Kinetics of Release of Serotonin from Isolated Secretory Granules. I. Amperometric Detection of Serotonin from Electroporated Granules

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ABSTRACT We developed a method for measuring the efflux of 5-hydroxytryptamine (5-HT, serotonin) from isolated intact granules of the mast cell of the beige mouse. This method combines electroporation of the vesicle membrane with amperometric detection of 5-HT. A single secretory granule is placed between two platinum electrodes (distance ~100 μm) and positioned adjacent (<1 μm) to a carbon fiber microelectrode. A short (~30 μs) high-intensity voltage pulse (electric field of ~5 kV/cm) is delivered to the electrodes to trigger the mechanical breakdown of the granule membrane, which activates the release of 5-HT. We observed concurrent swelling of the granule matrix with the oxidation of 5-HT at the carbon fiber electrode (overpotential + 650 mV). Similar to the release of secretory products during exocytosis, the oxidation current exhibits a spike-like time course with a noninstantaneous rising phase (time between onset of current and maximum flux, t_{max}) with ~25% of the molecules released during this period. When the current reaches its maximum, the granule matrix attains its maximum swollen state. We found that the rising phase depends on the initial cross-sectional area of the granule ($t_{max} \cong 21r^2$) and reflects the time required for membrane rupture. The average $t_{1/2}^{spike}$ of the amperometric spikes was found to be ≅150 ms, which is 3–7 times faster than the $t_{1/2}$ measured during cellular exocytosis.

INTRODUCTION

During exocytosis secretory products are released through a pore that connects the lumen of the secretory vesicle to the extracellular medium. Release from single vesicles can be monitored by amperometry with a carbon fiber electrode (Leszczyszyn et al., 1990; Chow et al., 1992; Jankowski et al., 1993; Alvarez de Toledo et al., 1993; Bruns and Jahn, 1995). Typically three different signals are recorded during cellular exocytosis: 1) a single amperometric spike, 2) a spike that is preceded by a small current (the "foot" of the spike); and 3) a small transient current (foot-like) not followed by a spike that is measured during transient fusion or "flicker." In the mast cell of the beige mouse the capacitance of the granule and the conductance of the fusion pore are monitored from changes of cell admittance measured during exocytosis, where the conductance correlates with the amperometric current of serotonin (5-HT) (Alvarez de Toledo et al., 1993). This suggests that "foot" signals that are recorded from many different cells (Chow et al., 1992; Alvarez de Toledo et al., 1993; Ureña et al., 1994; Bruns and Jahn, 1995; Zhou et al., 1996) represent the release of molecules through a small metastable fusion pore (diameter up to 20 nm; Spruce et al., 1990; Alvarez de Toledo et al., 1993) that can either close or irreversibly expand. If the pore expands irreversibly, a large amperometric current (the spike) follows the foot signal. This time-dependent geometry of the fusion pore is one of the factors modulating the rate of release. This rate is much slower than predicted from models in which the diffusivity of the secretory product within the vesicle and/or through the fusion pore is assumed to be the same as in bulk (Alvarez de Toledo et al., 1993; Wightman et al., 1995; Chow and von Rüden, 1995; Pihel et al., 1996). This suggests that the geometry at the site of exocytotic release is not the only factor limiting release, but the diffusivity of the secretory product within the vesicle and/or through the fusion pore is lower than in bulk.

At present it is difficult to model exocytotic release, especially during the main phase of release (i.e., the spike). This is because the irreversible expansion of the fusion pore cannot be readily monitored. In addition, the diffusivity of secretory products within the vesicle and the fusion pore are unknown for both the early and late phases of exocytotic release.

In this communication we report a method for measuring the efflux of serotonin from a secretory granule of a mast cell of the beige mouse. In the accompanying paper we determine the diffusivity within the granule and show that the efflux of 5-HT is controlled by ion exchange (Marszalek et al., 1997). In this method, electroporation of the vesicle membrane is used to trigger the efflux of serotonin from the granule, and this flux is then detected by amperometry at a carbon fiber electrode. We employ secretory granules of the mast cell extracted from the beige mouse because these granules are large (diameters up to ~7 μm) and are readily examined under the light microscope. In addition, because the vesicles are large and of cellular dimensions, their membranes can be readily electroporated at moderate electric field strengths (Tsong, 1991).

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MATERIALS AND METHODS

Isolation of intact secretory granules

Mast cell secretory granules were prepared from beige mice (bg^j/bg^j strain; Jackson Laboratories, Bar Harbor, ME), following the procedure described by Oberhauser and Fernandez (1993). Cells were obtained by peritoneal lavage by use of CO_2-independent medium (Life Technologies, Grand Island, NY) with 0.1% bovine serum albumin (fatty acid free; ICN Biomedicals, Aurora, OH) in solution (solution A). This suspension was then centrifuged at 100 × *g* for 10 min. Cells were resuspended in 1 ml of solution A and layered on 2 ml of 22.5% w/v metrizamide (dissolved in solution A) and centrifuged at room temperature for 20 min at 400 × *g* at the interface. The pellet was washed in 3 ml of solution A for 10 min at 100 × *g* and then resuspended in 1 ml of CO_2-independent medium. This suspension of purified mast cells was subjected to five or six sonication pulses at ~25% of the maximum power (sonifier model 450; Branson Sonic Power Co., Danbury, CT) and mixed by pipetting. Typically, sonication at this energy level breaks the cellular membrane, causing minimal damage to the granule membrane, and mixing of the suspension liberates granules from the partially broken cells. This suspension was plated onto glass-bottomed chambers and stored at 37°C under 5% CO_2 atmosphere. Unless otherwise stated, experiments were conducted in an external solution, either Hanks' balanced salt medium containing (in mM): 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 4 $NaHCO_3$, 0.3 Na_2HPO_4, 5.5 D-glucose with 0.01 g/liter phenol red (Sigma Chemical Co., St. Louis, MO), or a modified Ringer's medium containing (in mM): 150 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.5 NaOH, 2.8 KOH, 10 HEPES (300 mOsm/kg, pH 7.2). Typically, preparations always contain a mixture of granules, some with their membrane intact and others devoid of membrane. Intact granules are easily distinguished from granules devoid of membrane because they remain condensed and refractile when viewed with Nomarski optics. Secretory granules from the beige mouse mast cell are frequently nonspherical. We therefore calculate an equivalent radius for the granule (r_{eqv}) by use of $r_{eqv} = \sqrt{S/\pi}$, where S is the cross-sectional area of the granule measured from an image. Hereafter, when we refer to the granule radius, we actually mean its equivalent spherical radius.

