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Capillary electrophoretic study of individual exocytotic events in single mast cells

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Abstract

In this work we have demonstrated the application of on-column dynamic release of serotonin from individual granules within rat peritoneal mast cells (RPMCs). These granules are approximately 0.25 fl in volume and represent some of the smallest entities studied by capillary electrophoresis (CE). With the coupling of high-speed CE and laser-induced native fluorescence, a time resolution of 0.002 min and a detection limit of 0.9 amol ($S/N=3$, rms) were achieved. By using a secretagogue as the running buffer for CE, we resolved the peak profile of individual degranulation events released due to exocytosis. The average amount of serotonin released for five RPMCs analyzed was 33 amol ±16%. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Single cell; Serotonin

1. Introduction

Mast cells stimulated by secretogogues release a host of biologically active mediators by a common secretory mechanism called exocytosis. The rat peritoneal mast cells (RPMCs) have been widely used as a model to study the release of allergic mediators [1,2]. One such mediator is serotonin (5-hydroxytryptamine), a neurotransmitter that is central to intracellular communication in signal transduction systems.

The rapid and dramatic exclusion of secretory granule matrix of RPMCs due to exocytosis to the extracellular medium has been readily observed after secretagogue stimulation by both fluorescence and phase contrast microscopies [3–6]. Many groups have utilized microscale analytical instrumentation to separate and analyze secretion of single cells [6–9].

In particular, capillary electrophoresis (CE) has become a popular method due to its small sample volume requirements, extremely high separation efficiency and speed, and the ability to manipulate single cells (such as derivatization) on column with minimal dilution. Developments in microelectrode techniques have allowed for the extensive use of electrochemical detection of easily oxidizable compounds such as serotonin and histamine [9–11]. Laser-induced fluorescence (LIF) is another well-established detection method for single-cell analyses. Many neurotransmitters exhibit fluorescence when excited with suitable wavelengths [6,12,13]. As a direct detection method, laser-induced native fluorescence (LINF) avoids problems associated with derivatization reactions, such as incomplete or slow reactions, dilution of reaction products, and contamination.

In this article, we describe the on-column release of serotonin from individual granules of RPMCs using CE–LINF. This coupling has become a powerful tool for individual cell analyses. As noted previ-

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ously, here CE has a dual role as a separating and time-resolving scheme [6].

2. Materials and methods

The experimental setup used has been described elsewhere [6]. Briefly, a 20 μm I.D.×360 μm O.D. fused-silica capillary was used (Polymicro Technologies, Phoenix, AZ, USA) with a total length of 24 or 30 cm (14 or 20 cm effective, respectively). Voltages of −10 kV and −28 kV at the detection end were employed throughout. A high-voltage supply (EH Series; 0–40 kV; Glassman High Voltage, Whitehorse Station, NJ, USA) was used to drive the electrophoresis. The capillaries were conditioned with deionized water, 20 m*M* NaOH and running buffer (5 min each) before use. Data were collected at 10 Hz with a 24-bit A/D interface (ChromPerfect Direct, Justice Innovation, Palo Alto, CA, USA) and stored in an IBM/PC-compatible computer.

The 305-nm line from an argon-ion laser (Model 2045; Spectra-Physics, Mountain View, CA, USA) was isolated from other lines with a prism and focused with a 1-cm focal length quartz lens onto the detection window of the capillary. Fluorescence was collected with a 20× microscope objective (Edmund Scientific, Barrington, NJ, USA) and passed through a UG-1 (Schott Glass Technologies, Duryea, PA, USA) and a WG-320 (Melles Griot, Irvine, CA, USA) filters onto a photomultiplier tube (PMT). A resistor/capacitor (RC) circuit, consisting of a 12-kW resistor and a 1-μF capacitor was placed at the PMT output. Peak integration was performed with commercial software (Peakfit, Jandel Scientific, San Rafael, CA, USA).

