Quantal Release of Serotonin from Platelets

Shencheng Ge,† James G. White,‡ and Christy L. Haynes*,†

Department of Chemistry, and Department of Laboratory Medicine, Pathology and Pediatrics, School of Medicine, University of Minnesota, 207 Pleasant Street SE, Minneapolis, Minnesota 55455

Even though platelets are known to play a critical role in hemostasis, mediated in part by their uptake, storage, and release of serotonin, there are many unexplored aspects of this process. Herein, single-cell amperometry is employed to characterize the dynamic secretion of serotonin from platelet dense-body granules. On the basis of a three-dimensional random walk simulation that estimates detection efficiency with varied spacing between the carbon-fiber microelectrode and the platelet, it is clear that the detected charge likely represents complete oxidation of the released granule contents and, thus, is a good method to calculate the serotonin concentration in each granule. Using the measured charge and volume estimates based on transmission electron microscopy (TEM) data, the granular concentration of serotonin is approximately 0.5 M. The simulated spike widths are significantly narrower than most of the measured amperometric spikes, clearly indicating that the stored serotonin is highly associated with an aggregate rather than freely diffusible within the dense-body granule. Additionally, by varying extracellular buffer temperature and pH to adjust the driving forces for serotonin delivery from the dense-body granules to the extracellular space, it is clear that, although platelet chemical messenger storage and secretion is similar to that of other secretory cells, there are some important distinctions.

Platelets in the blood stream are widely known for their pivotal role in primary hemostasis, that is the arrest of bleeding after blood vessel breakage.1–3 These anuclear cells are discoid in shape, approximately 3 µm in diameter and 1 µm thick, significantly smaller than other types of blood cells. Their primary role in hemostasis is based on their function as secretory cells, and they are known to have multiple specialized compartments that coexist in the platelet cytoplasm, including α-granules and dense-body granules.1–3 These intracellular compartments store a myriad of chemical species such as adhesive and healing proteins in α-granules and small molecules and ions in dense-body granules.14 Upon exposure to collagen after vessel breakage, platelets initiate a cascade of cellular processes which culminate in secretion of contents from α- and dense-body granules. Released messengers recruit and activate additional platelets at the injury site through a positive feedback loop, eventually forming a platelet plug to cease blood loss, the essential step in primary hemostasis.7 Many techniques have been used historically to characterize the release process from platelets. For example, high-performance liquid chromatography and radiometric assays are extensively used to characterize the release of serotonin from platelets,5,6 however, chromatographic analysis and radiometric assays provide an assessment of average response from a population of cells, masking heterogeneity among individual cells. Transmission electron microscopy (TEM) has also been used extensively for decades to study the microstructural changes and granular fusion in individual platelets.7,8 Despite its ability to reveal numerous cellular features, TEM only provides a static picture of secretion from platelets. Until now, there is a paucity of information available to describe the dynamic nature of chemical messenger secretion in individual platelets. Measuring the dynamics of granular release in individual platelets will give insight into the basic process of hemostasis and may also reveal the mode of dysfunction in diseased platelets. Hitherto, due to their extremely small size and their propensity to be activated, measuring the dynamic secretion from the single platelets represented a highly challenging analytical problem. As with our previous study,9 the work presented herein exploits carbon-fiber microelectrochemistry to monitor the dynamic secretion of chemical messengers from dense-body granules in single platelets. Interpretation of the data presented herein reveals how perturbations to the extracellular environment, including temperature and extracellular pH, influence the molecular machinery that governs exocytosis in the platelets and the release kinetics of the chemical messengers from dense-body granules. Furthermore, the present study also demonstrates the potential of this method to explore malfunctioning platelets in diseases such as Hermansky–Pudlak syndrome.4,10

EXPERIMENTAL SECTION

Preparation of Washed Platelets. The protocol for preparation of washed rabbit platelets is approved by the University of Minnesota IACUC protocol 0802A27063. An amount of 10–15 mL of blood was drawn from the midear artery after a rabbit (New Zealand Rabbit, Bakkom Rabbitry, Wisconsin) was sedated using

butorphanol and acepromazine. The collected blood was mixed with the anticoagulant ACD at a ratio of 6:1 (v/v). The anticoagulated blood was then centrifuged at 500g for 15 min to separate platelet-rich plasma from the whole blood. Then, the platelets were separated from the isolated platelet-rich plasma and washed once via centrifugation at 750g for 4 min. Finally, the platelets were resuspended in a Ca²⁺-free bicarbonate buffered Tyrode’s medium (NaCl 136.9 mM, KCl 2.6 mM, MgCl₂ 1.0 mM, d-glucose 5.6 mM, and NaHCO₃ 12.1 mM with pH adjusted to 7.2). This preparation always yielded a platelet suspension contaminated by red blood cells; however, platelets are readily distinguishable from other cell types based on size and morphology. Thus, no further effort was made to prepare pure, washed platelets. The washed platelets prepared using this method were stable for several hours and provided ample time for the detailed experiments.

