growth hormone) are stored in secretory and synaptic vesicles. In mast cells the secretory products are associated with the heparin sulfate proteoglycan, and we present evidence that 5-HT is released by ion exchange. In chromaffin cells the secretory products are associated with

a proteinaceous matrix. Chromogranin A appears to be the protein responsible for binding catecholamines (Schroeder et al., 1996). It is likely that catecholamines are also released by ion exchange (Pihel et al., 1996). Ion exchange is the most likely mechanism for release of small charged transmitters and secretory products when they are stored within a charged matrix.

There is some evidence in other secretory systems, e.g., zymogen granules within acinar cells (Reggio and Palade, 1978; Reggio and Dagorn, 1978) and prolactin granules within the anterior pituitary gland (Zanini et al., 1980), that there are proteoglycan-like matrices within vesicles. This may also be true for the synaptic vesicles of neurons (Volknandt and Zimmerman, 1986; Carlson and Kelly, 1983; Scranton et al., 1993), but there is no direct measurement of the diffusivity of the charged transmitters within the matrices. For example, the transmembrane protein SV2 found in many synaptic vesicles (Buckley and Kelly, 1985) is a keratan sulfate proteoglycan (Scranton et al., 1993). The two forms of this protein, H and L, both contain large glycosaminoglycan side chains (mol wt $>$ 140,000; Scranton et al., 1993). Although there is some evidence to suggest that the protein could function to transport neurotransmitters (specifically acetylcholine; Feaney et al., 1992, and Bahr et al., 1992), it is not unlikely that it could also bind neurotransmitters and ATP electrostatically and release them by ion exchange.

If the interdiffusion of neurotransmitters and counterions within the lumen of a synaptic vesicle is slower than in bulk, the lumen of the vesicle is likely to limit the number of neurotransmitter molecules reaching the mouth of the fusion pore. If this scenario is true, both the diffusivity of neurotransmitters within a synaptic vesicle and the geometry of the fusion pore would determine quantal release, and may be involved in postfusion regulation of synaptic transmission (Marszalek et al., 1996; Rahamimoff and Fernandez, 1997).

CONCLUSIONS

We present several pieces of evidence that ion exchange is the mechanism controlling the release of 5-HT from the matrix of a secretory granule of the beige mouse mast cell. The most important are 1) the low diffusivity of 5-HT within the granule matrix ($\sim 10^{-8}$ cm^2 s^{-1}); 2) the reduction in the diffusivity of 5-HT when the counterion is changed from monovalent to divalent; and 3) the concomitant swelling and shrinking of the granular matrix. We suggest that both the geometry of the exocytotic fusion pore and the diffusivity of 5-HT within the granule matrix must affect the rate of release of amines during exocytosis in mast cells.

APPENDIX 1: MODEL USED TO DESCRIBE ION EXCHANGE WITHIN THE SECRETORY GRANULE

The fluxes of ions during ion exchange can be described by the Nernst-Planck electrodiffusion equation (Bockris and Reddy, 1977; Helfferich, 1962):

$$J_A = -D_A\left[\nabla C_A + C_A \frac{z_A e_o}{kT}\nabla\varphi_A\right] \qquad \text{(A1.1)}$$

where D_A is the diffusion coefficient of species A, C_A is the concentration of species A, φ is the electrostatic potential, T is the temperature, k is the Boltzmann constant, e_o is the charge of the electron, and z_A is the charge number of species A. The first term in square brackets is the pure diffusional flux as a result of the concentration gradient of ion A, and the second term describes ionic transport that is driven by the negative gradient of the electrostatic potential. Identical expressions can be derived to describe the flux of all participating counterions.

Imposing several initial boundary conditions (Helfferich, 1962), the Nernst-Planck equation is solved and describes the rate of ion exchange when it is limited by the diffusivity of counterions within an ion exchanger ("particle" diffusion) or within the "unstirred layer" at the interface between the exchanger and the surrounding medium ("film" diffusion; Helfferich, 1962). The expressions are outlined in Helfferich, (1962) for the case of two counterions (A and B) exchanging and are discussed briefly below.