Mast cell ringer buffer (MCR) was used as the cell suspension buffer. MCR was prepared by dissolving 5 m*M* glucose, 5 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 2.5 m*M* KCl, 140 m*M* NaCl and 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Fisher Scientific, Fair Lawn, NJ, USA) in deionized water (Milli-Q Synthesis and Elix 5, Millipore, Bedford, MA, USA); pH 7.4. All solutions were filtered with a 0.22-μm cutoff cellulose acetate filter (Costar, Cambridge, MA, USA) before use. Serotonin hydrochloride standard (Sigma, St. Louis, MO, USA) was dissolved in MCR to determine detection limit. Polymyxin B sulfate (Pmx) (Life Technologies, Grand Island, NY, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to strength in MCR buffer. Sodium dodecyl sulfate (SDS) (0.1%) (Fisher Scientific) was dissolved in MCR.

Isolation of RPMCs was based on the method of Parpura and Fernandez [14]. Mast cells were harvested by peritoneal lavage from male Sprague–Dawley rats (Laboratory Animal Resources, Iowa State University, IA, USA). The rats were anesthetized and then decapitated. Approximately 50 ml of the lavage saline consisting of CO_2-independent medium (Gibco, Gaithersburg, MD, USA) containing 0.175% fatty acid-free bovine serum albumin (ICN Biomedicals, Costa Mesa, CA, USA) was injected into the peritoneal cavity. The rat was inverted and massaged for ~5 min, an incision made into the cavity and the lavage saline withdrawn. The lavage was centrifuged for 10 min (700 rpm, 100 *g*, 10°C). The resulting pellet was resuspended in 1 ml of fresh lavage saline and layered over 2 ml of 0.22% metrizamide (dissolved in the lavage saline). After a 20-min (1400 rpm, 400 *g*, 10°C) centrifugation, the supernatant was discarded. The remaining pellet was resuspended and centrifuged for 10 min (700 rpm, 100 *g*, 10°C). Again, the supernatant was discarded and the cells were resuspended in the lavage saline until use. Prior to cell injection, the cells were washed twice in MCR and suspended in MCR.

The procedure for injecting individual cells is the same as described previously [6]. A small drop of cell solution (~15 μl) was placed on a microscope slide into which the inlet end of the capillary was inserted. About 5 mm of the polyimide coating of the capillary was removed so that an injected cell could be easily seen. A septum over the outlet buffer vial provided an air-tight seal. Applying suction with a syringe, one can inject and confirm a single-cell injection into the capillary. Cell adhesion to the capillary wall was tested by applying pressure with the syringe. The cell solution was then carefully wiped away and a larger drop (~1 ml) of Pmx (10 units/ml in MCR) was added. Electrophoresis was run directly on the microscope slide at −20 to −28 kV for a few seconds to migrate the Pmx over the cell. The voltage was then lowered to −10 to −12 kV and electrophoresis was continued for ~3 min more. Electrophoresis was stopped, the Pmx solution

was wiped away, and a large drop (~1 ml) of SDS was introduced into the capillary to lyse the cell by applying suction with a syringe. Electrophoresis was continued at low voltage in SDS.

3. Results and discussion

In our earlier study of exocytosis of single RPMC in capillary tubes [6], a broad peak with some small features was observed when Pmx was migrated over the immobilized cell. The time resolution there was too slow to sort out individual exocytotic events except for occasional bursts of serotonin away from the main event. The purpose of this study is to improve the temporal resolution so that the primary exocytotic events can be resolved and quantified. This way, there will not be any bias in interpreting the peaks as being derived from single granules.

Fast CE has been demonstrated before, with peak widths in the low ms to sub-ms regime. This is simply done by using very high applied potentials and short migration distances to limit axial diffusion of the analyte zones. In our on-column stimulation experiments, very short migration distances are not easily achieved due to the need to inject the cell under a microscope. With a moderate capillary length, the applied potential will have to be very high to produce the large field gradients needed for fast separations. We fixed the maximum applied voltage at 28 kV based on safety and arcing considerations.