Preparation of carbon fiber electrodes

Carbon fiber (Thornel P-55; Amoco Corp., Greenville, SC; nominal radius 5 μm) microelectrodes were prepared as described by Kawagoe et al. (1993). The electrodes were polished at a 45° angle on a micropipette beveler (model BV-10; Sutter Instruments, Novato, CA). 5-HT was detected at the carbon electrode that was held at +0.65 V versus a Ag/AgCl reference electrode (Alvarez de Toledo et al., 1993). The performance of the electrodes was tested by chronoamperometry. A step potential (0 to +0.65 V) was applied to an electrode immersed in a test solution containing 100 μM 5-HT, and a steady-state current (I_{total}) was measured. The current measured in the test solution without 5-HT ($I_{background}$) was subtracted from I_{total} to obtain the oxidation current of serotonin (I_{5HT}). I_{5HT} was compared with the magnitude of the oxidation current of serotonin in the external standard solution. Because the surfaces of carbon fibers deteriorate during an experiment, they were polished frequently (about every hour) to ensure maximum sensitivity.

Preparation of the electrode for electroporation of the granules

The granule membrane was electroporated with a platinum electrode; two platinum wires attached to a single glass pipette. The electrode was made by placing one of the wires (thickness ~80 μm) into a glass pipette with its end extending ~6 mm out of the pipette tip (diameter ~200 μm). The second wire was glued to the surface of the pipette in such a way that the ends (6 mm) of both wires were parallel and the distance between them was ~100 μm. The electrode was insulated and mechanically strengthened by coating the pipette with epoxy (Shell Epon resin 828; Miller-Stephenson Chemical Company, Sylmar, CA). The ends of the wires were exposed by removing the coating from the tip of the electrode. Before use, the electrode was cleaned by immersing the tip in bleach and rinsing in distilled water. The electrode was mounted in a holder and positioned (*x*, *y*, and *z*) by a micromanipulator.

RESULTS AND DISCUSSION

Description of the technique

Experiments were performed at room temperature on the stage of an inverted microscope (model IM-35; Carl Zeiss, Oberkochen, Germany) coupled to a CCD camera (model 4815–2000/0000; Cohu, Electronics Division, San Diego, CA). The experimental arrangement is outlined in Fig. 1. There are two main sections to the experimental apparatus, components required to 1) electroporate the granule membrane and 2) detect the release of amines from the granules. Electroporation is triggered by the discharge of a capacitor, C_p, through the platinum electrodes. C_p (2 μF) is charged to a desired voltage by the output of a high-voltage operational amplifier (Burr-Brown 3583). The output voltage (V_{out}) of the OP-AMP is determined by the input voltage (V_{in}) and the feedback loop (R_1: 994 Ω; R_2: 30,600 Ω, gain = 31.8). To minimize electrical interference in the amperometric circuit caused by the electroporation circuit, we separated the two circuits electrically by inserting switches SW1 and SW2. When C_p is being charged, switch SW1 is closed and SW2 is open; during electroporation, when C_p is discharging, SW1 is open and SW2 is closed. The variable resistor, R_3, controls the discharge time of the capacitor (30–300 μs). To analyze temporal changes of the granule size after electroporation, we required a time reference superimposed on the video recording. We used an auxiliary camera to

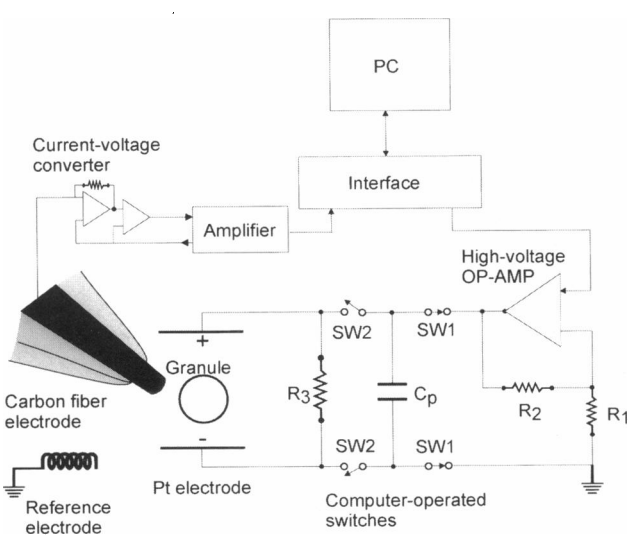


FIGURE 1 Schematic drawing of the experimental setup. A voltage pulse is delivered to the platinum electrodes (Pt) from a high-voltage operational amplifier, which is interfaced to a computer. The 5-HT released from the secretory granule is oxidized at a carbon fiber electrode. This current is amplified, sampled, and recorded on the computer. See text for more details.

monitor the signal that activated switches SW1 and SW2 (signal displayed on an oscilloscope) and mixed this video signal with the image obtained from the CCD camera attached to the microscope. Images were obtained at video rate (30 frames/s), digitized with a frame grabber (model Oculus-TCX; Coreco, St-Laurent, Québec, Canada), and analyzed with image-processing and analysis software (Ultimage; Graftek France, Mirmande, France).

