Biophysical Chemistry 137 (2008) 63–69

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

The effects of co-culture of fibroblasts on mast cell exocytotic release characteristics as evaluated by carbon-fiber microelectrode amperometry

Bryce J. Marquis, Christy L. Haynes *

Department of Chemistry, University of Minnesota, Minneapolis, MN, USA

ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 3 July 2008
Accepted 3 July 2008
Available online 9 July 2008

Keywords:
Mast cells
Microelectrodes
Amperometery
Fibroblasts
Degranulation
Co-culture

ABSTRACT

In this work, carbon-fiber microelectrode amperometry (CFMA) is employed to probe changes in the biophysical mechanism of exocytosis under varied cell culture conditions. Degranulation and serotonin exocytosis from mouse peritoneal mast cells (MPMCs) were measured both without and with co-cultured Swiss-albino 3t3 fibroblasts using CFMA. After 24 h in culture, there are distinct differences in the exocytotic characteristics of MPMCs cultured with and without fibroblast support cells, as detected by CFMA, including an increased number of secreted serotonin molecules, number of granule fusion events, secretion rate, and granule membrane tension. Beyond 48 h in culture, MPMCs cultured alone cannot be analyzed using CFMA due to decreased viability and membrane tension whereas MPMCs co-cultured with fibroblasts were maintained for up to 28 days in culture. Some secretion characteristics evolved over the long-term co-culture but the total amount of serotonin released per cell remained largely constant. This work quantitatively demonstrates that the MPMC/fibroblast co-culture system presents a promising model system for chronic exposure or disease model studies based on CFMA analysis.

1. Introduction

Mast cells are expressed abundantly in both mucosa and connective tissue, usually strategically poised at the interface with both the external milieu and nerves or blood vessels [1]. Thus, it is not surprising that these cells have long been recognized for their important roles in allergic and inflammatory reactions and have more recently been implicated in protection against bacteria and parasites [2], wound healing [3], pain sensing [4,5], and both innate and acquired immune system processes [6,7]. Many of the physiological actions of mast cells are tied to their piecemeal or anaphylactic degranulation functions, where they release their granular contents, including pro-inflammatory mediators (such as histamine and serotonin), lipid mediators, cytokines, chemokines, and growth factors. To further explore fundamental mast cell function or use mast cells for quantitative in vitro assays, culture conditions must produce stable or predictable degranulation behavior. Isolated MPMCs have been used extensively as a model cell for assessing acute immunotoxicity but these cells are not stable after 24 h in culture [8–10]. The in-culture lifetime of murine-harvested peritoneal mast cells can be greatly extended under co-culture conditions with 3t3 Swiss-albino fibroblasts; ensemble-averaged measurements indicate that granular contents and bulk degranulation properties of peritoneal-derived mast cells in co-culture with fibroblasts have been maintained for up to 30 days [11]. Stem cell factor, a growth factor known for regulation of mast cell development and prevention of mast-cell apoptosis, is produced by fibroblasts and is hypothesized to be primarily responsible for the prolonged culture [12]. While a limited number of in vitro immunotoxicity studies with chronic exposure have been performed using this MPMC co-culture system [13,14], result interpretation from bulk assays is difficult based on the mixture of cell types present [9]. Therefore, a direct single cell measurement technique such as carbon-fiber microelectrode amperometry (CFMA) is desirable to assess any time-evolving changes in degranulation function under the required heterogeneous culture conditions.

CFMA has been used to directly characterize exocytotic function from a wide variety of individual cells including neural [15], chromaffin [16], and mast cells [17]. This technique is able to provide sub-millisecond resolution information regarding the secretion of electroactive chemical messengers from individual granules of single cells. CFMA data analysis reveals in-depth information regarding the contents of the granules, the kinetics of intragranular matrix expansion, the stability of the granule-plasma membrane fusion pore, as well as the number of successful fusion events and the rate at which they occur. Previously, this technique has been recognized as a potent tool for evaluating the biophysical mechanisms of neurotoxicity [18] but CFMA on mast cells also presents an opportunity to study immunotoxicity. To date, CFMA experiments on MPMCs have used only isolated culture of MPMCs, limiting experiments to short-term studies due to compromised viability of MPMCs cultured alone.

The work presented herein characterizes the CFMA-determined exocytotic characteristics of degranulation from the long-term culture

* Corresponding author.
E-mail address: chaynes@umn.edu (C.L. Haynes).

0301-4622/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2008.07.002

of murine peritoneal mast cells with Swiss-albino 3t3 fibroblasts to facilitate future immunotoxicity and mast cell function studies. Specifically, CFMA is used to monitor the oxidation of serotonin as it is released via calcium ionophore-stimulated mast cell degranulation. When co-cultured with fibroblasts, significantly more granules undergo exocytosis and the number of serotonin molecules released from each granule is also increased. These characteristics are maintained for 21 days in culture but decline significantly by 28 days in culture. After 35 days in culture, membrane integrity is weakened to an extent that attempted CFMA measurements result in membrane rupture, preventing accurate measurements.

2. Experimental

2.1. Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) and used without additional purification.

2.2. Cell culture

3t3 Swiss-albino fibroblasts were obtained from American Type Tissue Collection (Manassas, MA) and grown in Dulbecco's Modified Eagle Media (HyClone, Logan, UT) formulated with 4.5 mM L-glutamine, 4.5 mg/mL L-glucose, 110 μg/mL sodium pyruvate and supplemented with 10% bovine calf serum, 10 U/mL penicillin and 10 μg/mL streptomycin (Gibco, Carlsbad, CA). Fibroblasts were plated on 35 mm Petri dishes and grown to confluence in an incubator held at 37 °C and 5% CO_2, changing media every two days.