**TEM Preparation.** Fixation was accomplished by combining the sample at the appropriate time with an equal volume of 0.1% glutaraldehyde in White’s saline (a 10% solution of a 1:1 mixture of (1) 2.4 mM NaCl, 0.1 mM KCl, 46 mM MgSO₄, and 64 mM Ca (NO₃)₂·4H₂O and (2) 0.13 M NaHCO₃, 8.4 mM NaH₂PO₄, and 0.1 g/L of phenol red, pH 7.4). After 15 min the samples were centrifuged to pellets, and the supernatant fixative removed and replaced with 3% glutaraldehyde in the same buffer. The samples resuspended in the second aldehyde fixative were maintained at 4 °C for 30 min and then sedimented to pellets. The supernatant was removed and replaced with either 1% osmic acid in Zetterquist’s buffer or 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for 1 h at 4 °C. All samples were dehydrated in a graded series of alcohols and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance contrast. All examinations were made in a Phillips 301 electron microscope.

**Electrochemistry Measurement.** The fabrication procedure for the microelectrodes followed a previously published protocol. Briefly, a carbon fiber (Thornel T650, Amoco Corp., Greenville, SC) was aspirated into a glass capillary (A-M system, Carlsborg, WA) and heated under the force of gravity using a micropipette puller (PE-21, Narishige, Tokyo, Japan) to create two microelectrodes with tapered tips, where the carbon fiber was tightly encased in a thin glass shell. The carbon fiber was trimmed as close to the end of the pulled glass capillary as possible using a scalpel. Then, the trimmed carbon-fiber electrodes were dipped into a preheated mixture of EPON resin 828 (Miller-Stephenson, Morton Grove, IL) and 15% weight % hardener 1,3-phenylenediamine (Sigma-Aldrich, St. Louis, MO) and allowed to cure at 25, 100, and 150 °C for overnight, 4 and 4 h, respectively. One day before the experiment, 5–7 microelectrodes were polished on a diamond-embedded beveling wheel (BV-10 microelectrode beveler, Sutter Instrument Company, Novato, CA) at a 45° angle to maximize cell–electrode contact area. Each polished electrode had a freshly exposed elliptical carbon-fiber electroactive area surrounded by a thin glass insulating perimeter. After the electrodes were back-filled with 4.0 M potassium acetate and 150.0 mM potassium chloride solution for electrical contact, the tips were soaked in isopropyl alcohol until use. One electrode is typically used for measurements from five platelets before repolishing.

All electrochemistry measurements were conducted in a temperature-controlled chamber (Warner Instruments, Hamden, CT) on an inverted microscope (Eclipse TE2000U, Nikon, Tokyo, Japan) with phase-contrast optics. A 40× phase-contrast objective is typically required for viewing platelets. The experiments used an Axopatch 200B potentiostat (Molecular Device, Sunnyvale, CA) and were controlled by a locally written LabView program (National Instruments, Austin, TX). A two-electrode configuration was employed where a Ag/AgCl electrode (MF 2052, BASi, West Lafayette, IN) was used as the reference electrode. The working electrode, a carbon-fiber microelectrode, in a headstage was mounted on a piezo-controlled micromanipulator (Burleigh PCS-5000, EXFO life science and industrial division, Ontario, Canada) allowing fine three-dimensional (3D) positioning. An additional glass capillary (orifice 10–15 μm) containing stimulating solution was placed adjacent to the working electrode and was controlled by a picospritzer (Parker Hannifin Corp., Cleveland, OH) in order to deliver stimulant to the target cells underneath the working electrode when needed. An optical micrograph showing the relative positions and sizes of the working electrode and the stimulant-containing glass capillary is shown in Figure 1A.

After the working electrode, reference electrode, and the stimulant-containing glass capillary were properly positioned, the washed platelets could be loaded into the experimental chamber; however, the loading procedures were different for temperature- and pH-controlled experiments. Briefly, for temperature-controlled experiments, the medium (Tyrode’s solution, pH 7.2) in the chamber was first heated to the desired temperature and then one small droplet of room-temperature platelet suspension was added to the warmed medium. The mixture was quickly stirred by a plastic pipet to ensure the rapid mixing of platelets suspension with the warmed medium. Platelets were allowed to sediment onto the polylysine-coated coverslip briefly before electrochemistry measurements. For pH-controlled experiments, the procedure was identical except that the washed platelets in storage were resuspended in pH-adjusted medium before they were loaded into the chamber. Meanwhile, the pH of the stimulating solution was adjusted to match the medium pH. All pH-controlled experiments were conducted at room temperature, and all temperature-controlled experiments were conducted at physiological pH. Under these conditions, it took approximately 10–15 min for all the platelets to sediment onto the coverslip. If left for a longer than a few minutes after sedimentation, the platelets would be activated based only on contact with the coverslip. Accordingly, all electrochemical measurements were completed within several minutes after chamber loading. Repeated loading could be easily achieved to collect enough data from a population of platelets for statistical analysis.