Particle diffusion control

The Nernst-Planck electrodiffusion equations are solved for particle-diffusion controlled ion exchange (Helfferich, 1962) when the number of fixed charges is assumed to be constant and there are a minimal number of coions present in the ion exchanger. Both are reasonable assumptions for the matrix of the secretory granule of the mast cell. For the case of only two counterions exchanging, the flux (J_A) can be derived by writing out an expression identical to A1.1 for counterion B and combining both expressions under the restrictions that $z_A J_A + z_B J_B = 0$ (no electric current) and $z_A C_{A(i)} + z_B C_{B(i)} = -C_{fixed}$ (electroneutrality). The flux of species A (J_A) is then

$$J_A = -\left[\frac{D_{A(i)}D_{B(i)}(z_A^2 C_{A(i)} + z_B^2 C_{B(i)})}{D_{A(i)}z_A^2 C_{A(i)} + D_{B(i)}z_B^2 C_{B(i)}}\right]\nabla C_{A(i)} \qquad \text{(A1.2)}$$

where $C_{(i)}$ denotes the concentration of species A or B inside the ion exchanger and C_{fixed} the concentration of the fixed ionic groups within the exchanger. In the secretory granule of the mast cell, the fixed charges arise from the negatively charged heparin sulfate proteoglycan. The term in square brackets is the interdiffusion coefficient, D_{AB}. It is not a constant, as the concentrations of A and B change during the course of ion exchange (the concentration of A decreases and the concentration of B increases). Therefore the flux of counterion A depends not only on its diffusivity, but also on the diffusivity of counterion B; this interdiffusion of the counterions is what controls the rate of ion exchange. Therefore, from Eq. A1.2 it is obvious that to determine D_{AB} requires knowing the diffusivities of both counterions and their time-dependent relative concentrations within the ion exchanger.

It was not possible to measure the relative time-dependent concentrations of all exchanging counterions and their individual diffusivities within the micrometer-size matrix of a secretory granule. We could determine the efflux of serotonin at the surface of the matrix ($J_{5\text{-HT}}$) from the measured oxidation current of 5-HT at a carbon fiber electrode. Therefore, to estimate a diffusivity for 5-HT within the granule matrix, we use a simplified approach and assumed that the exchange of 5-HT with the external cation is isotopic (Helfferich, 1962) throughout the course of ion exchange, i.e., the external cation is an isotope of 5-HT, and $D_{A(i)} \cong D_{B(i)}$. This reduces Eq. A1.1 to Fick's first law. Of course, $D_{5\text{-HT}}$ estimated using the isotopic exchange model is not a self-diffusion coefficient of 5-HT, but rather the effective diffusion coefficient $D_{5\text{-HT}}$ that approximates D_{AB}.

Isotopic exchange

An expression describing the rate of isotopic ion exchange for a spherical exchanger of radius r, where the flux is radial at $t > 0$ and when exchange

is limited by diffusion within the sphere, is derived by Boyd et al. (1947) and Helfferich (1962) and is

$$1 - \frac{Q(t)}{Q^0} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-D_A t\pi^2 n^2}{r^2}\right) \qquad \text{(A1.3)}$$

where $Q(t)$ represents the number of counterions A (5-HT) within the ion exchanger at time t, Q_0 represents the initial number of counterions within the ion exchanger at time $t = 0$, and D_A is the diffusion coefficient of the counterion within the exchanger and is constant throughout the process. The time at which a fraction of the counterions are exchanged can be calculated from Eq. A1.3. For example, the time at which half of the counterions are exchanged ($t_{1/2}$) is given by

$$t_{1/2} = 0.03\frac{r^2}{D_A} \qquad \text{(A1.4)}$$

This equation can be used to estimate the diffusivity of 5-HT within the granule matrix.

Isotopic exchange and film diffusion control

Helfferich (1962) derives expressions for the rate of ion exchange when it is limited by interdiffusion of counterions within an unstirred layer of liquid at the interface between the ion exchanger and the bulk solution (so-called film). Two analytical expressions are derived for two limiting cases; one is restricted to ions of equal valence, and the second is restricted to counterions of equal mobility. Both have limited applicability, and we therefore resort to the simpler case, also derived by Helfferich, where the exchange of 5-HT with counterion B is isotopic. In this derivation the "film" or the "unstirred" layer around the exchanger is treated as a planar layer of thickness (δ), and interdiffusion in the film is treated as quasistationary, i.e., it is assumed that diffusion across the film is fast compared with the concentration changes at the film boundaries. In this case the rate of ion exchange is described by