Mouse mast cells were obtained by peritoneal lavage. Briefly, 5 ml of cold growth media was injected into the peritoneal cavity of C57BL/6J mice (Jackson labs, Bar Harbor, ME) immediately following euthanasia by CO_2 asphyxiation according to protocol #0509A75006 approved by the University of Minnesota Institutional Animal Care and Use Committee. The peritoneal cavity was massaged for two minutes before removal of the lavage fluid. Cells were isolated from the lavage fluid by centrifugation at 300*xg* for five minutes, resuspended in growth media and plated at 1.5×10^5 cells/mL on top of confluent fibroblasts. One-half volume media replacements were conducted every 24 h over the course of the long term co-culture. For experiments involving mast cells cultured alone, mast cells were harvested as described above and plated directly onto bare Petri dishes.

Tris Buffer solution for amperometry experiments was prepared with a final osmolarity of 335.2 mOsM, including 300 mM sodium chloride, 12.5 mM trishydroxymethylaminomethane, 8.4 mM potassium chloride, 5.6 mM α-D-glucose, 4.5 mM calcium chloride, and 4.2 mM magnesium chloride. Culture medium was replaced with warm tris buffer immediately before amperometry experiments.

2.3. Electrochemistry experiments

Beveled carbon fiber microelectrodes and micropipettes were constructed following a previously published method yielding carbon fiber microelectrodes with an estimated active surface area of ~54 μm^2 [19]. An optical micrograph of a microelectrode, cell, and stimulating micropipette is shown in Fig. 1A. In a typical electrochemistry experiment, growth media was removed from mast cell plates, the cells were washed twice, and the media was replaced with tris buffer solution (pH 7.4, 37 °C). During the course of the experiment, the cell plate was held at 37 °C using a temperature-control stage (Warner Inst., Hamden, CT). For identification of serotonin during exocytosis, cyclic voltammetry was performed by lowering the microelectrode into contact with a mast cell with a stimulating micropipette placed 20–100 μm from the cell. The electrode potential was cycled from 0.2 V to 1.2 V at a scan rate of 300 V/s for thirty seconds before stimulating the cell with a 3 s bolus of 10 μM A23187. Data acquisition began 5 s before the stimulation pulse, continued for 90 s, and the recorded background-subtracted cyclic voltammogram revealed the signature oxidation and reduction

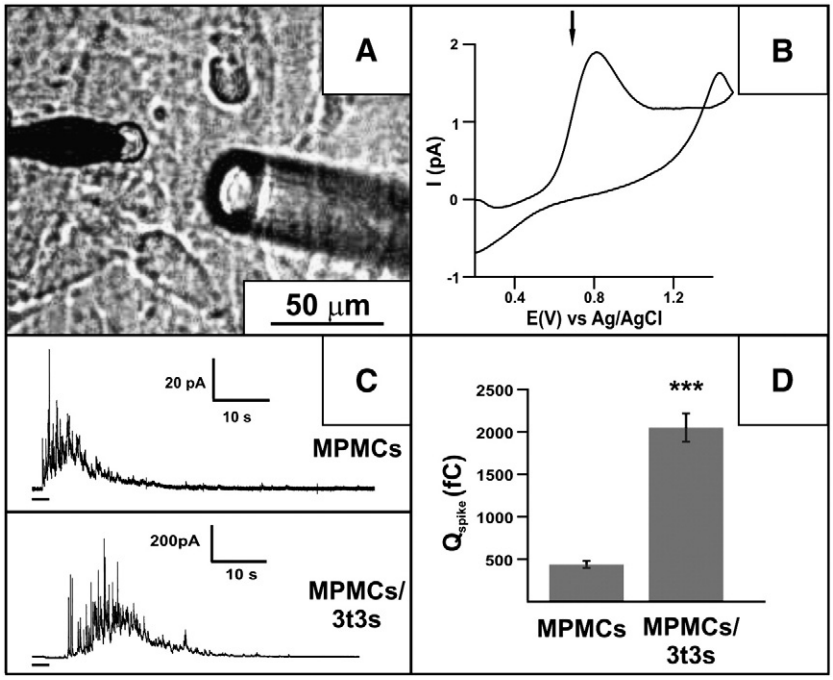

Fig. 1. (A) Image displaying carbon-fiber microelectrode placed on mast cell (left) and stimulating pipette (right). (B) Cyclic voltammogram recorded during mast cell exocytosis, showing characteristic oxidation spike of serotonin, arrow indicates potential applied during amperometry experiments. (C) Representative traces measured after 24 h culture of MPMCs alone (top) and MPMC 3t3 co-culture (bottom). Note ten-fold difference in *y*-axis scale. (D) Bar chart showing mean spike areas from 24 h culture of MPMCs alone and MPMC/3t3 co-culture. Error bars represent ±SEM; ***$p<0.001$.

features for serotonin (Fig. 1B), indistinguishable from those seen when injecting standard solutions.

For CFMA measurements, the microelectrode was held at a constant potential sufficient to oxidize serotonin (+700 mV vs. Ag/AgCl) and lowered into contact with a mast cell. Only serotonin can be measured at the applied potential using a bare carbon-fiber microelectrode; detection of other redox-active species such as histamine, ADP, ATP, or adenine either require significantly larger potential excursions or alternate electrode materials. The micropipette is placed 20–100 μm from the cell and a 3-second-bolus of 10 μM A23187 solution was delivered to the target mast cell, projected by a 1–3 psi nitrogen pulse. The A23187 secretagogue was chosen to enable comparison to the extensive literature-precedent of mast cell CFMA experiments. Data acquisition began 3 s before the stimulation pulse and continued for 90 s. Electrodes were pre-treated as previously described [20] between every trace and used a maximum of six times to minimize effects of serotonin fouling of the electrode surface. Experiments were conducted on individual cell culture plates for a maximum of 2 h — yielding between 5–15 individual cell traces per cell plate (Fig. 1C).