The amperometric current collected at the carbon fiber was amplified by an Axopatch-1B patch-clamp amplifier (Axon Instruments, Foster City, CA). The fastest amperometric spikes were filtered at 500 Hz and sampled at 1 ms/point. Slower events were filtered at 200 or 100 Hz and sampled at 1.5–5 ms/point. A computer program written in LabView for Windows v3.1 (National Instruments, Austin, TX) controlled V_{in} and synchronized the triggering of the electroporating pulse (opening SW1, closing SW2) with the acquisition of the amperometric current with an AT-MIO-16X interface (National Instruments) (Fig. 1). The electroporating pulse usually generates a transient current (I_{art}) at the carbon fiber electrode (Fig. 2 *B*). The total current measured (I_{total}) is a sum of the oxidation current (I_{5HT}) and I_{art} (Fig. 2 *A*). The magnitude of the transient current varies among fibers, but is fairly reproducible for a fiber when subjected to the same electroporating voltage. We determined I_{art} by repeating the pulse after a granule was electroporated (Fig. 2 *B*), and subtracted I_{art} from I_{total} (Fig. 2 *C*) to calculate I_{5HT} (Fig. 2 *C*). The origin of I_{5HT} can be determined to an accuracy of 5–10 ms, because of the small transient left after this deconvolution routine (Fig. 2 *C*).

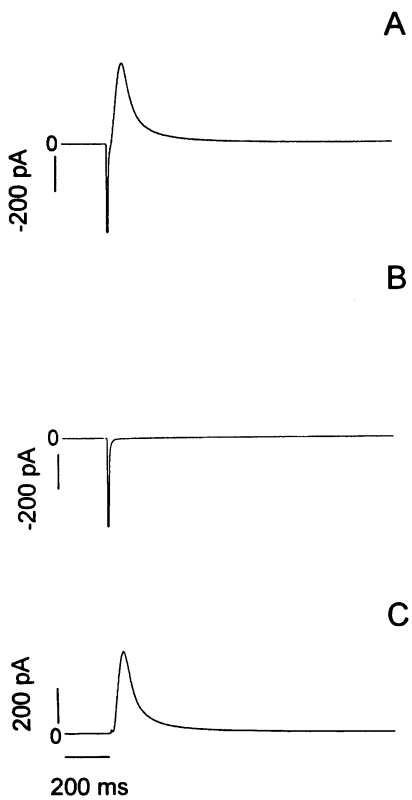


FIGURE 2 Current generated at the carbon fiber electrode. This current consists of the oxidation current of serotonin (I_{5HT}) and a transient current produced by the electroporating pulse (I_{art}). (*A*) Spike showing the total current (I_{total}) recorded upon electroporating the membrane of a secretory granule. (*B*) Spike showing transient current reproduced by repeating the electroporating pulse. (*C*) Spike showing the oxidation current of serotonin (I_{5HT}) obtained by subtracting *B* from *A*.

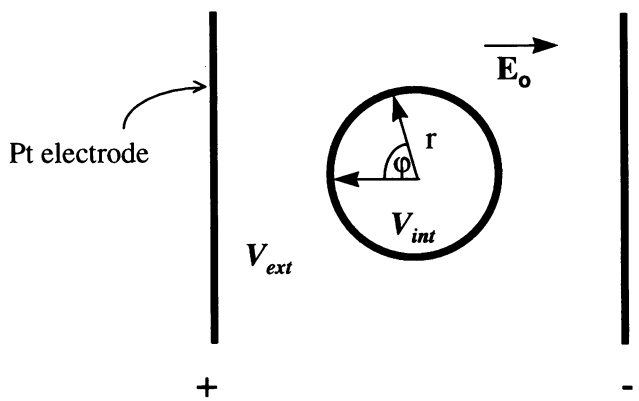
Electroporation of the membrane of the secretory granule of the beige mouse mast cell

Electroporation of cells is a standard technique that is used to transiently permeabilize plasma membranes (Neumann et al., 1989; Tsong, 1991). Numerous studies show that electroporation results from the dielectric breakdown of the lipid bilayer in supracritical electric fields. This field induces a membrane potential (ΔV_m) according to Eq. 1 (Cole, 1972; Fig. 3):

$$\Delta V_m = 1.5\, rE \cos \varphi$$

where r is the radius of a cell (granule) that is assumed to be spherical, E is the electric field strength, and φ is the angle between the surface normal and the field direction (Fig. 3). This equation is valid for conductive spheres that are surrounded by nonconductive membranes where the thickness of the membrane (d) is much less than r and the time constant for charging the membrane is small compared with the duration of the electric pulse (Hibino et al., 1991).

We determined that electroporation of the granule membrane occurred 1) if changes in the cross-sectional area of a granule were observed (Fig. 4) and/or 2) 5-HT was detected at the carbon fiber electrode (see next section). Condition 1) was used because secretory granules of mast cells of the beige mouse swell when their core is exposed to sodium saline. This swelling occurs because the core is a highly charged heparin sulfate proteoglycan matrix that expands in



$$\Delta V_m = V_{ext} - V_{int} = 1.5\, r\, E_o \cos \varphi$$

FIGURE 3 Schematic drawing showing the relationship between the electric field strength (E_o) and the potential induced across the granule membrane (ΔV_m), assuming the granule is a sphere with radius r.

