Correlating Ca\textsuperscript{2+} Responses and Secretion in Individual RBL-2H3 Mucosal Mast Cells*

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The role of Ca\textsuperscript{2+} in stimulus-response coupling in non-excitable cells is still not well understood. The Ca\textsuperscript{2+} responses of individual cells are extremely diverse, often displaying marked oscillations, and almost nothing is known about the specific features of these Ca\textsuperscript{2+} signals that are important for the functional response of a cell. Using the RBL-2H3 mucosal mast cell as a model, we have studied the temporal relationship between changes in intracellular Ca\textsuperscript{2+} and serotonin secretion at the single-cell level using simultaneous indo-1 photometry and constant potential amperometry. Secretion in response to antigen never occurs until intracellular Ca\textsuperscript{2+} is elevated, nor is it seen during the first few oscillations in Ca\textsuperscript{2+}. Exocytotic events tend to be clustered around the peaks of oscillations, but excellent secretion is also seen in cells with sustained elevations in Ca\textsuperscript{2+}. Ca\textsuperscript{2+} release from stores in the absence of influx fails to elicit secretion. If refilling and continued release of Ca\textsuperscript{2+} from stores is prevented with thapsigargin, Ca\textsuperscript{2+} influx can still trigger secretion, suggesting that store-associated microdomains of Ca\textsuperscript{2+} are not required for exocytosis. Our findings demonstrate the importance of an amplitude-encoded Ca\textsuperscript{2+} signal and Ca\textsuperscript{2+} influx for stimulus-secretion coupling in these nonexcitable cells.

Although it is generally agreed that an increase in Ca\textsuperscript{2+} is both necessary and sufficient for the initiation of secretion in most excitable cells, the role of Ca\textsuperscript{2+} in nonexcitable cells is less clear (1). The individual Ca\textsuperscript{2+} responses of nonexcitable cells are often extremely heterogeneous, and many models have been proposed for the generation of these complex and often oscillatory patterns (2). Both amplitude-encoded and frequency-encoded Ca\textsuperscript{2+} signals have been proposed (3, 4), and the availability of both mechanisms would allow multiple signaling pathways to be activated by Ca\textsuperscript{2+} in a single cell (5). Hepatocytes, with their repetitive oscillations of constant amplitude but variable frequency are prime candidates for frequency-modulated Ca\textsuperscript{2+} signaling (6), and it has now been shown that mitochondrial NAD(P)H production in these cells is indeed regulated by the frequency of Ca\textsuperscript{2+} oscillations (7, 8). In contrast, it seems clear that the secretory response of single rat salivary acinar cells is tightly coupled to the amplitude of the Ca\textsuperscript{2+} response (9), as is ciliary beating in tracheal epithelial cells (10). Furthermore, it has recently been shown that differential activation of transcription factors in B lymphocytes is achieved via non-oscillatory Ca\textsuperscript{2+} signals of different amplitudes and durations (11).

In most cases, however, it has not been easy to determine the specific features of the Ca\textsuperscript{2+} signal that are important for a physiological response. This is because sensitive methods for detecting function at the single-cell level and with high temporal resolution are not readily available. Furthermore, it now seems clear that additional signals, such as the activation of protein kinase C (12) or one or more G-proteins (13), are often required for the initiation of secretion in nonexcitable cells. We have therefore examined the Ca\textsuperscript{2+} dependence of secretion at the single-cell level using the RBL-2H3 mucosal mast cell. The relatively slow time course of secretion (14) and the complex Ca\textsuperscript{2+} signals seen in response to antigen (5, 15, 16) make this an excellent model system for studying the temporal relationship between Ca\textsuperscript{2+} and exocytosis in nonexcitable cells.

EXPERIMENTAL PROCEDURES

RBL-2H3 mucosal mast cells (17) seeded at low density on glass coverslips were sensitized with dinitrophenyl-specific immunoglobulin E and allowed to incorporate exogenous serotonin as described previously (14). Cells were incubated with 0.5 μM indo-1/acetoxymethyl ester for 20 min at 37 °C in modified Tyrode’s solution (185 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5.6 mM glucose, 10 mM Na-HEPES, pH 7.4) containing 250 μM sulfinpyrazone and 0.1% bovine serum albumin (16). Experiments were conducted in modified Tyrode’s solution containing 250 μM sulfinpyrazone and 0.05% gelatin. Cells were maintained at 35–37 °C throughout the experiment and were stimulated by perfusing the sample chamber with 3 ml of warm solution containing 1 μg/ml antigen (bovine γ-globulin to which 10–20 dinitrophenyl groups/molecule had been coupled (18)). Fluorescence of the intracellular Ca\textsuperscript{2+} indicator, indo-1, was excited using light from a Hg lamp filtered at 365 nm. Emitted light from a single cell was split using a 455 nm dichroic mirror and measured with a pair of photomultiplier detectors after filtering at 405 and 485 nm. Data were acquired, stored, and analyzed using Oscar or FeliX software (PTI Inc., South Brunswick, NJ) on a microcomputer equipped with an A/D interface board. The indo-1 ratio \((F_{405}/F_{485}) - R_{\text{mean}}\) was calculated after correction for background fluorescence, and the intracellular Ca\textsuperscript{2+} concentration was determined assuming a \(F_{\text{GFP}}\) of 250 nM for indo-1 (19). Microelectrodes for amperometry were prepared by inserting an 8-μm diameter carbon fiber into a glass capillary tube, which was then drawn in a jet pipette puller and sealed with cyanoacrylate glue. Once the glue had cured, the carbon fiber was trimmed back as close as possible (\(\sim 5 \mu\text{m}\)) to the glass, and the pipette was back-filled with 3 mM KCl. Unless otherwise stated, the electrode potential was set to +550 mV, and the fiber tip was placed \(\sim 4 \mu\text{m}\) from the cell surface (20). Amperometric responses were filtered at 500 Hz, digitized, and stored on video tape. Amperometric signals were never detected in unstimulated cells or in cells that had not been loaded with exogenous serotonin (14), thus confirming that they are due to exocytotic release of serotonin. The total amount of serotonin detected was calculated from the integral of the current associated with all amperometric events (21) observed prior to and immediately after detergent lysis for a number of individual cells. From this we estimated that we can detect about 25% of the total cell serotonin (14), which is similar to other estimates for the efficiency of amperometric detection of exocytosis (21, 22).