$$1 - \frac{Q(t)}{Q^0} = 1 - \exp\left(\frac{-3D_A C_B t}{r\delta C_{A(i)}}\right) \qquad \text{(A1.5)}$$

where $C_{A(i)}$ is the concentration of counterion A (5-HT) within the exchanger; C_B is the bulk concentration of counterion B, and D_A is the diffusion coefficient of counterion A in the film. The time at which a fraction of the counterions are exchanged can be calculated from Eq. A1.5, where the time at which half of the counterions are exchanged ($t_{1/2}$) is given by

$$t_{1/2} = 0.23\frac{r\delta C_{A(i)}}{D_A C_B} \qquad \text{(A1.6)}$$

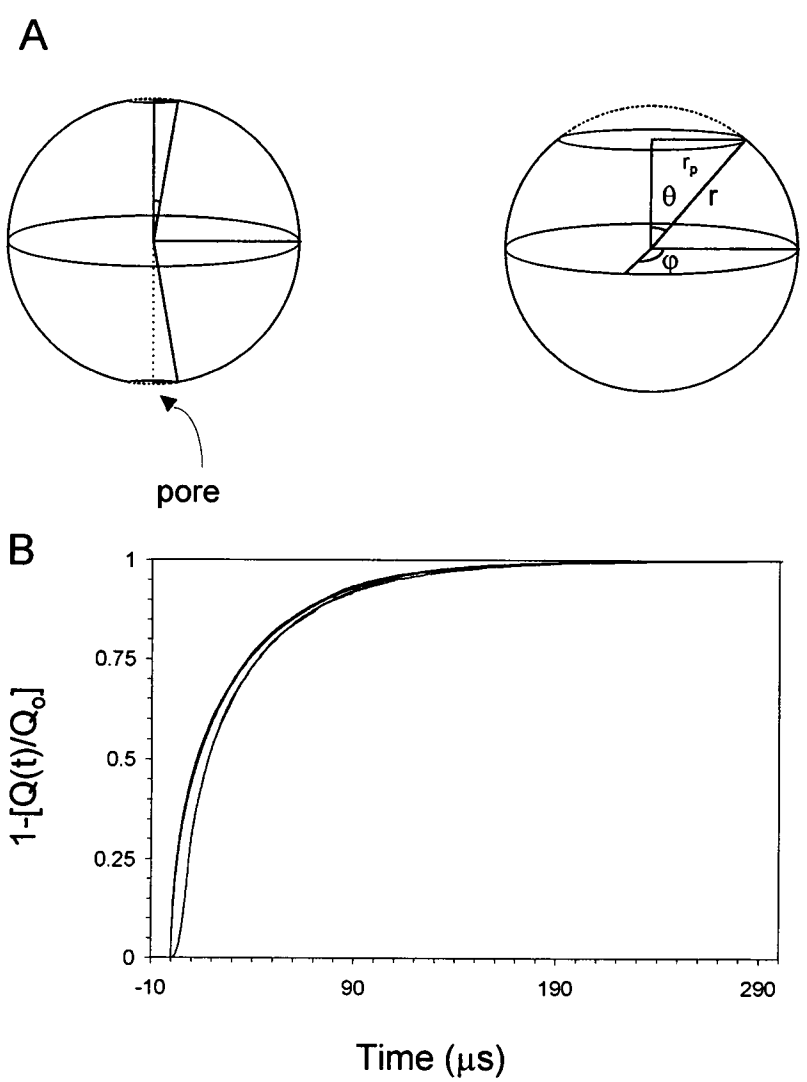

FIGURE 9 Geometry (*A*) and results (*B*) of the Monte Carlo simulation of the efflux of particles from a spherical vesicle. Comparison of the time course of diffusion from a sphere of radius $r = 20$ nm when there was no membrane limiting the efflux throughout the process with the time course measured when there was a membrane covering all of the surface except for two pores of radius ($r_p = 0.25$ nm) that were positioned at the poles of the sphere at $t = 0$. At $t > 0$ the pores expanded and no membrane was present after 8.5 μs. In the simulation the number of particles was 32,000 or 8000; step length (l), 0.2 or 0.5 nm; and D, 1.5×10^{-8} cm^2 s^{-1}. The $t_{1/2}^{int}$ was 18.04 μs (membrane present) and 12.4 μs (no membrane present). Q_o is the initial number of particles within the vesicle, and $Q(t)$ is the number of particles left at time t.