For amperometry experiments, a positive potential, +700 mV versus Ag/AgCl, was first applied to the carbon-fiber working electrode. Then it was tightly positioned onto an unactivated platelet. The precise spacing between beveled tip and the platelet on the coverslip is difficult to measure due to the relatively large size of the carbon-fiber microelectrode in comparison to the

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The carbon-fiber microelectrode, on the right, is shown to be positioned close to a glass capillary, on the left, containing stimulant, 10 µM ionomycin. Platelets appear as dots on the coverslip and as a light ring when they are suspended in solution and out of focus. Inactivated platelets are usually round and light-refractive, whereas the activated platelets typically change their shape from round to spiky and appear dark when viewed using phase-contrast optics. A red blood cell is shown as well slightly below the glass capillary. (B) Electron micrograph showing the microstructure of the platelet interior. Dense-body granules, marked by arrows on one of the platelets, are scattered throughout the cytoplasm. The less dark and slightly bigger granules are α-granules where numerous proteins are stored. (C) A typical amperometric trace showing multiple spikes detected as serotonin is released from the dense-body granules of a single platelet. A magnified view of an individual spike reveals a rapid rising phase before the maximum and a relatively slow decay afterward. The inset cyclic voltammogram (solid line) obtained from the maximum of a spike (represented by the box on the amperometry spike maximum) overlaps that (dotted line) from 10 µM standard serotonin solution and confirms the secreted and detected molecules are serotonin. (D) Histogram of dense-body and granular size obtained from TEM study. Data measured from 284 identified dense-body granules were used for the construction of these plots. The best-fit Gaussian distributions are superimposed on the histograms. The mean and standard deviation for both best-fit curves are (expressed in mean ± standard deviation): 64.45 ± 17.39 nm for dense bodies and 87.52 ± 20.72 nm for granules. (E) Histogram of \( Q \) obtained from the amperometry study. A total of 460 spikes from 25 platelets were used to construct this plot. The best-fit Gaussian distribution is superimposed on the histogram. The mean and standard deviation for the best-fit curve are 5.47 ± 2.14 fC1/3.

Data Processing and Statistical Analysis. The amperometric data were exported, digitally filtered at 1000 Hz, and then analyzed using commercially available software (Minianalysis, Snyptosoft Inc., Decatur, CA). The threshold for spike detection was set to 5 times the root-mean-square of the background current. Spike analysis included the determination of the following parameters: \( I_{amp} \), amplitude of spike; \( T_{rise} \), the time from 10% to 90% of the spike maximum on the rising phase of the spike; \( T_{decay} \), the time from 100% to 20% of the spike maximum on the decaying phase of the spike; \( T_{1/2} \), spike width at the half-maximum; and \( Q \), the spike area integrated from 0.5% of the rising peak amplitude to 20% of the decaying peak amplitude. An automatic analysis function within the software was first used to detect spikes. A second visual check was followed to ensure correct evaluation of the detected spikes and rule out any possible errors. Mean spike characteristics were calculated for each platelet, and then data from platelets exposed to identical experimental conditions were subsequently pooled together for statistical comparison to other platelet populations. This approach was selected, instead of pooling all spikes from that population of platelets, to minimize overrepresentation of spikes with large areas.\(^{13}\) Two additional parameters, \( N_{spike} \) and largest percentage within 2 s window, were analyzed for each platelet. \( N_{spike} \) represents the total number of spikes detected for each

individual platelet. The largest percentage within 2 s window indicates the percentage of spikes detected within the 2 s window where spike frequency is highest within a given amperometry trace. No outliers were excluded in the analysis presented herein; however, two additional constraints were imposed after the initial data analysis: (1) excluding traces with $N_{\text{spike}} < 10$ or (2) excluding nonburst-like release. The reanalysis did not change the overall trend for the parameters above (data not shown). All data are reported as mean ± standard error of the mean (mean ± SEM) and subject to the unpaired and two-tailed Student’s $t$ test.