RESULTS AND DISCUSSION

The response of a typical mast cell when immunoglobulin E receptors on the cell surface are aggregated by antigen is shown in Fig. 1A (16). The Ca\textsuperscript{2+} response was measured using

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the fluorescent indicator, indo-1 (19), and the release of serotonin from secretory vesicles in the same cell was monitored electrochemically (23) using constant potential amperometry (24). All amperometric events have the same asymmetric shape (Fig. 1B), which is characteristic of exocytotic release of single secretory vesicles (24). As has been shown in other cell types (21, 25), some of the amperometric spikes are preceded by a small rising phase or “foot” (data not shown), which is thought to be due to the slow release of vesicle contents through a narrow fusion pore that forms before full vesicle fusion.

Secretory events do not occur until intracellular Ca<sup>2+</sup> is elevated, and in cells where Ca<sup>2+</sup> is oscillating, amperometric events tend to be clustered around the peaks of the oscillations, and are infrequent or absent in the troughs between oscillations (Fig. 1, A and B). In contrast to pituitary gonadotrophs, where exocytosis is largely confined to the rising phase of a Ca<sup>2+</sup> oscillation (26), secretory events in the mast cell do not appear to be specifically linked to either the rising or falling phase of the oscillation or to the peak itself (Fig. 1B). There is no obvious threshold concentration of Ca<sup>2+</sup> for the initiation of secretion, and secretion is not detected during every increase in Ca<sup>2+</sup> (Figs. 1 and 4). Histogram analysis of the Ca<sup>2+</sup> concentrations at which secretion is observed in oscillating cells (Fig. 2, narrow hatch marks) shows that the majority of amperometric events have the same asymmetric shape (Fig. 1B), which is characteristic of exocytotic release of single secretory vesicles (24). As has been shown in other cell types (21, 25), some of the amperometric spikes are preceded by a small rising phase or “foot” (data not shown), which is thought to be due to the slow release of vesicle contents through a narrow fusion pore that forms before full vesicle fusion.

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Correlating Ca²⁺ and Secretion in Individual Mast Cells

Little or no secretion is detected in cells that oscillate from base-line levels of Ca²⁺, but excellent secretion is seen during sustained non-oscillatory increases in intracellular Ca²⁺. A, antigen-induced oscillations from resting Ca²⁺ levels fail to initiate secretion. The mean intracellular Ca²⁺ (± S.D.) after the addition of antigen was 162 ± 135 nM. Lysis of the cell with saponin at the end of the experiment (460 s) confirmed that the electrode could detect release of serotonin from this cell. B, secretion occurs as soon as there is a more sustained increase in intracellular Ca²⁺ in an antigen-stimulated cell. The mean intracellular Ca²⁺ during oscillations prior to secretion (210–400 s) was 211 ± 137 nM; during oscillations after the onset of secretion (400–490 s) it was 257 ± 83 nM; and during the plateau where secretion was occurring (490–600 s) it was 784 ± 115 nM. The mean intracellular Ca²⁺ during the entire secretory period (400–600 s) was 324 ± 111 nM. C, excellent secretion is observed in a cell showing a sustained, non-oscillatory increase in intracellular Ca²⁺ in response to antigen. D, thapsigargin (Tg, 100 nM) also induces a sustained increase in intracellular Ca²⁺ that is accompanied by serotonin secretion. Amperometric data were digitized at 60 Hz (A–C) or 20 Hz (D).

Fig. 4.
Correlating Ca\(^{2+}\) and Secretion in Individual Mast Cells

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**Fig. 5.** Lag time distribution for the onset of the Ca\(^{2+}\) response to antigen and the initiation of secretion. Data from a total of 26 cells are shown. The dotted line shows the one-to-one relationship; all points lie above this line, indicating that secretion never occurs until Ca\(^{2+}\) has increased. The solid line is the fit for 22 of the cells (circles) and has a slope (± S.E.) of 1.17 ± 0.15, an intercept of 38 ± 9 s, and a correlation coefficient of 0.87. The four cells with secretion lag times >350 s (triangles) were excluded from the fit; two of these cells initially had oscillations from base-line levels of Ca\(^{2+}\), whereas only a few secretory events were detected in the other two cells.