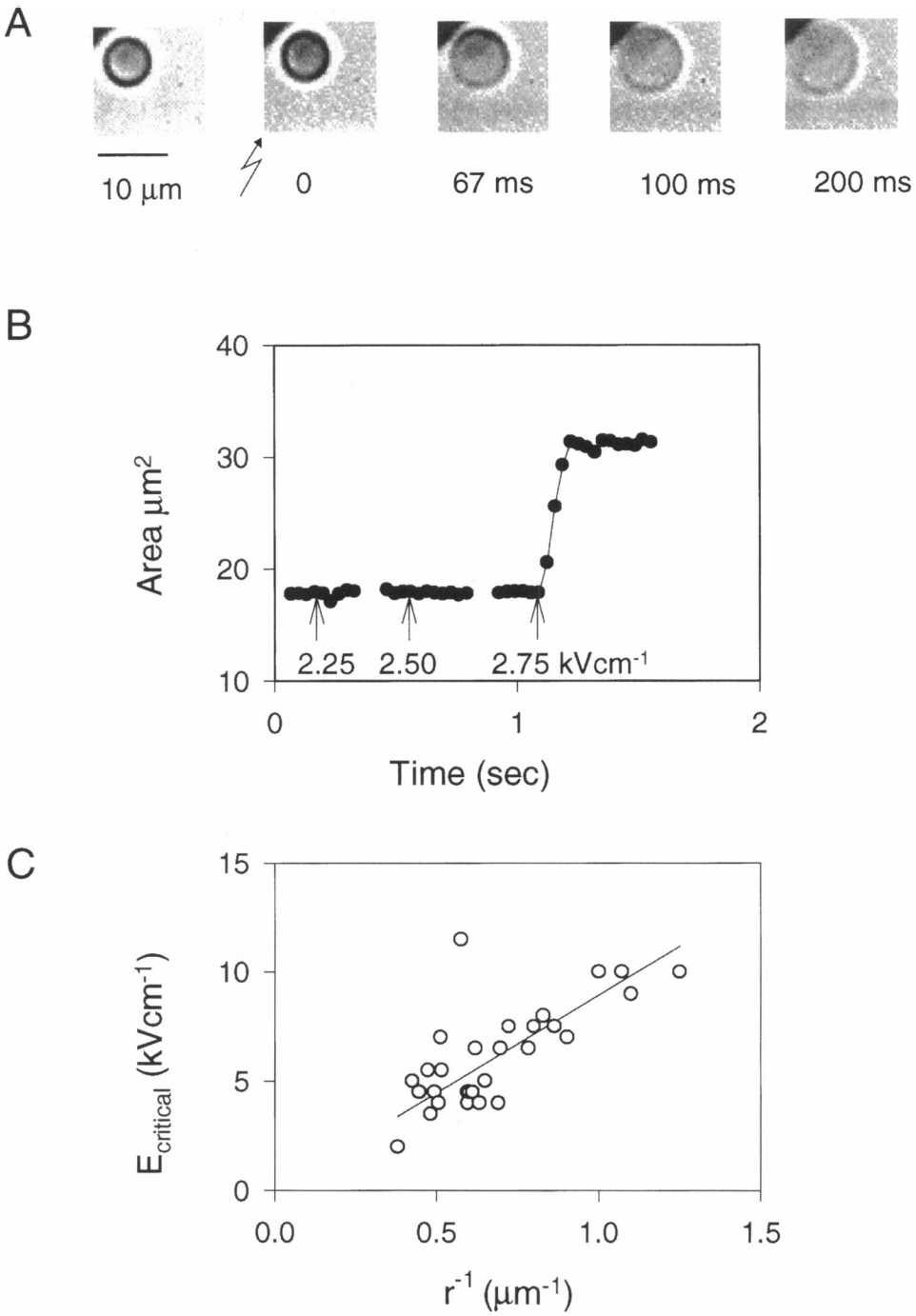


FIGURE 4 (*A*) Typical series of images showing a secretory granule before and after electroporating the membrane in external sodium saline. The duration of the pulse was ~30 μs. $E_{critical}$, the initial radius (r_o), and the final radius (r_f) of the granule were 3.3 kV cm^{-1}, 2.6 μm, and 3.6 μm, respectively. The edge of the carbon fiber (*black triangle*) is visible in the upper left corner of each image. (*B*) The critical electric field ($E_{critical}$) required to porate the granule membrane in external sodium saline was determined by monitoring the swelling of the matrix. The degree of swelling was monitored by measuring the change in the cross-sectional area of a secretory granule. The three arrows indicate the time when a pulse was delivered to the electrodes. The radius, $E_{critical}$, and ΔV_m^{crit} for this granule are 2.4 μm, 2.75 kV cm^{-1}, and 0.99 V, respectively. (*C*) The $E_{critical}$ required to porate the granule membrane is correlated with the radius of the granule. The critical membrane voltage ΔV_m^{crit} is 1.3 V, and it was calculated from the slope with Eq. 1.

electrolyte containing ~100 mM monovalent ions (e.g., Na and K) and recondenses in electrolyte containing the same concentration of divalent ions (Ca, histamine, and serotonin at an acidic pH of ~4; Curran and Brodwick, 1991; Fernandez et al., 1991).

We determined the critical membrane voltage ΔV_m^{crit} required to electroporate the membrane of an intact secretory granule by subjecting granules to brief (300-μs) pulses of an electric field and monitoring the cross-sectional area of a granule as the field strength was increased. A typical series of images of an intact secretory granule taken before and after electroporation of the membrane in sodium saline is shown in Fig. 4 *A*. Poration of the granule membrane at an electric field strength of 3.3 kV cm^{-1} caused the cross-sectional area of the granule to increase by about twofold within 100 ms. Outlined in Fig. 4 *B* is a detailed plot of the cross-sectional area measured from a second intact secretory granule when subjected to an electric field of increasing strength. Fields of 2.25 and 2.5 kV/cm had no visible effect on the surface area, but a pulse of 2.75 kV/cm immediately triggered swelling of the granule. Repeating this experiment many times with granules of various sizes, we found that the