2.4. Data analysis

Detected current was filtered using a low-pass Bessel filter (5 kHz) and amplified with a gain of 10–20 mV/pA through an Axon Instruments Axopatch™ 200B (Molecular Devices, Inc., Sunnyvale, CA). Experimental parameter control and data acquisition are accomplished using a home-built breakout box and custom software modules created using the LabVIEW™ (National Instruments, Austin, TX) programming language. Data was digitally filtered at 200 Hz and exported for analysis in MiniAnalysis Software (Synaptosoft Inc., Fort Lee, NJ). The spike detection threshold was set to five times the root mean square current noise measured from each amperometric trace and all spikes with an area less than 70 fC were excluded. Mean values for the spike characteristics in each trace were combined among cells treated with the same experimental conditions and averaged to provide mean experimental values. Spike frequency and spike number were exceptions to this procedure because each trace yielded a single value which was then averaged over the series of cells. In all cases, $n \geq 15$ cells and the total number of amperometric spikes ranged from 918 to 4949. To minimize bias towards larger values when removing outliers, logarithms of standard deviations and experimental means were taken, and logged experimental values more than two logged standard deviations from the log mean were considered statistical outliers. The significant differences between release from different culture conditions (Fig. 1D) were determined using unpaired two-tailed student's Student's *t*-tests assuming equal sample variances (α=0.05) using Microsoft Excel's data analysis toolkit (Microsoft Inc., Seattle, WA).

2.5. Interpretation of amperometric data

Analysis of amperometric spike characteristics reveals rich details about the secretory granule's intracellular movement toward the cell membrane, fusion with the cell membrane, and ion-exchange between the extracellular buffer and the intragranular matrix. The area under a spike (Q) can be directly related to the number of molecules secreted by using Faraday's Law: $Q=znF$ where Q is the charge, z is the number of electrons lost in the oxidation, F is Faraday's constant, and n is the number of moles of electroactive molecule secreted. Spike integrals are calculated from the offset baseline when necessary. The cumulative Q for the entire cell is used to estimate the total amount of serotonin released per cell. To determine cumulative Q, calculations are done assuming that the electrode covers 10% of the cell surface area (assuming a spherical cell with an average radius of 6.5 μm) and that degranulation characteristics are uniform across the surface area of the cell. The spike half-width ($t_{1/2}$) value is a measure of the rate of expulsion of chemical messengers via ion-exchange following cell-granule fusion [21]. During spike analysis, both the baseline and the peak current are identified, and the half-maximum current is calculated based on these values; then the width of the spike is measured at this half-maximum current. Spike number reveals the number of granules that successfully fuse with the cell membrane to initiate exocytosis and spike frequency is measured by counting the number of spikes that occur divided by the time lapsed between the first and last spike. Cube root of charge histograms reveal a Gaussian distribution corresponding to the normal distribution of granule size exocytosed from a single cell [22]. Histograms from 24 h and 4 week co-culture conditions were made using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA) and fit to the sum of two Gaussian distributions. Goodness of fit was compared to single Gaussian distributions, and in all cases, the distributions were best fit by the summation of two Gaussians (in all cases $p \leq 0.0016$). A minority of amperometric events (up to 10%) are preceded by a pre-spike feature, also known as a foot [23]. The pre-spike foot (PSF) reveals an arrested state of granule-cell membrane fusion where vesicle contents leak through a small (viz., 5 nm) fusion pore.

3. Results

3.1. Effect of culture time on isolated MPMC secretion characteristics

Consistent with previously published results, trypan blue assays (data not shown) revealed that MPMCs experience decreased viability under isolated culture conditions, resulting in total death of population in 72 h [11]. CFMA analysis of serotonin exocytosis was conducted for isolated MPMCs after 3 h (n=15) and 24 h (n=18) in culture. After 48 h in culture, valid CFMA measurements were not possible due to compromised MPMC membrane integrity as was revealed by mechanically stimulated exocytosis upon placement of the electrode onto the cell surface. Due to the different stimulation method that initiates exocytosis from MPMCs cultured for 48 h without fibroblasts, spike parameters from this time point could not be included in comparative analysis [24]. There were few statistically significant differences between spike parameters obtained from 3 h and 24 h cultured isolated MPMCs, with the exception being number of spikes ($p<6\times10^{-6}$) (Table 1). The longer culture conditions resulted in a 60% decrease in the number of detected serotonin spikes with a 49% decrease in spike frequency ($p<0.002$), signaling a time-dependent degradation in granule trafficking, docking, priming, or fusion. This resulted in a 65% decrease in the total serotonin released per cell ($p<7\times10^{-5}$) after 24 h in culture.