The initial Ca\(^{2+}\) oscillations have been attributed to release of Ca\(^{2+}\) from intracellular stores because they do not depend on Ca\(^{2+}\) influx (16); influx is, however, necessary to replenish the stores, sustain the Ca\(^{2+}\) response (16), and support secretion (27). Thus, a possible explanation for the lack of secretion during the first few oscillations is that secretion does not occur until stores have been depleted sufficiently to activate Ca\(^{2+}\) influx. However, using the manganese influx technique to monitor the antigen-induced activation of the Ca\(^{2+}\) influx pathway (32, 33), we were unable to dissociate the initial increase in Ca\(^{2+}\) from the activation of influx (Fig. 6; n = 5 cells). Thus the delay in secretion cannot be attributed to slow activation of Ca\(^{2+}\) influx.

It is possible that the first few Ca\(^{2+}\) oscillations are required for the transport or docking of secretory vesicles (34). In excitable cells, both the recruitment of secretory vesicles and exocytosis itself are Ca\(^{2+}\)-dependent (34). However, our finding that secretion is immediately halted when intracellular Ca\(^{2+}\) is reduced to resting levels but resumes as soon as intracellular Ca\(^{2+}\) increases again (Fig. 3) suggests that Ca\(^{2+}\)-dependent vesicle docking may not be the rate-limiting step in the initiation of secretion from mucosal mast cells. Another
possibility is that the initial Ca\(^{2+}\) response is necessary for the production of other intracellular messengers (12, 35). A detailed biophysical study of the Ca\(^{2+}\) dependence of exocytosis in peritoneal mast cells diazylated with a nonhydrolyzable analogue of guanosine triphosphate, GTP\(_\gamma\)S,\(^1\) also found little or no secretion during the initial increase in Ca\(^{2+}\) (36). It was suggested that this might be due to the slow activation of protein kinase C (36), which is also required for secretion in mast cells (12), and this may also be an explanation for our results. Consistent with this, we found that addition of the protein kinase C activator, phorbol myristate acetate (50 nM), 5–8 min before antigen reduced the delay between the initial increase in Ca\(^{2+}\) and the initiation of secretion from 34 ± 17 s (mean ± S.D., n = 9 cells) to 16 ± 8 s (n = 10 cells; p < 0.025, Student’s unpaired t test).

It is well established that Ca\(^{2+}\) influx is required for antigen-induced secretion in RBL-2H3 mucosal mast cells (27, 37), and Fig. 7A confirms that secretion does not occur until extracellular Ca\(^{2+}\) is restored to an antigen-stimulated cell. However, the role played by Ca\(^{2+}\) influx is not clear. Because the Ca\(^{2+}\) response due to release from stores in the absence of influx is relatively transient and often consists of oscillations to baseline levels of Ca\(^{2+}\) (Fig. 7A and Ref. 16), it may be that influx is simply required to sustain the Ca\(^{2+}\) response for long enough for secretion to occur. Alternatively, it is possible that microdomains of high Ca\(^{2+}\) close to the site of exocytosis, which are known to be important in excitable cells (38), might also occur in mast cells (39). Because the conductance of the calcium release-activated Ca\(^{2+}\) channel found in mast cells and lymphocytes is much smaller (40) than that of the voltage-sensitive Ca\(^{2+}\) channels found in excitable cells, it may not be possible to generate such steep gradients of Ca\(^{2+}\) close to the plasma membrane in mast cells. In contrast, the Ca\(^{2+}\) conductance of inositol triphosphate receptor channels is high (41), so Ca\(^{2+}\) microdomains in the vicinity of the stores may be more important for exocytosis in nonexcitable cells. If this is the case, then depleting stores of Ca\(^{2+}\) and preventing them from refilling using the Ca\(^{2+}\)-ATPase inhibitor thapsigargin should prevent secretion even when Ca\(^{2+}\) influx is restored. However, as Fig. 7B shows, exocytotic events still occur under these conditions, provided that extracellular Ca\(^{2+}\) is present. These results clearly demonstrate that Ca\(^{2+}\) influx across the plasma membrane can directly trigger exocytosis and that refilling and continued release of Ca\(^{2+}\) from stores and any store-associated microdomains of Ca\(^{2+}\) are not essential for exocytosis in mucosal mast cells.

To our knowledge, this is the first study to examine in detail the temporal relationship between individual secretory events and Ca\(^{2+}\) oscillations in nonexcitable cells, and it clearly demonstrates that the Ca\(^{2+}\) signal in individual mucosal mast cells is amplitude-encoded. We have also confirmed at the single-cell level that there is an absolute requirement for elevated intra-

\(^{1}\) The abbreviation used is: GTP\(_\gamma\)S, guanosine 5′-3-O-(thio)triphosphate.