The small quantities of material in each granule and the relatively low fluorescence efficiency provide a challenge for detection. The S/N can be improved by using longer integration times. This however is at odds with our need to provide high temporal evolution for following the cellular events. We were able to resolve this conflict by introducing the secretagogue at a high linear velocity (applied potential) but switching to a lower velocity (voltage) during detection. The effect of going from −28 kV to −10 kV is to increase the temporal resolution 3-fold. The S/N was increased by more than the standard $\sqrt{3}$ factor because at the slower speed one can use an integrating A/D converter rather than a sample-and-hold A/D converter.

Pmx was migrated over the cell at a larger negative voltage (−28 kV) than the running voltage (−10 kV). Since electropherogram data were collected at the lower negative voltage, a conversion was applied so that the observed electropherograms and related calculations show events occurring at the larger negative voltage, i.e. in real time. Typical observed and converted electropherograms are depicted in Fig. 1a and c, respectively. Serotonin is stored in granules that are 2.5×10^{-16} l in volume. Therefore, each peak in Fig. 1a and c corresponds to amol amounts of serotonin inside single granules released from the cell. Binding proteins are also released with each granule. However, these are not observed in Fig. 1a or c because they are in much lower amounts and migrate much slower than serotonin. Also in Fig. 1, we see that the abundance of peaks occurs during the initial stages of stimulation (Fig. 1a) and the small peaks disappear after the cell lysis event (Fig. 1b, 11.2 min). This confirms that we are observing exocytosis events in Fig. 1a. The broad peak at 13.2 min in Fig. 1b corresponds to proteins dissolved during cell lysis, identical to observations in our earlier studies [6]. Presumably, proteins are also released during exocytosis, but the amounts are much smaller for the individual events (ratio of the peaks at 13.2 min and at 11.2 min in Fig. 1b) and are not detected here.

A plot of FWHM (full width at half maximum, min) vs. point in time for each of the five cells analyzed does not increase significantly as a function of time (data not shown). This tells us that axial diffusion is not the primary source of band broadening in our experiments. In addition, comparing diffusion calculations to FWHM (converted to length) we see that the observed FWHM is greater than diffusion. This demonstrates that the detection system time constant is the limiting factor. However, as shown below, this technique is appropriate for obtaining sufficient temporal resolution of exocytotic events.

The positive skew observed in the histogram of granular concentrations (Fig. 2) is typical of biological parameters that are related to age. The distribution also agrees with the results of histochemical studies by Mellbolm and Eberback [15]. An average of 14.9±3.6 fmol (RSD) of total serotonin per cell was calculated by combining all the release and lysis peak areas. Previous studies of