This equation can be used to estimate the diffusivity of 5-HT within the "unstirred" layer.

APPENDIX 2: MONTE CARLO SIMULATION OF THE EFFLUX OF PARTICLES FROM A VESICLE WITH AND WITHOUT A MEMBRANE INITIALLY PRESENT

The diffusion coefficient of 5-HT within the granule was calculated, assuming the efflux is radial from the onset. This is an approximation, because at $t = 0$ the granules are covered by a membrane that is not permeable to 5-HT, and it takes a finite time t_f to rupture it. To determine how much we underestimate $D_{5\text{-HT}}$ as a result of this assumption, we use the Monte Carlo technique to simulate the efflux of 5-HT out of a vesicle initially covered with an impermeable barrier and compare it with the efflux simulated when there was no membrane present. We simulated the efflux measured when a vesicle was porated in Ringer's medium and NaCl, CsCl, $LaCl_3$, and histamine dihydrochloride solutions.

Conditions of the simulation

Because poration of the granule membrane is most likely to occur at the poles of the granule (Marszalek et al., 1997), we placed two pores of radius $r_p = 0.25$ nm at the poles of the vesicle (Fig. 9) of radius r, at $t = 0$. We chose 0.25 nm for the pore radius, because 0.5 nm is the approximate size of a serotonin molecule, and used vesicles of radii 5–20 nm to reduce computation time. Use of the small vesicles is justified because we do not require absolute values of $t_{1/2}^{int}$, but only the relative change in $t_{1/2}^{int}$. We showed (see Marszalek et al., 1997, and Fig. 2 *B*) that the rising phase of the amperometric spike (t_{max}) reflects the time for membrane rupture and is proportional to the initial surface area of the vesicle, $t_{max} = Ar^2$, where A is a constant that depends on the composition of the external electrolyte (Table 1). We also found that the amperometric current increases linearly with time during this phase, indicating that membrane rupture proceeds at a constant rate. We set $t_f = t_{max}$ and simulate membrane rupture by allowing the pores to expand at a constant rate. For example, simulating the efflux from a vesicle (radius = 20 nm) in Ringer's medium ($A = 21$ ms/μm^2; Table 1), the rising phase $t_{max} = 8.4$ μs.

In the simulation, the particles have infinitely small volume and are assigned spherical coordinates $[r_i, \theta_i, \phi_i]$. At $t = 0$, up to 500, 4000, or 32,000 particles are placed uniformly within a sphere of radius 5, 10, or 20 nm, respectively. At $t > 0$ the particles move together and the time (t) is calculated with $jl^2/6D$, where j is the number of steps, D the diffusion coefficient, and l the step length. If a particle moves into the "membrane," it is internally reflected. A particle escapes when $r_i > r$, and the program stops tracking its position. The simulation terminates when all of the particles diffuse out of the vesicle. To avoid errors arising from finite size effects, the step length was always at least 40 times less than the radius of the sphere. We used the diffusion coefficients determined experimentally in the various electrolytes (see Table 1) to calculate t.

The simulations were performed on a Silicon Graphics Server (SGI) at the Biomedical Imaging Research Center (Rochester, MN). This server is equipped with eight 200-MHz R4400 CPUs, each with 4 MB of cache, 512 MB of memory, and 10 GB of disk space.

Results of the simulation

When we simulated the conditions observed in Ringer's medium ($D = 1.5 \times 10^{-8}$ cm^2 s^{-1}, the equivalent rising phase $t_{max} = 8.5$ μs), we found that the rate at which particles escaped from the vesicle decreased and the $t_{1/2}^{int}$ increased by a factor of $\lambda = 1.45$ when there was a membrane initially present, as compared to when there was no membrane (Fig. 9). The time to reach maximum flux was 8.13 μs, and 23% of the particles escaped during this period. This is in agreement with experimental measurements; ~25% of the 5-HT molecules were released during the rising phase when the granular membrane was electroporated in Ringer's medium. The curve obtained from the simulation is similar to that observed experimentally (compare Fig. 9 with Figure 7 *A* in Marszalek et al., 1997); both curves are initially concave towards the y axis. We found similar results when we simulated the conditions in the other electrolytes. The λ and the percentage of particles that escaped were 1.38 and 22% (histamine dihydrochloride), 1.6 and 26% (CsCl), 1.58 and 25% (NaCl), and 1.3 and 23% ($LaCl_3$).

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