critical electric field $E_{critical}$ is inversely related to the size of the granule. This suggests that Eq. 1 can be used to calculate ΔV_m^{crit} for the granule membrane, presumably because the granule membrane is subjected to a fairly uniform field (Fig. 3) and the matrix of the granule is conductive (Nanavati and Fernandez, 1993). The ΔV_m^{crit} was found to be 1.3 V (Fig. 4 *C*). This is in reasonable agreement with studies on electroporation of cells (spherical) where the ΔV_m^{crit} is ~1 V for pulses in the microsecond to millisecond range (Zimmermann, 1986; Tsong, 1991) and is within the range required to electroporate giant lipid vesicles (1.1–1.8 V) (Needham and Hochmuth, 1989). A voltage pulse of ~400 mV was sufficient to porate the membrane of the secretory granule of the mast cell of the beige mouse when it was attached to a patch pipette in "whole-granule" configuration (Oberhauser and Fernandez, 1993). In that setup the pulses were of much longer duration (~10 ms, compared to 300 μs), and the micropipette focused the electric field on a patch of the granule membrane.

Intact granules remain condensed in sodium saline solutions (Fig. 5). It is only when the membrane becomes permeable to the external electrolytes that the granule swells. To verify that swelling is induced by porating the membrane and is not a direct effect of the electric field on the matrix without the membrane becoming permeable, we repeated the electroporation experiments in 150 mM histamine dihydrochloride at pH 3.0 (Fig. 5). No swelling was observed when the intact granule was subjected to an electric field pulse (2.5 kV/cm, 300 μs), but the granule was observed to swell spontaneously upon the replacement of histamine solution with sodium saline. This implies that the primary effect of a brief and intense electric field is to porate the membrane; swelling only occurs when the matrix is exposed to sodium saline.

Characteristics of the oxidation current of serotonin measured after electroporation of the granule membrane

In a typical experiment, an intact secretory granule was positioned between two platinum wires (electroporating electrode) and adjacent (within 1 μm) to a carbon-fiber electrode (Figs. 1, 4 *A*, and 6). An image of the granule was recorded before the fiber was brought close to it, and this image was used to determine the initial size of the granule (Fig. 6, *leftmost image*). A second image was recorded once the granule was positioned adjacent to the fiber and is a record of the fiber-granule geometry at the site of release (Fig. 6, *second images from left*). We routinely achieved electroporation of the granule membrane by using electric fields that were within the range $E_{critical} < E < 2E_{critical}$, unless otherwise stated. After electroporation in sodium saline, rapid swelling of the granule was observed (Fig. 6, *third image from left*), and the oxidation current of 5-HT was measured (Fig. 6, *right*) simultaneously. To determine the maximum size of the granule, the fiber was moved away and an image of the granule (Fig. 6, *fourth image from the left*) was obtained.

Outlined in Fig. 6 (*rightmost panels*) and Fig. 7, *A* and *B*, are traces of the oxidation current of 5-HT obtained from the granules. The efflux of 5-HT exhibits a spike-like time course with a noninstantaneous rising phase and a current that decays exponentially from its peak amplitude. We characterize this current (Fig. 7 *A*) by measuring the integral of the spike (Q), the total charge detected, the peak amplitude (I_{max}), the rising phase (t_{max}), the time from the onset of the current until I_{max} is reached, the width of the spike at half of its maximum amplitude ($t_{1/2}^{spike}$), and the time at which half of the 5-HT molecules are released ($t_{1/2}^{int}$, determined from the time course of the integral). Only a fraction of the total efflux is collected by the carbon fiber (~10-μm diameter), and this amount depends on the granule-fiber geometry at the site of release (Figs. 4 and 6). We estimate a lower limit for this fraction at ~25% for large granules (r = 3 μm) and ~40% for smaller granules (r = 1 μm). The efflux may also be affected by the carbon fiber, which acts like a sink and modifies the concentration gradient of 5-HT at the side of the granule facing the fiber. Although we do not collect the total efflux, we do assume that the time course of the amperometric spike reflects the time course of this total flux. This is a reasonable assumption, provided that the concentration of 5-HT is uniform within the granule

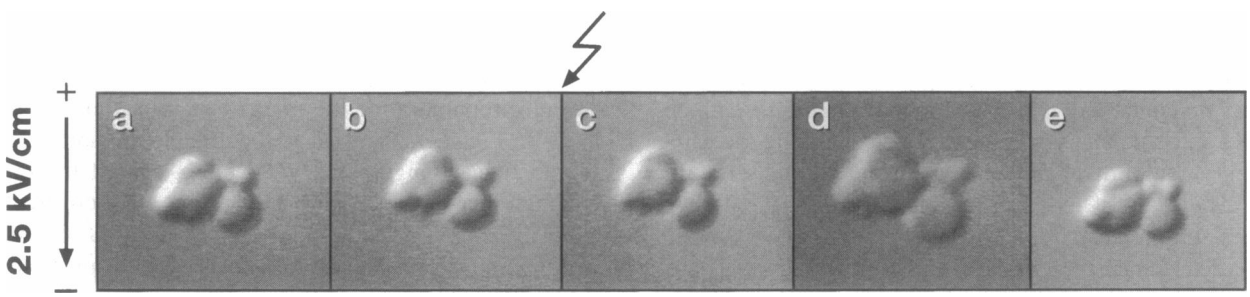


FIGURE 5 A transient electric field (~300 μs) porates the membrane, but does not directly induce swelling of the granule matrix. A sequence of images shows a single granule in standard sodium saline (*a*). The solution was then replaced by 140 mM histamine dihydrochloride (pH 3.5) (*b*). In this medium the granule was subjected to an electroplating pulse (2.5 kV/cm), but the matrix of the granule did not swell (*c*). The granule swelled spontaneously when the solution was replaced by sodium saline (*d*) and recondensed when the solution was replaced by histamine dihydrochloride (*e*).