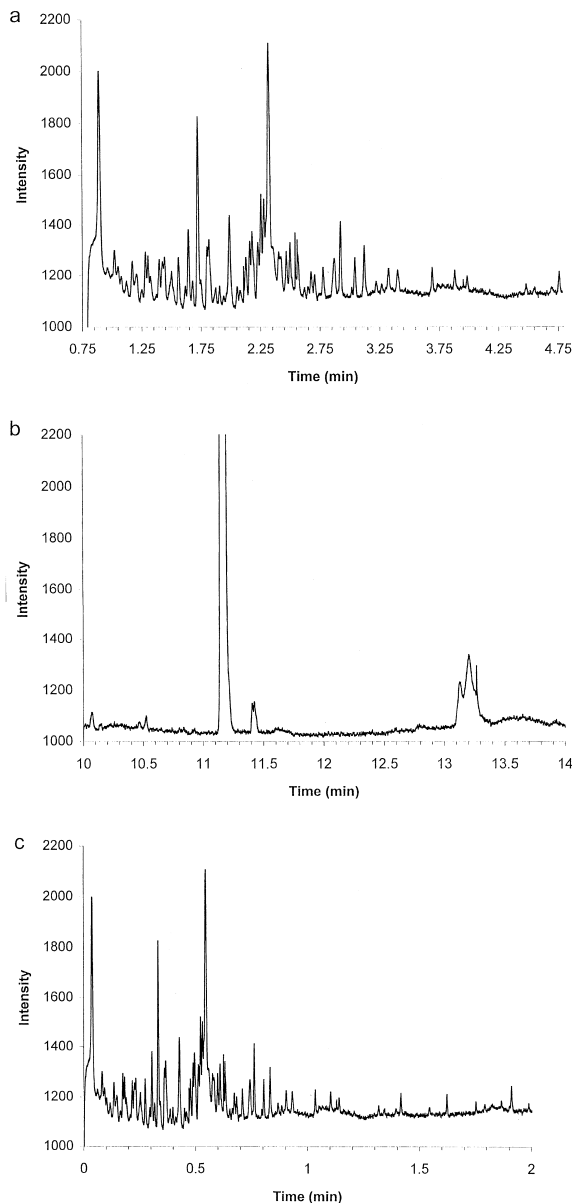

Fig. 1. Electropherogram for a typical RPMC. (a) Release region, (b) lysis region of the same run, and (c) converted electropherogram from (a). Conditions were −28 kV for Pmx (50 units/ml) injection for 40 s, −10 kV for running voltage. Capillary length was 30 cm (20 cm effective). The release of serotonin from individual granules gives rise to distinct peaks in the electropherograms (a) and (c). These peaks are not observed when the cell is lysed, as shown in electropherogram (b). There, the peak at 11.2 min corresponds to serotonin left inside the cell after release while the peak at 13.2 min corresponds to proteins in the cell.

rat mast cells have reported an average of 2–24 fmol of serotonin per cell [16].

However, even though there is no obvious clustering in the histogram in Fig. 2, these peaks may not correspond to single exocytotic events. To provide further insight, a unimodal distribution of the combined FWHM of 355 peaks obtained from five cells is shown in Fig. 3. This indicates that the FWHMs are quite homogenous and that peaks with FWHM <0.01 min are mostly single-granule peaks. The most probable amount of serotonin in a granule, according to Fig. 2, is around 4 amol. This falls within the range reported by Lillard and Yeung [6]. Summing up the peak areas of five RPMCs analyzed, an average of 33 amol±16% of serotonin released per cell was calculated.

Taking into consideration that all degranulation events were obtained approximately within 1 min from the start of secretagogue stimulation (Fig. 1c) and our time resolution of 0.002 min, it is possible to observe up to 500 peaks per cell. This represents approximately 50% of the total granules in a single mast cell. We observed an average of only 71.2 peaks per cell such that the average percent of serotonin released was 33±16%. These observations indicate incomplete degranulation of cells after stimulation. The extent of degranulation is of course due to duration, type and concentration of stimulation, as well as the state of health of the cells.

4. Concluding remarks

We have demonstrated the application of on-column dynamic release of serotonin from RPMCs with a time resolution of 0.002 min. With the coupling of CE–LINF, sensitive detection is accomplished with high temporal resolution. By using a secretagogue as the running buffer for CE, the profile of individual release events due to exocytosis was observed for single RPMCs. An average of 33±16% of serotonin was released from five RPMCs.