**Digital Simulation.** A three-dimensional random walk simulation was used to evaluate the diffusional broadening from an instantaneous point source release of molecules situated on an inert plane with a carbon-fiber microelectrode positioned above. The geometry for the simulation is shown in Figure 2A. The differences between the actual experimental setup and the simulation are the electrode orientation and tip shape. Instead of the actual angle of 45° with respect to the plane of coverslip, the simulated electrode is perpendicular to the inert plane. Also, the simulated electrode tip is assumed to be circular rather than elliptical. To simulate the signal collected during an amperometric measurement, the electrode tip acts as a molecular sink where all electroactive molecules are instantaneously oxidized and consumed upon collision with the active part of the electrode. In a typical simulation, each of 50 000 molecules was allowed to complete a random walk process as described below. Initially, one molecule originating from the point source was allowed to step over one unit length, $x$, in all three dimensions simultaneously with the time interval determined by $2(D_{\text{serotonin}})(t) = x^2$, where $D_{\text{serotonin}}$ is the diffusion coefficient for serotonin in free solution, $5.4 \times 10^{-6}$ cm$^2$/s. The step size is always less than $1/10$ of the separating distance between the electrode and the inert plane, $h$. The specific direction for each step in one dimension was randomly determined. In cases where the molecule collided with the inert plane or the insulating layer, it would bounce back to its original position. When the step resulted in a collision between the molecule and the carbon-fiber surface or the number of steps exceeded a critical number, the random walk for that molecule was stopped. The critical number was selected to ensure that the longest time a molecule could wander did not exceed $10T_{1/2}$, where $T_{1/2}$ is the full time width at the half-maximum of the response by the electrode under the conditions simulated. The detection efficiency was calculated from the ratio of detected molecules to total molecules.

**RESULTS AND DISCUSSION**

**Quantal Secretion of Serotonin.** Small-molecule chemical messengers, including serotonin, ATP, ADP, and Ca$^{2+}$ ions that are stored in dense-body granules, are among the species secreted after platelets are activated. Serotonin, an electroactive molecule, is known to be stored in the dense-body granules of platelets across a variety of species including humans. In the

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blood stream, serotonin is taken up, stored, and released only by platelets, and accordingly, understanding the fundamental characteristics and dynamic behavior of serotonin in platelets is critical. Single-cell carbon-fiber electrochemistry measurements can accomplish this task as our previous work has already demonstrated. Figure 1C shows a typical amperometric trace collected from a platelet due to oxidation of serotonin after activation by ionomycin, an ionophore that transports extracellular Ca$^{2+}$ across the cell membrane to trigger the secretion machinery. Using the same electrode, a cyclic voltammogram, Figure 1C inset, from a single release event confirms the molecular identity as serotonin.

Electrochemical data provides a unique perspective on dense-body secretion. Analysis of individual spikes reveals a wealth of information on this dynamic process. The full width at the half-height ($T_{1/2}$) indicates that serotonin stored in the dense-body granules complete the release process within 7.2 ± 0.6 ms (Figure 2E, averaged from 24 platelets), that is the average time for serotonin to escape from the granule. Measurement of the area under each spike facilitates accurate quantification of the serotonin released from individual dense-body granules. Clearly, dense-body granules display size heterogeneity within both single and populations of platelets. The electrochemical data exhibit a similar heterogeneity in the amount of serotonin released from individual granules. The quantitative evaluations of the diameter and area distribution are shown in Figure 1, parts D and E, respectively. Since the measured quantity of serotonin released from individual granules is directly correlated with the volume of the granules by means of Faraday’s law, $Q = F n C (4/3) R^3$, where $F$, $n$, $C$, and $R$ represent Faraday’s constant, the number of electrons lost during oxidation, granular concentration, and radius of the granule, respectively, if there is an equal concentration of serotonin in each granule, a histogram of a cubic root of $Q$ from a group of platelets (Figure 1E) should match the radius distribution. This distribution is indeed very similar to the radius distribution obtained from TEM data as shown in Figure 1, parts B and D. Both histograms fit well to Gaussian distributions. For the purpose of comparison, the dimensionless ratio of the standard deviation to the mean of the best-fit Gaussian distribution is defined as a measure of the extent of dispersion. For the electrochemical data, this ratio is 0.39 indeed close to 0.23 ratio calculated from the TEM data, especially considering the variation induced by the random thin slicing in the TEM study. This observed similarity suggests that serotonin is stored at approximately the same concentration in all granules, as is common in many other secretory cell types. In fact, carbon-fiber microelectrochemistry data is the first to experimentally demonstrate that platelets, as with other secretory cell types, exhibit a similar “quantal phenomenon”. Using the average values for the size of dense-body granules from TEM data and quantity of serotonin per granule presented herein, this concentration is calculated to be approximately 0.5 M, which agrees with the quantal size, 0.6 M, estimated using literature values of granular size in our previous study.