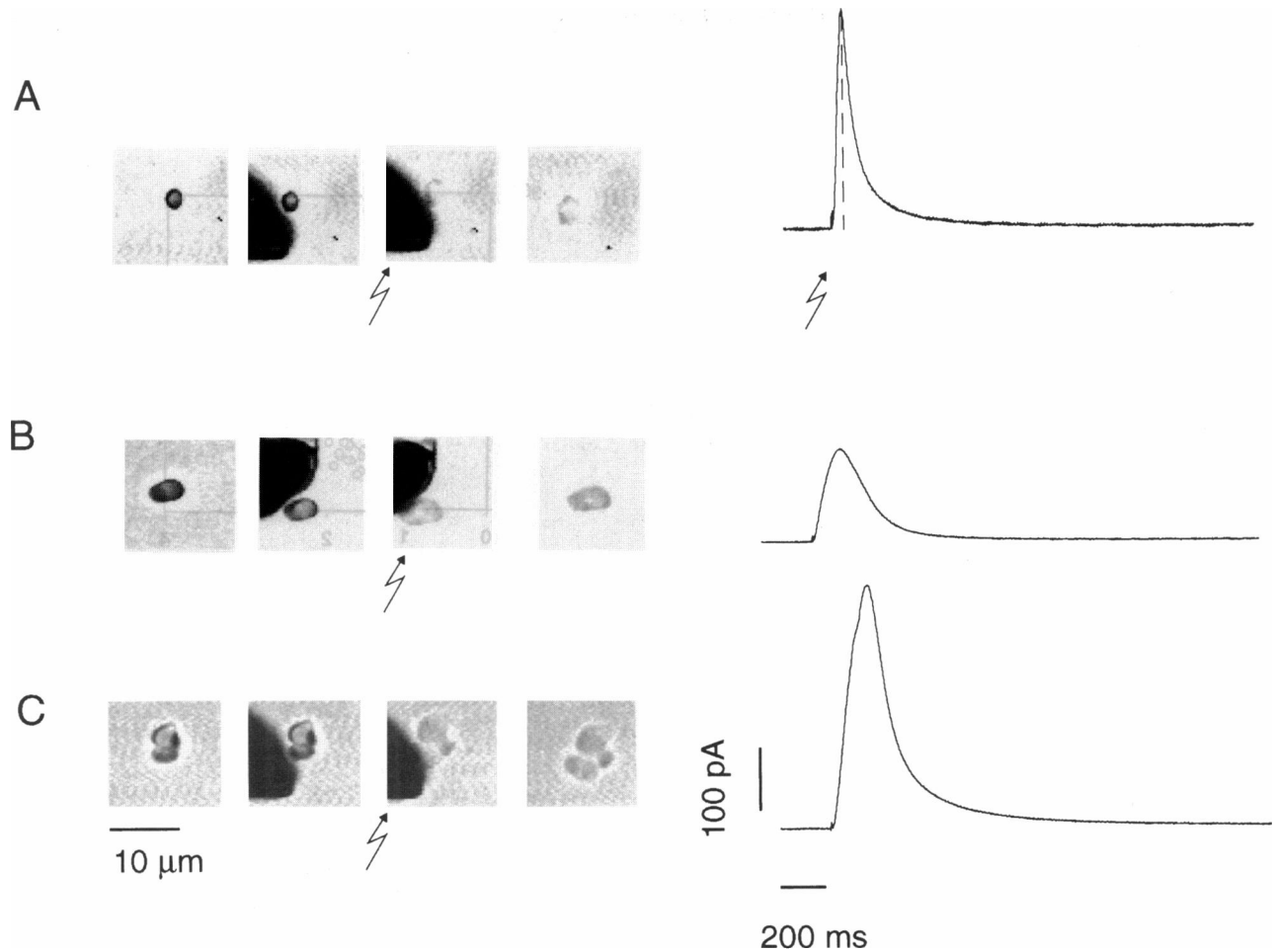


FIGURE 6 The size and shape of the granules from the mast cell of the beige mouse are variable and generally not spherical, and this variability is reflected in the amperometric current collected at the carbon fiber electrode after the membrane is electroporated (*rightmost panels*). The images left of the arrow were obtained before the granule membrane was electroporated. The Q, I_{max}, $t_{1/2}^{spike}$, $t_{1/2}^{int}$, t_{max}, r_o, and r_f for the first to third spike are 50 pC, 405 pA, 68 ms, 102 ms, 49 ms, 1.7 μm, and 2.5 μm (*A*); 29 pC, 128 pA, 186 ms, 159 ms, 112 ms, 2.3 μm, and 2.8 μm (*B*); and 92 pC, 367 pA, 173 ms, 196 ms, 152 ms, 2.85 μm, and 4.2 μm (*C*), respectively.

and the flux is approximately isotropic. Amperometric spikes are, of course, broadened by the diffusion of serotonin to the detector. Because the distance between the edge of the granule and the detector is less than or equal to 1 μm, this additional diffusion, which takes ~1 ms (assuming a diffusion coefficient in the external solution of 1×10^{-5} $cm^2\ s^{-1}$), has a minimal effect on the width of the spike, which has an average $t_{1/2}^{spike}$ of ~150 ms.

In the majority of cases (99%, $n = 190$) there was no measurable delay between the electroporating pulse and the onset of the amperometric spike, and none of the spikes were preceded by a small current indicative of the "foot" signal (Chow et al., 1992). This observation suggests that the electroporating pulse triggered the irreversible breakdown of the granule membrane, i.e., no stable pores formed. Even in the few events (1%) where the amperometric spike was delayed relative to the electroporating pulse, a foot-like signal was not observed (data not shown). This suggests that the granule membrane was in a metastable state for a few hundred milliseconds before it ruptured, but during this time it was not permeable to serotonin.