3.2. Effect of fibroblast co-culture on MPMC secretion characteristics

Comparative CFMA analysis of isolated MPMCs and MPMCs co-cultured on Swiss-albino fibroblasts for 24 h yielded significant changes in the exocytosis characteristics following stimulated degranulation (Table 1). The mean Q_{spike} increased nearly five-fold from 434±43 fC to

Table 1
Mean±SEM parameters acquired from CFMA studies on MPMCs alone or co-cultured with Swiss albino 3t3 fibroblasts, $^{**}p<0.01$ from t-test vs. 24 h MPMCs alone; $^{***}p<0.001$ *t*-test vs. 24 h MPMCs alone

Parameter	MPMC	MPMC	MPMC/3t3
	3 h	24 h	24 h
	(n=15)	(n=18)	(n=20)
Q_{spike}	579±59 fC	434±43 fC	2051±169 fC***
Number of spikes	125±12***	51±3.4	143±9.7***
Frequency	5.39±.74 Hz***	2.74±.35 Hz	3.09±.20 Hz
Total serotonin per cell	3.57±.058 fmol***	1.24±.014 fmol	10.65±.20 fmol***
$t_{1/2}$	21.3±1.7 ms	26.6±3.1 ms	38.5±1.2 ms***
% of spikes with feet	2.89±0.06%	2.67±0.32%	3.19±0.24%**
Q_{psf}:Q_{spike} ratio	0.229±0.018	0.262±0.032	0.170±0.011**

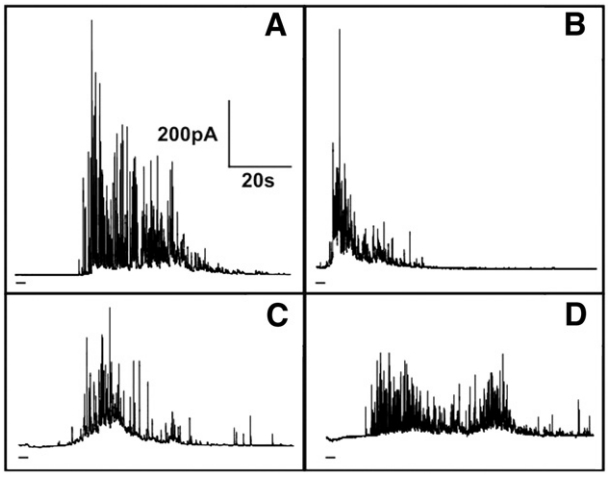

Fig. 2. Representative traces recorded from MPMCs co-cultured with fibroblasts over the course of 4 weeks. Three second stimulation pulse is represented by bold line below each trace. The panels show traces obtained after (A) 24 h, (B) 72 h, (C) 2 weeks, and (D) 4 weeks in culture. The scale bars shown in panel A apply to all panels.

2051 ± 169 fC ($p<9\times10^{-11}$), Fig. 1D. This indicates an increase in the number of serotonin molecules released per granule from 1.35×10^6 to 6.40×10^6 per granule. Additionally, a nearly three-fold increase in the number of spikes per cell is observed ($p<4\times10^{-9}$), indicating an increase in the number of successful granule fusion events. Overall, the cumulative number of serotonin molecules released from individual mast cells increased 1400% from 6.65×10^8 molecules from isolated cells to 9.97×10^9 molecules from co-cultured cells. Studies by Siraganian and coworkers of immortalized mast cell lines cultured on fibronectin-coated dishes also show increased secretion [25], suggesting that this enhancement arises from mast cell surface receptor interactions with fibronectin in the extracellular matrix of fibroblasts. To investigate this possibility, CFMA was carried out on MPMCs cultured for 24 h on coverslips coated with fibronectin instead of fibroblasts. This culture condition yielded increased release of serotonin molecules to 6.25×10^9 molecules per cell (data not shown); while this is statistically higher than MPMCs alone ($p<6\times10^{-7}$), it is still statistically lower than secretion measured from cells co-cultured with fibroblasts ($p<0.004$).

Further analysis comparing 24 h cultured isolated MPMCs and co-cultured MPMCs revealed significant changes in spike $t_{1/2}$ as well as PSF characteristics. Comparison of $t_{1/2}$ between isolated MPMCs and fibroblast co-cultured MPMCs reveal a 45% longer $t_{1/2}$ under co-culture conditions ($p<7\times10^{-4}$). Additionally, the Q_{PSF}/Q_{spike} ratio decreased by 35% when MPMCs were co-cultured with fibroblasts ($p<2\times10^{-3}$) and the percentage of spikes proceeded by a PSF increased from 2.6% to 3.2% ($p<4\times10^{-4}$).

3.3. Long term co-culture of MPMCs with fibroblasts

CFMA analysis was also conducted for MPMCs co-cultured with fibroblasts at 1–4 days, 1 week, 2 weeks, 3 weeks and 4 weeks (Fig. 2). CFMA was attempted after a fifth week in culture but placement of the electrode on the membranes of MPMCs resulted in mechanically stimulated exocytosis, invalidating these measurements. Spike parameters were compared over time to detect cell behavior variability during long-term culture (Fig. 3). Average Q_{spike} and $t_{1/2}$ were