**Simulated Secretion of Serotonin.** Digital simulations, such as the random walk simulation, are useful in simulating vesicular or granular secretion and have been applied successfully in studies of secretion processes in multiple cell types. First, the simulation provides a feasible means to examine different parameters affecting measured electrochemical signals. For example, the effects of an altered distance separating the release site from the sensing surface can be readily evaluated. Once the effects of these parameters are determined and reasonable values are chosen, the simulation can provide insight into the storage mechanism of molecules within vesicles or granules, which are otherwise not easily obtained.

Herein, a random walk simulation is employed to examine the collection efficiency of the carbon-fiber microelectrode used in this experiment. In order to accurately quantify the quantal size, the collection efficiency needs to be critically evaluated because, as demonstrated earlier, a significant portion of molecules released from a granule or vesicle may not be detected due to their distant release from the electrode, thus resulting in an underestimated quantal size. To take this effect into account, the distance-dependent detection efficiency is carefully evaluated for the experimental setup used for the present study. The collection configuration is modeled as a symmetric cylinder positioned perpendicular to an infinite plane as depicted in Figure 2A. The only electroactive sensing area is the bottom of the cylinder with a diameter of 5 $\mu$m in the center. Experimentally, tight positioning between the platelet and the electrode is achieved based on gentle sliding of the electrode on the coverslip; accordingly, the separation distance, $h$, is approximated based on geometry to be 0.5 $\mu$m. The release site, corresponding to a dense-body granule fusing with cytoplasmic membrane, is treated as a point source at a position defined axial. Using the random walk simulation, the percentage of molecules detected out of total molecules released is plotted versus the axial position of the release site as shown in Figure 2B. The shaded area corresponds to the space beneath the active sensing area. Obviously from the simulation, over 80% of the released molecules are detected if the releasing site is covered by the sensing area of the electrode. Nearly 100% detection efficiency is achieved when the releasing site falls in approximately 1.5 $\mu$m radius from the symmetric center. The size of a typical platelet is approximately 2–3 $\mu$m in diameter, assuming a discoid morphology. Even more convenient in this particular cell type is that the platelet initiates a centralization, mobilizing granules toward the cytoplasmic center during the exocytotic process. Thus, it is fairly reasonable to assume that the majority of dense-body granules fuse with membranes lining channels of the open canalicular system and covering the exposed surface early in this process following activation. As a result, the present measurements achieve nearly 100% efficiency in measuring serotonin released from dense-body granules. This high efficiency ensures the highest possible accuracy in calculating granular concentration of serotonin.

Although the random walk simulation provides an excellent way to estimate detection efficiency, because of the finite number of molecules considered, it usually gives a less smooth, though identical curve when compared to the obtained using a formula directly derived from Fick's law. With nearly 100% detection efficiency, the previously described configuration is equivalent to a more simplified system where a point source is confined to an inert plane which is in parallel with an infinite detection plane separated by a distance \( h \). In such a system, Chow et al. have derived an analytical equation to describe the flux of molecules into the detection plane after instantaneous point source occurrence.\(^{22}\) The flux, \( J \), is given by

\[
J = \sum_{i} (-1)^{i} \frac{M(h - 2ih)}{2\pi h^2Dt} \exp\left(-\frac{(h - 2ih)^2}{4Dt}\right)
\]

where \( M \) is the number of molecules being released instantaneously from a point source, \( h \) is the distance between the two infinite planes, \( D \) is the diffusion coefficient for serotonin, \( t \) is time, and \( i \) is the iteration number. This mathematical sum gives a convenient way to accurately calculate the time course after instantaneous point source occurrence. Comparison between the predicted and measured time course of secretion gives some insight into the mechanisms of molecular storage and release. In Figure 2C, eq 1 is used to model the dependence of \( T_{rise}, T_{1/2}, \) and \( T_{decay} \) on the distance \( h \) with \( h \) varying from \( h = 0.2 \mu m \) to \( h = 1 \mu m \), accounting for the possible distance variations between the release site and the detection plane. Although the detection efficiency decreases from \( h = 0.5 \mu m \) to \( h = 1 \mu m \), the equation predicts similar time courses for \( T_{rise}, T_{1/2}, \) and \( T_{decay} \) as compared to results predicted from the random walk simulation. On the basis of the model of instantaneous point source release, the predicted \( T_{rise}, T_{1/2}, \) and \( T_{decay} \) range from 0 to 1.5 ms for \( h \) between 0.2 and 1 \( \mu m \). Strikingly, the predicted time courses only match the measured time course from a very small population of spikes when compared to the experimental results in Figure 2D–F. For example, for \( h = 1 \mu m \), the predicted time courses for \( T_{rise}, T_{1/2}, \) and \( T_{decay} \) are 0.14, 0.78, and 1.29 ms, respectively. In comparison to the experimental results, 100%, 96%, and 96% of the spikes have larger values than those predicted values of \( T_{rise}, T_{1/2}, \) and \( T_{decay} \), respectively. For \( h = 0.5 \mu m \), all of the experimental values of \( T_{rise}, T_{1/2}, \) and \( T_{decay} \) are larger than those predicted. This disagreement reveals that a continuous and slow, rather than instantaneous, release process is responsible for the great majority of serotonin secretion. These results clearly imply that serotonin is not freely diffusible within the dense-body granules, and this finding is in line with conclusions from previous studies by Berneis and co-workers.\(^{24,25}\) These authors demonstrated that serotonin and ATP could form large molecular aggregates in artificial mixtures of those two constituents at molar ratios similar to those occurring in vivo.\(^{24}\) The same authors also showed that the existence of divalent cations found within the same granule could have a stabilizing effect on the aggregate structure.\(^{24,26}\)