The size and shape of the granules from beige mouse mast cells are highly variable, and these characteristics are reflected in the amperometric spikes shown in Fig. 6. The $t_{1/2}^{spike}$ measured from the spike generated by the medium-sized granule of radius 1.7 μm is 68 ms (Fig. 6 *A*), compared to a $t_{1/2}^{spike}$ of 186 ms measured from the spike of the bigger granule (Fig. 6 *B*) with a radius of 2.3 μm. Similarly, the rising phase of the spikes increased with granule size from 49 to 112 ms. Giant granules of the mast cell of the beige mouse are formed from several smaller granules, and frequently the granule subunits can still be discerned. Typical examples are shown in Figs. 5 and 6 *C*. The spikes are similar to those observed from granules with a more uniform appearance, and sometimes they display small anomalies in the rising phase of the spike (Fig. 6 *C*).

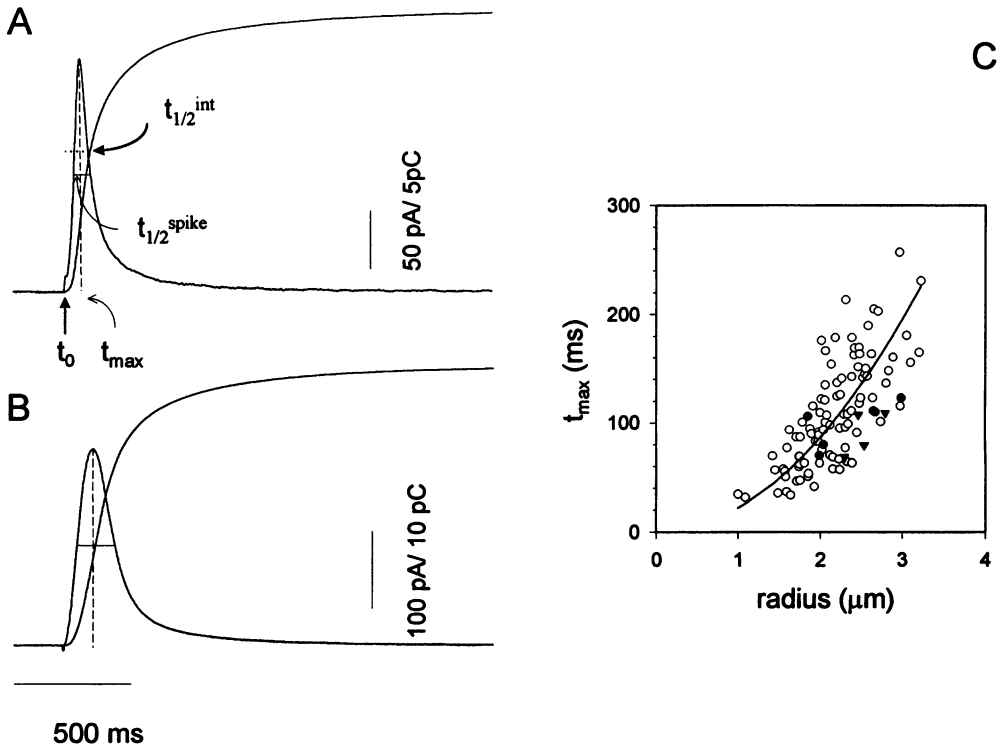


FIGURE 7 Amperometric spikes exhibiting fast (*A*) and slow (*B*) rising phases and the integral of the spikes. The Q, I_{max}, $t_{1/2}^{spike}$, $t_{1/2}^{int}$, t_{max}, r_o, and r_f for the upper and lower spikes are 24 pC, 202 pA, 71 ms, 108 ms, 54 ms, 1.9 μm, and 2.9 μm (*A*) and 54 pC, 255 pA, 159 ms, 162 ms, 116 ms, 3.0 μm, and 3.44 μm (*B*), respectively. The area under the spike from the onset to t_{max} represents 19% and 30% of the total area of the spike, i.e., 19% and 30% of the molecules were released before the current attained its maximum value. (*C*) The duration of the rising phase of the amperometric spike of serotonin depends on the size of the granule. ○, ▼, Efflux triggered by electroporation; ●, efflux triggered by the addition of Triton X-100. Solid triangles represent granules that were porated at 2–3 times the $E_{critical}$. The solid line represents a fit of the data to $t_{max} = Ar^2$, where $A = 21$. The data obtained in Triton X-100 were not included in the fit.

The rising phase of the amperometric spike reflects the time required for membrane rupture

Similar to the cell undergoing exocytotic release, the amperometric spikes obtained from the electroporated granules exhibit a noninstantaneous rising phase (Figs. 6 and 7, *A* and *B*). In Fig. 7 examples of spikes that exhibit a fast (t_{max} of 54 ms) and slow (t_{max} of 116 ms) rising phase are shown; ~19% (Fig. 7 *A*) and ~30% (Fig. 7 *B*) of the 5-HT molecules were released during this phase. We found for granules immersed in saline ($n = 100$) that between 15% and 35% of the molecules were released during the rising phase. We also found that the rising phase of the amperometric spike obtained from isolated granules is correlated with the radius of the granule (Fig. 7 *C*). We fitted t_{max} against the initial radius of the granule to the equation $t_{max} = Ar^x$ and found a reasonable correlation ($\rho = 0.75$, $n = 100$) with A and x equivalent to 27.5 and 1.7, respectively. This suggests that the rising phase depends upon the initial area of the granule ($t_{max} \approx 21r^2$, $\rho = 0.71$) and reflects the time required for membrane rupture (Fig. 7 *C*). This time is determined by the kinetics of the electroporation and the rate of expansion of the pores. Poration is a rapid process (microsecond time scale; Hibino et al., 1991), whereas the expansion of the pores that leads to the irreversible breakdown of the membrane is slower (millisecond time scale; Chernomordik et al., 1987). The magnitude of the rising phases (~30–260 ms) measured from the amperometric spikes (Fig. 7, *A* and *B*) are on the same order of magnitude as the time required for pore expansion in erythrocyte membranes (~100 ms; Chernomordik et al., 1987) and slower than the time required for pore expansion in planar lipid bilayers (~1 ms). Wilhelm et al. (1993) studied the kinetics of membrane rupture in planar lipid bilayers and found that the conductance of the pore(s) increases linearly with time. The current of the rising phase also increases linearly with time (between 10% and 90% of the peak amplitude), supporting our hypothesis that this phase reflects the time for membrane rupture. The current increased at a constant rate of 6 pA/ms (Fig. 7 *A*) and 3 pA/ms (Fig. 7 *B*), which is ~3–17 times faster than that observed during exocytosis (Alvarez de Toledo et al., 1993). To establish if we could decrease the time required for rupture, we increased E two- to threefold above $E_{critical}$ (Fig. 7 *C*, *solid triangles*), but found that t_{max} was not significantly faster. This suggests that the time required for membrane rupture does not critically depend upon the electric field strength, at least for pulses 100 μs in range. This is in agreement with results obtained from planar lipid bilayers, where the kinet-