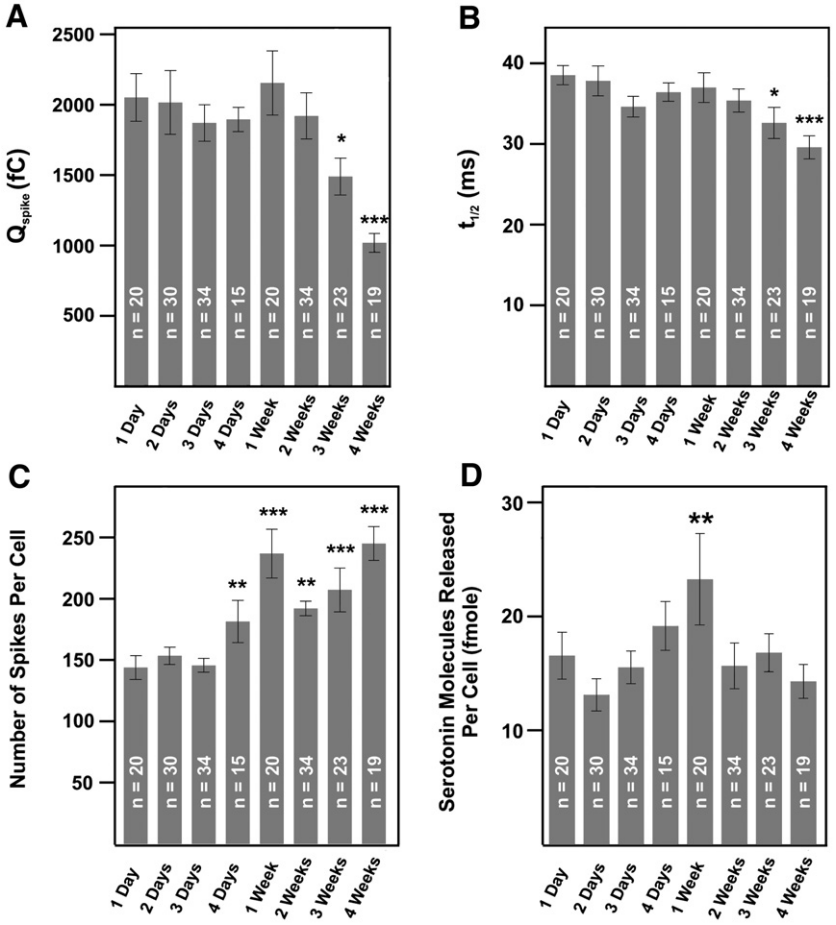

Fig. 3. Effects of long term co-culture with 3t3 fibroblasts on amperometric spike parameters obtained from MPMCs where n is the number of traces analyzed to yield data. Bar charts show mean spike parameters ± SEM *$p<0.05$ from t-test; **$p<0.01$ from t-test; ***$p<0.001$ from t-test.

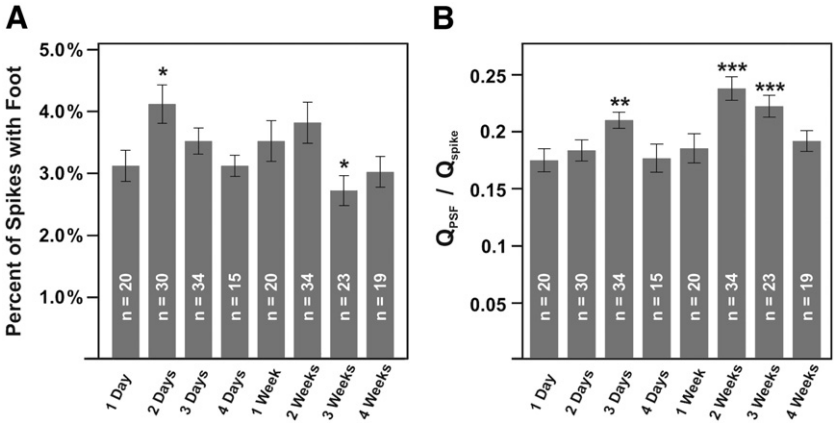

Fig. 4. Effects of long term co-culture with 3t3 fibroblasts on amperometric pre-spike foot parameters obtained from MPMCs where n is the number of traces analyzed to yield data. Bar charts show mean spike parameters ± SEM, *$p<0.05$ from *t*-test; **$p<0.01$ from *t*-test; ***$p<0.001$ from *t*-test.

maintained for up to two weeks with no significant deviation from 24 h culture behavior. During the third and fourth week in culture, both Q_{spike} and $t_{1/2}$ decreased, finally resulting in a 48% decrease in Q_{spike} ($p<2\times10^{-5}$) and a 20% decrease in $t_{1/2}$ ($p<3\times10^{-4}$) after 4 weeks in culture. Spike number did not change for the first 72 h in culture but increased from 145 to 245 spikes per cell after four weeks in culture ($p<3\times10^{-14}$). The decrease in Q_{spike} between 24 h and 4 weeks in culture was apparently offset by the increase in number of fusion events, resulting in a statistically indistinguishable ($p>0.38$) total amount of serotonin molecules released per cell. While the ability to measure from individual granules and individual cells, as is done here, reveals an apparent change in cell's mechanistic exocytotic behavior, the variation would be completely masked in the standard ensemble-averaged assays.

PSF analysis (Fig. 4) revealed statistically significant deviations from 24 h characteristics for the percentage of spikes proceeded by a PSF at 48 h ($p<0.02$) and 3 weeks ($p<0.04$) in culture but yielded no significant differences from other culture times. Furthermore, Q_{PSF}/Q_{spike} ratios were found to vary significantly at 72 h ($p<0.02$), 1 week ($p<2\times10^{-7}$) and 2 weeks ($p<7\times10^{-4}$) from the rest of the time points. During these deviations, Q_{PSF}/Q_{spike} increased by up to 32.5%.

4. Discussion

4.1. Time evolution of exocytosis behavior in isolated MPMCs

CFMA studies of MPMCs have revealed changes in degranulation behavior due to the influence of co-culture with fibroblasts. Without co-culture, mast cell viability decreases rapidly over a period of 72 h. After 24 h, a change in exocytotic function is apparent from CFMA data and by 48 h, viability is decreased such that CFMA measurements are not possible. Decreased spike number after 24 h results in decreased overall chemical messenger secretion from the MPMCs cultured without fibroblasts. This change signals either compromised exocytotic machinery (cytoskeletal elements, docking components, or sensor proteins) or enhanced compound degranulation occurring over the 24 h in culture. The latter is unlikely, however, based on previous EM studies that have shown little evidence of such degranulation occurring under similar culture conditions [26]. The secretion behavior recovers partially upon addition of 10 mg/mL fibronectin, a major product of confluent fibroblasts, to MPMCs, consistent with the enhanced overall secretion found in previously published results using RBL-2H3 cells [25]. However, our results indicate that, while adherence to fibronectin at this concentration increases MPMC degranulation, it does not fully simulate the effects of the fibroblast support cells. Mast cells and fibroblasts are known to interact via gap-junction intercellular communication [27] allowing for the exchange of chemical species; one or many of these exchanged chemical species may account for the further increases in co-cultured degranulation. Detailed analysis of the effects of fibronectin and other major fibroblast secretory products on degranulation behavior will be the subject of future studies.