Experimental and simulated data presented herein provides the first dynamic biophysical evidence that serotonin is stored in dense-body granules as an aggregate structure as proposed previously.\(^{27}\)

The digital simulation method not only provided interesting insights into the mechanism of serotonin storage, but combined with TEM study, it also offers interesting information about release of serotonin from dense-body granules. From the amperometry experiments, the measured spikes usually show a rapid rising phase and an exponential decay from the peak maxim, (Figure 3B), which presumably correspond to the initial rupture of dense-body granule and the late stage of fusion where granular membrane is fully incorporated into plasma membrane, respectively.\(^{28,29}\) In fact, the predicted spike based on instantaneous release is much sharper and narrower than most of the measured spikes; this observation is consistent with the supposition that serotonin is stored in a highly aggregated state. Amperometry traces also include a small population of spikes (approximately 11%, 51 out of 452 spikes analyzed from 24 platelets) with a clearly discernible foot feature prior to the full fusion spike (Figure 3C). The full fusion portion of the spike has similar characteristics as other normal spikes without the foot feature, indicating a similar slow-release process. The foot phenomenon, however, is generally believed to arise when, soon after the initial granular rupture, a semistable fusion pore is maintained with limited flux of intragranular molecules to the extracellular space.\(^{28,29}\) The first serotonin molecules likely to escape the granule during the fusion pores maintenance are any unassociated, free molecules; thus the foot current likely reveals the number of molecules that are not associated with the aggregate complex. In fact, this supposition is support by TEM images which reveals the presence of a halo region between the dense-body core and the granular membrane in Figure 3A. Serotonin molecules exist in this region in the fluid state and are more freely diffusible through the fusion pore to reach the extracellular space than those associated with the dense body.

A rarer phenomenon observed, termed "kiss–run–kiss", is shown in Figure 3D; several small, extremely sharp spikes occurred prior to the presumed full fusion spike, though all of the spikes have similar amplitudes. When this type of release events occur (in less than 1% of the recorded traces), they always occur tens of seconds after the activation of the platelet and as a cluster, temporally separated from other release events. This unusual secretion behavior could be a result of the changes in the cytoplasmic membrane after multiple fusion events with α- and/or dense-body granules. Presumably, a dense-body granule with a halo region fuses during the late stages of secretion, when the cytoplasmic membrane has incorporated a significant amount of granular membrane character, and a fusion pore can open briefly and then reseal as the granule retracts. In the example shown in Figure 3D, this particular granule repeated this "kiss and run" process six times before executing full fusion. Using a predetermined spike area, separation distance, \( h \), of approximately


had a higher frequency of spikes at physiological temperature than at room temperature.\(^{31}\) In the work of Haynes et al., temperature is demonstrated to exert different effects on readily releasable and reserve pool vesicles in chromaffin cells.\(^{32}\) Herein, the effect of temperature on platelet secretion is examined in detail from room temperature to physiological temperature to explore how temperature influences the secretion process from dense-body granules. As shown in Figure 4 and Table 1, temperature had a similar effect as observed in other secretory cells, with an elevated frequency at physiological temperature compared to room temperature.\(^{31,32}\) Statistical comparisons of the cell populations reveal that higher temperature promotes higher frequency of granular fusion which is attributable to temperature influence on granular trafficking, docking, and/or fusion machinery. However, extracellular temperature variation does not appear to have any significant effect on either the number of molecules secreted per granule or the rate at which they are secreted. This is in stark contrast

**Temperature Effect.** Temperature is known not only to alter the efficiency or the exocytotic machinery, but it could also directly influence the release of molecules from vesicles and granules. Previous work by Pihel et al. showed that both mast and chromaffin cells released more molecules per spike and