ics of membrane rupture was found not to be controlled by the strength of the electric field (Wilhelm et al., 1993).

We also compared the time course of swelling with the concurrent efflux of serotonin and found that the granule matrix reaches its maximum swollen state when the current attains its maximum value (Fig. 8). For the typical example shown in Fig. 8, the surface area of the granule (see Fig. 4 *A*) increased 1.9 times upon electroporation of its membrane in sodium saline, and during the rising period only ~25% of the 5-HT molecules diffused from the granule. This is further evidence that the irreversible breakdown of the membrane must be complete before I_{max} is reached, because membranes can only be stretched by a small amount (~2–3%) before they rupture (Kwok and Evans, 1981; Needham and Hochmuth, 1989).

To test whether the efflux of serotonin critically depends on the way the membrane breaks down, we dissolved the granule membrane by the addition of surfactant (Triton X-100, final concentration 0.005%; *solid circles* in Fig. 7 *C*). The amperometric spikes are presented in Fig. 9, *A* and *B*; two patterns were observed. In one case the amperometric spikes were similar to those obtained when the granule membrane was electroporated (Fig. 9 *A*); in the second case, a small, slowly increasing current preceded the main spike (Fig. 9 *B*). However, the t_{max} measured in both cases is similar and close to that observed when the membrane was electroporated (Fig. 7 *C*). The small signal preceding the main spike resembles the "foot" signal measured during cellular exocytosis. This observation is interesting and indicates that foot signals not only arise when two membranes fuse (i.e., cellular exocytosis), but also occur when surfactant molecules interact with the granule membrane.

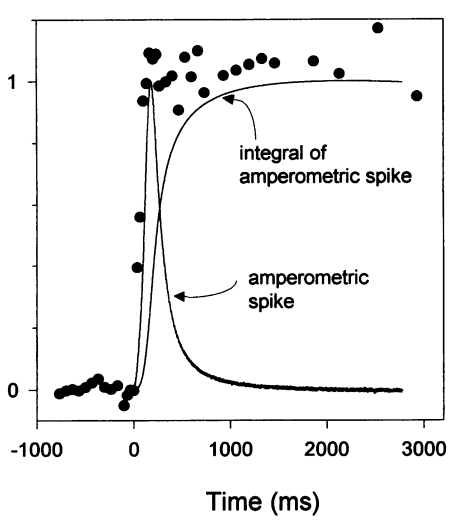


FIGURE 8 Comparison of the time course of swelling of the granule matrix and the efflux of 5-HT. The solid circles represent the change of the granule radius at a given time (t) normalized by the largest change in the radius, i.e., $(r(t) - r_o(t))/(r_f(t) - r_o(t))$, where r_o and r_f are the initial and final radius of the granule. The solid lines represent the time course of the amperometric current and the fraction of molecules released over this same time; these are normalized by the maximum current and integral, respectively.

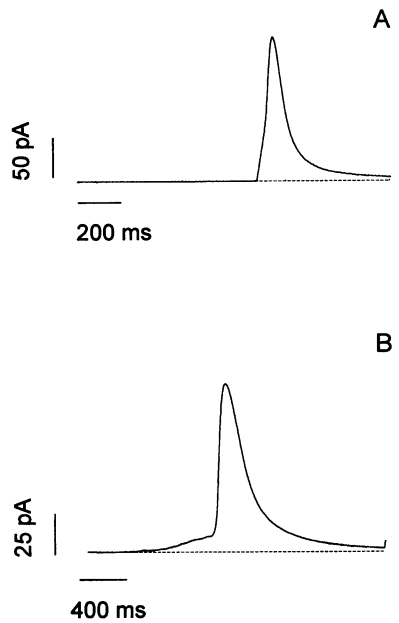


FIGURE 9 Typical amperometric spikes of serotonin obtained when the efflux was triggered by addition of surfactant to the external saline (Triton X-100). Triton X-100 was added to Ringer's medium (final concentration ~0.005%), the current at the carbon fiber electrode was continuously monitored, and the granule was continuously imaged. It took several seconds for Triton X-100 to diffuse to the granule, after which a spike-like current of serotonin was recorded at the fiber in *A* and *B*. In *B* the main spike is preceded by a small, slowly increasing current that is similar to the "foot" signal measured during cellular exocytosis (Chow et al., 1992). The Q, I_{max}, $t_{1/2}^{spike}$, t_{max}, $r_{initial}$, r_{final} are, respectively, 29.8 pC, 210 pA, 89 ms, 80 ms, 2.0 μm, and 2.9 μm for the main spike in *A*, and 38.5 pC, 97 pA, 225 ms, 106 ms, 1.85 μm, and 2.8 μm for the spike in *B*.

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