4.2. Comparison of exocytotic behavior in isolated and co-cultured MPMCs

Our experiments show a large increase in the total amount of serotonin released per granule (increased Q_{spike}) as well as a large increase in the total number of granules released (increased number of spikes) from MPMCs co-cultured with fibroblasts compared to isolated MPMCs. When combined, these data reveal enhanced degranulation, resulting in a greater total amount of serotonin released per cell. These results provide the first direct, quantitative evidence of increased degranulation and are qualitatively consistent with previously published work showing increased degranulation from an ensemble of MPMCs co-cultured with fibroblasts using indirect measurements [11,28]. The dramatic change in MPMC behavior when co-cultured with fibroblasts is likely more representative of in vivo cell function based on mast cell proximity to fibroblasts in the body.

The PSF arises from an arrested state during the vesicle fusion process, where a fusion pore has formed, allowing for the escape of chemical messengers before full fusion and matrix expansion occurs. The Q_{PSF}/Q_{spike} ratio has been previously correlated to the ratio of serotonin located in the granule halo and the heparin proteoglycan matrix core [29]. The decrease in Q_{PSF}/Q_{spike} ratio, as found in our co-culture data, suggests that there is either a decrease in serotonin stored in the halo region of the granules or an increase in serotonin located in the matrix region when MPMCs are co-cultured with fibroblasts. Previous studies with mouse mast cells have revealed increased heparin production by mast cells in the presence of fibroblasts or fibroblast-derived stem cell factor, suggesting that co-cultured MPMC granules contain a larger volume of dense core than isolated MPMCs [11,30]. Recent studies show that serotonin storage in mast cells granules is dependent on the amount of dense core matrix material synthesized [31]. Accordingly, these co-cultured mast cells are capable of storing a larger serotonin payload, and this difference is manifested as an increased average *Q*, a longer $t_{1/2}$, an increased cumulative *Q*, and a decrease in Q_{PSF}/Q_{spike} ratio, as is measured here. The increased $t_{1/2}$ is due to the slow diffusion of serotonin through the highly associated dense core matrix [32]. The percentage of spikes proceeded by a PSF has been found to correlate with changes in the cellular membrane tension, with increases in membrane tension resulting in increased occurrences of PSF as the granule membrane

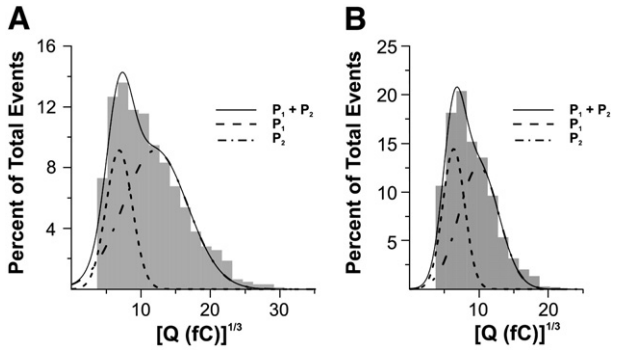

Fig. 5. Cube root of charge histograms after 24 h (A) and 4 weeks (B) from MPMCs co-cultured with 3t3 fibroblasts. Data is fit as the sum of two Gaussians revealing a $Q^{1/3}$ distribution with a smaller mean (P1) and a $Q^{1/3}$ distribution with a larger mean (P2) and the resultant sum (P1 + P2) for both the 24 h (R^2 = 0.972) and 4 week (R^2 = 0.985) co-culture experimental conditions.

resists the dense core matrix expansion [33]. Co-cultured MPMCs demonstrate an increase in the percentage of spikes with a PSF, revealing enhanced membrane stability in the presence of fibroblasts.

4.3. Time evolution of exocytosis behavior in co-cultured MPMCs

Even in co-cultured MPMCs, variations in exocytotic behavior occur over a 28 day culture period, and these alterations may be important in interpreting chronic immunotoxicity studies. There is little change in measured spike parameters over the first three days in co-culture. However, an increase in spike number occurs after four days in culture, indicating more individual granule fusion events, and after three weeks, there is a consistent decline in both $t_{1/2}$ and Q_{spike}, indicating that each fusing granule is releasing less chemical messenger content. With the exception of the one week culture data, the total amount of serotonin molecules detected does not change as the decrease in Q_{spike} is counteracted by the increase in total spikes. Cubic root of charge histograms are fit well by the sum of two Gaussian distributions;Fig. 5 shows fitted histograms for co-cultured mast cells after 24 h (R^2 = 0.972) and 4 weeks (R^2 = 0.985). The successful fit of two Gaussians to each data set suggests two distinct populations of exocytosed granules, as was previously reported for chromaffin cells [34]. TEM studies of mast cells at rest reveal a single Gaussian distribution of granule size [35]; thus, we hypothesize that the bimodal $Q^{1/3}$ distribution arises from a change in the granule population that undergoes multigranular exocytosis [36]. Comparing the two Gaussian fits from 24 h co-culture experiments to those of 4 week co-culture experiments reveals a statistically significant decrease in the mean $Q^{1/3}$ of the larger "multigranular" distribution ($p < 0.004$) over time whereas there is no significant difference in the mean of the smaller distribution ($p > 0.81$). Integrating the areas under the Gaussian fits shows a decrease in the relative amount of the larger $Q^{1/3}$ granule population from 71% to 62% when comparing the cells cultured for 24 h to those cultured for 4 weeks. The most likely explanation for these results is that, during long-term culture, the extent of multigranular exocytosis decreases. This change results in the detection of more granules exocytosed with less serotonin release per granule and the corresponding shift in the detected $Q^{1/3}$ distribution from multigranular exocytosis to single granular exocytosis. These changes would go undetected using ensemble-averaged assays for bulk degranulation.