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**Figure 3.** Simulation of amperometric spikes. (A) A TEM micrograph of a platelet. Three dense-body granules are shown with an arrow pointing to the possible site for a fusion pore to form when fusion occurs. (B and C) Comparisons between simulated and experimental spikes. The solid lines represent the measured spikes while the dotted lines are the simulated spikes. The spikes are from a platelet tightly positioned under the electrode. The conditions used for simulations are spike area = 107 fC, distance \(h = 0.5 \, \mu m\) in panel B and spike area without foot area = 235 fC, distance \(h = 0.5 \, \mu m\) in panel C. \(D_{\text{serotonin}}\) is \(5.4 \times 10^{-6} \, \text{cm}^2/\text{s}\). (D) A rarely observed phenomenon, termed “kiss–run–kiss”. Multiple sharp spikes occur prior to the major spikes. A schematic shows the likely source of these features when the granular membrane, initially in close juxtaposition to the cytoplasmic membrane, fuses briefly and retracts several times before its final full fusion. The intermediate state is marked by ‡. The spikes marked by * and # are shown to the right with the simulated curve superimposed on them. The simulated spikes have identical current maximum. The conditions used for the simulations are spike area = 80 fC, distance \(h = 0.638 \, \mu m\) for the peak marked by the * symbol and spike area = 52 fC, distance \(h = 0.520 \, \mu m\) for the spike marked by the # symbol. Spikes between these two on the left panel have a similar goodness of superimposition to the spike marked by the # symbol. The downward arrow on the right panel points to a transition where a small sharp spike merges into the major spike.
to the behavior measured from chromaffin and mast cells, especially due to the structural differences between the granular interior in platelets compared to chromaffin and mast cells. Both chromaffin and mast cell have a proteinaceous matrix that associates with the stored chemical messengers, and upon granular fusion, the temperature-dependent behavior of the exposed matrix controls the rate and extent of secretion. As a consequence, more molecules are released faster when the extracellular temperature is raised. However, this type of temperature dependence is apparently not relevant for the protein-free aggregate structure inside dense-body granules of platelets. Thus, both the rate and extent of secretion for dense-body granules remains unchanged at the altered temperatures examined herein. In conclusion, compared to other well-characterized cell models such as mast and chromaffin cells, the secretion behavior of platelets share similar characteristics, such as elevated release frequency at high temperature; however, platelets do exhibit significant differences, such as no temperature effect on the number of secreted molecules or vesicles. In stark contrast to the aforementioned secretory cell types, platelets are the only cell type that store signaling molecules in the absence of a protein scaffold, accordingly, variation of extracellular pH facilitates the exploration of chemical messenger storage in or release from dense-body granules. Herein, the effect of altered extracellular pH on secretion of serotonin from the protein-free aggregate complex is evaluated. An acidic buffer, pH 6.0, was chosen to be close to the intragranular pH; however, this pH is not too low to inhibit the activity of secretagogue, ionomycin. The high pH values are chosen from the same pH range studied in the previous work. In Table 2, the Nspike values, a measure of the number of dense-body granules that fuse successfully, do not change significantly with varied extracellular pH. A similar analysis of “largest percentage within 2 s window”, as was performed when examining the temperature effect, also reveals no statistical difference for all pH conditions studied herein (data not shown). Both observations indicate neither the low nor the high extracellular pH condition compromises the exocytotic machinery. Although Nspike remains unchanged, the effects of pH on amplitude, Q, T_{rise} and T_{decay} are significant (p < 0.01). A larger Q is observed when the pH is raised to 8.5; however, the Q value does not change significantly when the extracellular pH is lowered to 6.0. Concomitantly, T_{rise} and T_{decay} values are larger at pH 8.5 than pH 6.0 or 7.4. Among the kinetic parameters, only T_{rise} remains unchanged for all pH conditions (p > 0.70), suggesting neither the kinetics of the initial granular rupture following the fusion nor the sensitivity of the electrode changes under these conditions. Cumulatively, a platelet releases 69% and 96% more serotonin molecules under alkaline conditions (4.9 pC/platelet, 1.53 × 10^6 molecules/platelet) than neutral (2.9 pC/platelet, 9.05 × 10^7 molecules/platelet) or acidic (2.5 pC/platelet, 7.80 × 10^6 molecules/platelet) conditions, respectively. The results suggest that the alkaline extracellular pH provides an additional driving force to facilitate the dissolution and dissociation of serotonin from the acidic aggregate complex, thus leading to the observations of increased Q and altered release kinetics. On the basis of the pH studies done on chromaffin cells where increased pH led to larger Q and larger T_{rise/decay} to indicate more efficient dissociation of the proteinaceous storage matrix, the present study reveals similar results for the release from a protein-free matrix, suggesting that dense-body granules have a similar capacity to maintain high chemical messenger flux at basic pH environments.