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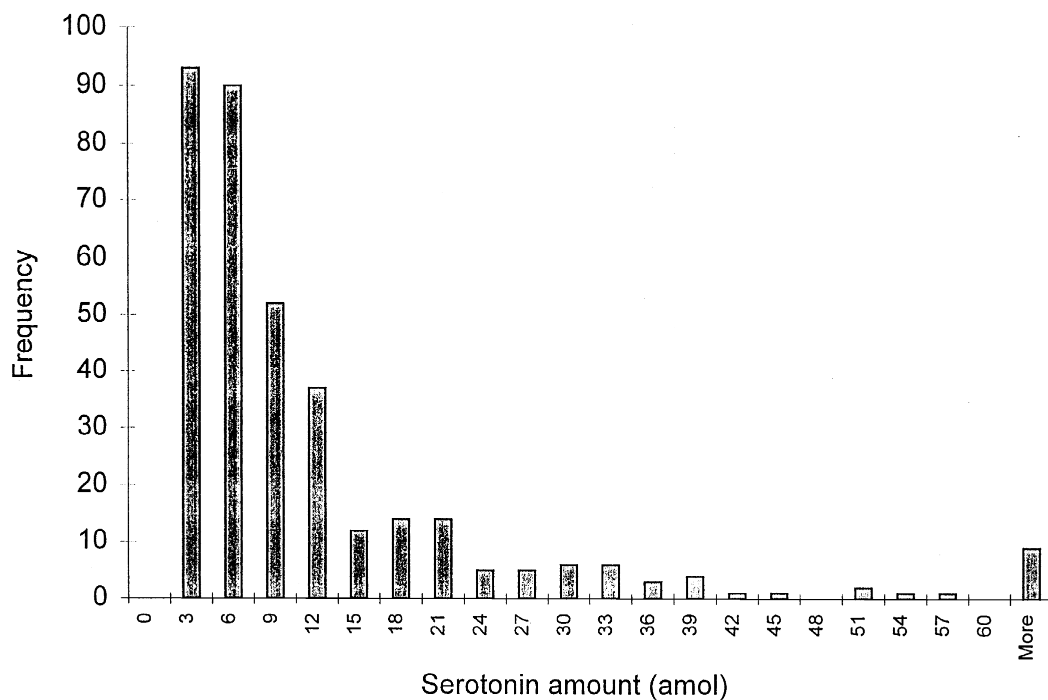

Fig. 2. Histogram of amounts of serotonin released per event from five different RPMCs.

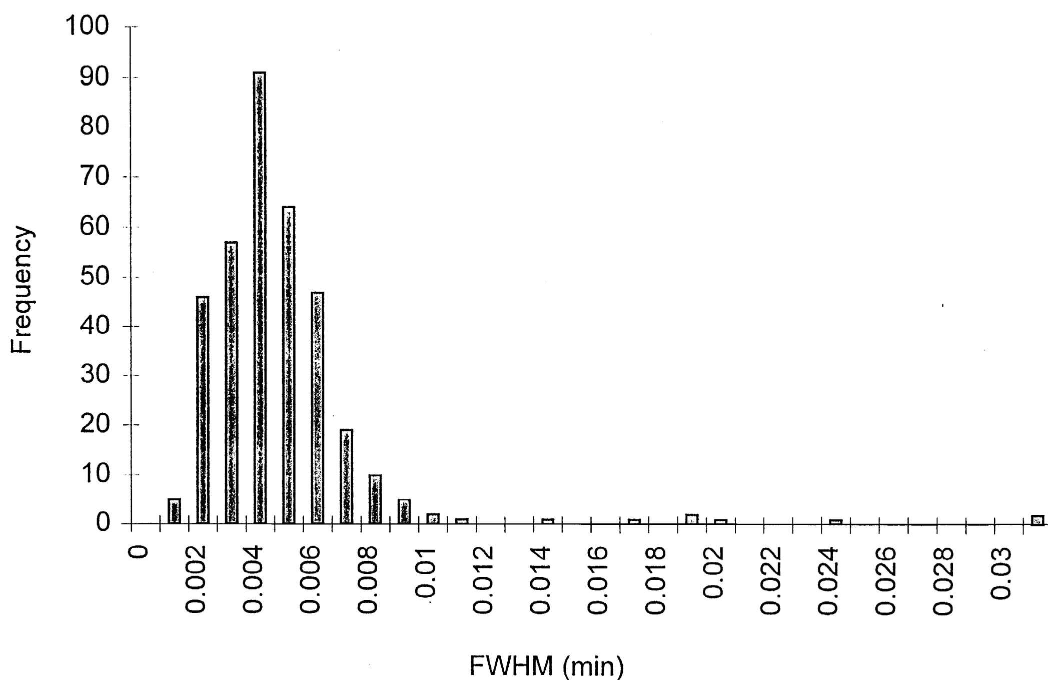

Fig. 3. Histogram of the FWHM (min) of release events recorded for five different RPMCs.

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