5. Conclusions

MPMC co-culture with fibroblasts maintains MPMC viability and reveals significant changes in CFMA detected exocytosis of serotonin. The simultaneous increase in the amount of serotonin molecules released per granule and the decrease in the effective diffusion rate of those molecules from the granule suggest that co-cultured MPMCs contain significantly more serotonin and proteoglycan matrix than MPMCs alone, making co-cultured MPMCs a better model of the in vivo cells. Additionally, the amperometry assay reveals that during long-term (viz., 4 week) co-culture, MPMC secretion behavior evolves to include fewer multigranular release events and more individual granule release events. PSF parameters were also found to greatly vary over the course of long-term culture. These PSF parameters reveal a constantly changing balance between membrane tension and granule composition as MPMCs age in culture, and thus, should be taken into consideration during chronic immunotoxicity studies, especially when considering chronic exposure to agents that may influence granular or cellular membrane integrity. Overall, this work quantitatively demonstrates that the co-culture system of MPMCs on fibroblasts is a good model for chronic exposure or toxicity studies for up to 21 days, with minimal change in basic cellular function. After 21 days, fundamental MPMC exocytotic behavior changes, highlighting the importance of time-based controls in experiments using this model system.

Acknowledgments

Financial support for this work was generously provided by a 3M Non-Tenured Faculty grant to CLH and a National Science Foundation grant (#CHE-0645041).

References

[1] C. Prussin, D.D. Metcalfe, IgE, mast cells, basophils, and eosinophils, Journal of Allergy and Clinical Immunology 111 (2003) S486–S494.
[2] M.F. Gurish, P.J. Bryce, H. Tao, A.B. Kisselgof, E.M. Thornton, H.R. Miller, D.S. Friend, H.C. Oettgen, IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*, Journal of Immunology 172 (2004) 1139–1145.
[3] C. Noli, A. Miolo, The mast cell in wound healing, Veterinary Dermatology 12 (2001) 303–313.
[4] V. Anaf, C. Chapron, I. El Nakadi, V. De Moor, T. Simonart, J.C. Noel, Pain, mast cells, and nerves in peritoneal, ovarian, and deep infiltrating endometriosis, Fertility and Sterility 86 (2006) 1336–1343.
[5] D. Levy, R. Burstein, V. Kainz, M. Jakubowski, A.M. Strassman, Mast cell degranulation activates a pain pathway underlying migraine headache, Pain 130 (2007) 166–176.
[6] P.B. Hill, R.J. Martin, A review of mast cell biology, Veterinary Dermatology 9 (1998) 145–166.
[7] M.K. Church, F. Levi-Schaffer, The human mast cell, Journal of Allergy and Clinical Immunology 99 (1997) 155–160.
[8] E. Ulleras, D. Trzaska, J. Arkusz, T. Ringerike, V. Adamczewskaa, M. Olszewski, J. Wyczolkowska, A. Walczak-Drzewlecka, K. Al-Nedawi, G. Nilsson, U. Bialek-Wyrzykowska, M. Stepnik, H. Van Loveren, R.J. Vandebriel, M. Lovik, K. Rydzynski, J. Dastych, Development of the "Cell Chip": a new in vitro alternative technique for immunotoxicity testing, Toxicology 206 (2005) 245–256.
[9] D.B.G. Oliveira, K. Gillespie, K. Wolfreys, P.W. Mathieson, F. Qasim, J.W. Coleman, Compounds that induce autoimmunity in the brown-Norway rat sensitize mast cells for mediator release and interleuklin-4 expression, European Journal of Immunology 25 (1995) 2259–2264.
[10] E.E. Graevskaya, A. Yasutake, R. Aramaki, A.B. Rubin, Effect of methylmercury on histamine release from rat mast cells, Archives of Toxicology 77 (2003) 17–21.
[11] F. Levi-Schaffer, K.F. Austen, J.P. Caulfield, A. Hein, W.F. Bloes, R.L. Stevens, Fibroblasts maintain the phenotype and viability of the rate heparin-containing mast-cell in vitro, Journal of Immunology 135 (1985) 3454–3462.
[12] T.J. Lin, A.D. Befus, Differential regulation of mast cell function by IL-10 and stem cell factor, Journal of Immunology 159 (1997) 4015–4023.
[13] F. Levi-Schaffer, M. Shalit, Long-term cultures of mast-cells — a new model for studying the allergic response, Pharmacological Research 24 (1991) 307–317.
[14] B.J. Marquis, A.D. McFarland, K.L. Braun, C.L. Haynes, Dynamic measurement of altered chemical messenger secretion after cellular uptake of nanoparticles using carbon-fiber microelectrode amperometry, Analytical Chemistry 80 (2008) 3431–3437.
[15] D.S. Koh, B. Hille, Modulation by neurotransmitters of catecholamine secretion from sympathetic ganglion neurons detected by amperometry, Proceedings of the National Academy of Sciences of the United States of America 94 (1997) 1506–1511.
[16] D.J. Leszczyszyn, J.A. Jankowski, O.H. Viveros, E.J. Diliberto Jr., J.A. Near, R.M. Wightman, Secretion of catecholamines from individual adrenal medullary chromaffin cells, Journal of Neurochemistry 56 (1991) 1855–1863.
[17] K. Pihel, E.R. Travis, R. Borges, R.M. Wightman, Exocytotic release from individual granules exhibits similar properties at mast and chromaffin cells, Biophysical Journal 71 (1996) 1633–1640.
[18] R.H.S. Westerink, Exocytosis: using amperometry to study presynaptic mechanisms of neurotoxicity, Neurotoxicology 25 (2004) 461–470.