Table 1. Spike Characteristics for Release Events Collected at Different Temperatures

<table>
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<th>pH</th>
<th>N_spike</th>
<th>Q/IC</th>
<th>I_max/pA</th>
<th>Trise/ms</th>
<th>T_1/2/ms</th>
<th>T_decay/ms</th>
<th>largest percentage within 2 s window</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>21 ± 2</td>
<td>262.9 ± 36.2</td>
<td>52.68 ± 9.89</td>
<td>4.11 ± 0.32</td>
<td>6.78 ± 0.56</td>
<td>11.47 ± 0.87</td>
<td>39.62 ± 3.45</td>
</tr>
<tr>
<td>31 °C</td>
<td>20 ± 2</td>
<td>343.6 ± 26.4</td>
<td>55.53 ± 7.17</td>
<td>4.27 ± 0.55</td>
<td>6.43 ± 0.42</td>
<td>10.92 ± 0.77</td>
<td>57.98 ± 4.66</td>
</tr>
<tr>
<td>37 °C</td>
<td>17 ± 2</td>
<td>388.7 ± 62.4</td>
<td>47.25 ± 7.81</td>
<td>3.92 ± 0.32</td>
<td>7.92 ± 0.75</td>
<td>13.43 ± 1.17</td>
<td>67.30 ± 4.66</td>
</tr>
</tbody>
</table>

* The number of platelets measured at 25, 31, and 37 °C is 24, 25, and 25, respectively. All data are reported as mean ± SEM. 

Table 2. Spike Characteristics for Release Events Collected at Different pHs

<table>
<thead>
<tr>
<th>pH</th>
<th>N_spike</th>
<th>Q/IC</th>
<th>I_max/pA</th>
<th>Trise/ms</th>
<th>T_1/2/ms</th>
<th>T_decay/ms</th>
<th>largest percentage within 2 s window</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.0</td>
<td>16 ± 2</td>
<td>175.9 ± 21.8</td>
<td>27.15 ± 3.55</td>
<td>4.40 ± 0.50</td>
<td>7.18 ± 0.70</td>
<td>12.25 ± 1.21</td>
<td></td>
</tr>
<tr>
<td>pH 7.4</td>
<td>19 ± 2</td>
<td>186.9 ± 34.8</td>
<td>36.08 ± 6.45</td>
<td>4.20 ± 0.44</td>
<td>7.21 ± 0.59</td>
<td>12.08 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>pH 8.5</td>
<td>15 ± 2</td>
<td>405.7 ± 58.3</td>
<td>63.32 ± 12.19</td>
<td>4.23 ± 0.52</td>
<td>10.88 ± 1.14</td>
<td>17.76 ± 1.68</td>
<td></td>
</tr>
</tbody>
</table>

* The number of platelets measured at pH 6.0, pH 7.4, and pH 8.5 is 25, 24, and 25, respectively. All data are reported as mean ± SEM. 

pH conditions and retain a significant amount of chemical messenger following fusion in physiological conditions. However, these measurements cannot rule out the possibility that the quantal size changes prior to platelet activation and secretion based on the influence of altered extracellular pH on intracellular processes, such as protein phosphorylation, as shown in an earlier study. At acidic pH conditions, the release kinetics in chromaffin cells are slowed (increased T_1/2) because of the diminished pH gradient and the pH-induced structural change of the protein constituent, chromogranin A, in chromaffin granules. However, this pH-dependent effect is minimal (unchanged T_1/2) in platelets probably due to the protein-free nature of the granular interior. Taken together, the present work clearly indicates that serotonin molecules are highly associated within the intragranular compartment and the extracellular space has a fundamental effect on the secretion behavior of the dense-body granules from platelets.

**CONCLUSION**

In the present study, the carbon-fiber microelectrochemistry method was successfully applied to study the secretion of dense-body granules from platelets and provided a dynamic picture of the secretion of chemical messengers from platelets. With its capability to measure individual release events on the single-cell level, the carbon-fiber microelectrochemistry method offers the first experimental evidence to support the quantal phenomenon in platelets. In combination with TEM and digital simulation studies, this novel method enabled the accurate estimation of quantal size in platelets and provided unique insight into the storage and release mechanisms of chemical messengers in platelet granules. Comparison between the experimental and the digital simulation results not only supported that serotonin is not freely diffusible, rather highly associated within granular interior, but also revealed dynamic features of the dense-body secretion in platelets, such as “kiss–run–kiss” events that have not been measured in other types of secretory cells. Lastly, the microelectrochemistry method also permits the detailed study of the influences of altered temperature and extracellular pH on the chemical messenger secretion from platelets. Clearly, the present study demonstrated that high temperature promotes more efficient exocytic machinery and that altered pH gradient between intragranular compartment and extracellular space changes the rate and extent of chemical messengers secreted.

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