[19] K.T. Kawagoe, J.B. Zimmerman, R.M. Wightman, Principles of voltammetry and microelectrode surface-states, Journal of Neuroscience Methods 48 (1993) 225–240.
[20] F.G. Gonon, C.M. Fombarlet, M.J. Buda, J.F. Pujol, Electrochemical treatment of pyrolytic carbon-fiber electrodes, Analytical Chemistry 53 (1981) 1386–1389.
[21] C. Amatore, Y. Bouret, E.R. Travis, R.M. Wightman, Interplay between membrane dynamics, diffusion and swelling pressure governs individual vesicular exocytotic events during release of adrenaline by chromaffin cells, Biochimie 82 (2000) 481–496.
[22] J.M. Finnegan, K. Pihel, P.S. Cahill, L. Huang, S.E. Zerby, A.G. Ewing, R.T. Kennedy, R.M. Wightman, Vesicular quantal size measured by amperometry at chromaffin, mast, pheochromocytoma, and pancreatic beta-cells, Journal of Neurochemistry 66 (1996) 1914–1923.
[23] K.P. Troyer, R.M. Wightman, Temporal separation of vesicle release from vesicle fusion during exocytosis, Journal of Biological Chemistry 277 (2002) 29101–29107.
[24] S.E. Zerby, A.G. Ewing, The latency of exocytosis varies with the mechanism of stimulated release in PC12 cells, Journal of Neurochemistry 66 (1996) 651–657.
[25] M.M. Hamawy, C. Oliver, S.E. Mergenhagen, R.P. Siraganian, Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin coated surfaces enhances secretion, Journal of Immunology 149 (1992) 615–621.
[26] E. Crivellato, L. Candussio, F. Mallardi, D. Ribatti, Recombinant human alpha-2a interferon promotes an atypical process of mast cell secretion with ultrastructural features suggestive for piecemeal degranulation, Journal of Anatomy 201 (2002) 507–512.
[27] S.R. Au, K. Au, G.C. Saggers, N. Karne, H.P. Ehrlich, Rat mast cells communicate with fibroblasts via gap junction intercellular communications, Journal of Cellular Biochemistry 100 (2007) 1170–1177.
[28] F. Levi-Schaffer, E.T. Dayton, K.F. Austen, A. Hein, J.P. Caulfield, P.M. Gravallese, F.T. Liu, R.L. Stevens, Mouse bone marrow-derived mast cells cocultured with fibroblasts. Morphology and stimulation-induced release of histamine, leukotriene B4, leukotriene C4, and prostaglandin D2, Journal of Immunology (Baltimore, Md.: 1950) 139 (1987) 3431–3441.
[29] C. Amatore, S. Arbault, I. Bonifas, Y. Bouret, M. Erard, A.G. Ewing, L.A. Sombers, Correlation between vesicle quantal size and fusion pore release in chromaffin cell exocytosis, Biophysical Journal 88 (2005) 4411–4420.
[30] M. Tsai, T. Takeishi, H. Thompson, K.E. Langley, K.M. Zsebo, D.D. Metcalfe, E.N. Geissler, S.J. Galli, Induction of mast cell proliferation, maturation, and heparin synthesis by rat c-kit ligand, stem-cell factor, Proceedings of the National Academy of Sciences of the United States of America 88 (1991) 6382–6386.
[31] M. Ringvall, E. Rönnberg, S. Wernersson, A. Duelli, F. Henningsson, M. Abrink, G. García-Faroldi, I. Fajardo, G. Pejler, Serotonin and histamine storage in mast cell secretory granules is dependent on serglycin proteoglycan. Journal of Allergy and Clinical Immunology 121 (2008) 1020–1026.
[32] P.E. Marszalek, B. Farrell, P. Verdugo, J.M. Fernandez, Kinetics of release of serotonin from isolated secretory granules .2. Ion exchange determines the diffusivity of serotonin, Biophysical Journal 73 (1997) 1169–1183.
[33] C.L. Haynes, L.N. Siff, R.M. Wightman, Temperature-dependent differences between readily releasable and reserve pool vesicles in chromaffin cells, Biochimica et Biophysica Acta-Molecular Cell Research 1773 (2007) 728–735.
[34] C. Amatore, S. Arbault, I. Bonifas, F. Lemaitre, Y. Verchier, Vesicular exocytosis under hypotonic conditions shows two distinct populations of dense core vesicles in bovine chromaffin cells, ChemPhysChem 8 (2007) 578–585.
[35] I. Hammel, D. Lagunoff, M. Bauza, E. Chi, Periodic, multimodal distribution of granule volumes in mast cells, Cell and Tissue Research 228 (1983) 51–59.
[36] G. Alvarez de Toledo, J.M. Fernandez, Compound versus multigranular exocytosis in peritoneal mast cells, Journal of General Physiology 95 (1990) 397–409.