Single-Cell Analysis of Ca++ Changes in Human Lung Mast Cells: Graded vs. All-or-Nothing Elevations after IgE-mediated Stimulation

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Abstract. Human lung mast cells were examined by digital video microscopy for changes in cytosolic free ionized calcium ([Ca++]) after stimulation with anti-IgE antibody or specific antigens. These studies sought to determine whether the mast cell response resembled a graded or an all-or-nothing process. Preliminary experiments indicated that labeling mast cells with fura-2 did not alter their response to IgE-mediated stimulation. Subsequent experiments established that an IgE-mediated stimulus evoked an elevation of [Ca++] from a baseline value of 85 nM to an average of 190 nM (range 60--450 nM, n = 23), with an average histamine release of 26%. There was a good correlation (Rs = 0.67) between the average net [Ca++] change and the subsequent histamine release (regression equation: %HR = 0.189(net[Ca]-52)). [Ca++] elevations were found to precede histamine release (t1/2 for [Ca++], of 35 s vs. t1/2 for histamine release of 110 s). Single-cell analysis found that even for very low values of histamine release, nearly all cells demonstrated a [Ca++] response. However, this response was markedly heterogeneous, ranging from no response to responses two to three times the mean. Comparative studies of mast cells stimulated under optimal and suboptimal conditions established that there was a graded [Ca++] response dependent on the strength of the stimulus. An all-or-nothing reaction for the [Ca++] response was ruled out.

SECRETION in mast cells is brought about by cross-linking cell surface-bound IgE antibody with specific antigens. However, it is well known that challenge of these cells in vitro, even with optimal concentrations of antigen, does not always lead to a total response; i.e., 100% histamine release. A frequently posed question is whether the less-than-total release is a consequence of only a fraction of the cells releasing all of their histamine or whether there is a graded (fractional) response in all the cells. There is evidence to support both possibilities, although it has accumulated from different cell sources—i.e., human and murine basophils and/or mast cells. Lawson et al. found that exposure of rat mast cells to Con A-coated Sepharose beads resulted in the extrusion of granules restricted to the regions of mast cell–bead contact (9). One interpretation of this data is that the cell has the capacity for graded responses. Electron micrographic studies of human basophil and mast cell degranulation by Dvorak et al. (4, 5), while not addressing the question in a strictly quantitative manner, have also suggested a graded response. In these studies, most basophils or mast cells could be found to be in some state of degranulation (14). However, at concentrations of antigen that generally induce a release reaction, which requires 15-60 min to complete, the asynchronous elevations in single-cell [Ca++], in these studies occurred during the first 1-3 min. This appears to represent too small a fraction of the total reaction to properly reinterpret the studies by Dvorak et al. On the other hand, counting human basophils by alcian blue stain uptake during the release reaction led Pruzansky et al. to conclude that human basophil degranulation was an all-or-nothing response (18).

These two viewpoints suggest grossly different underlying mechanisms. In the all-or-nothing model, once a certain threshold is reached, the cell would be committed to a total response. This particular characteristic describes the action potential in the nerve cell and thus finds some biological precedent. The mechanisms underlying the transduction of the cross-linking stimulus into granule extrusion in the mast cell are still unclear but have long been thought to include elevations of [Ca++] (3, 6, 8, 13, 15). Thus, it is at the common level of ion translocation that an all-or-nothing response in the mast cell could resemble the nerve cell. To test for this possibility, [Ca++] changes in stimulated human lung mast

1. Abbreviations used in this paper: BPO, benzylpenicilloyl; [Ca++], cytosolic free ionized calcium; HSA, human serum albumin.
cells have been examined at the single-cell level using the technique of digital video microscopy.

**Materials and Methods**

**Buffers**

PAG buffer is composed of 25 mM Pipes (Sigma Chemical Co., St. Louis, MO), 140 mM NaCl, 6 mM KCl, 0.003% human serum albumin (HSA) (Miles Laboratories, Inc., Elkhart, IN), 0.1% glucose. PAGCM buffer is made of PAG buffer with 1 mM CaCl₂ and 1 mM MgCl₂.

**Reagents**

Purified penicillin-specific IgE antibody and mouse anti-DNP IgE were obtained from Molecular Probes Inc. (Junction City, OR). Bovine serum albumin (BSA) and DNP-HSA were purchased from Sigma Chemical Co. (St. Louis, MO), 140 mM NaCl, 6 mM KCl, 0.003% human serum albumin (HSA) (Miles Laboratories, Inc., Elkhart, IN), 0.1% glucose. PAGCM buffer is made of PAG buffer with 1 mM CaCl₂ and 1 mM MgCl₂.

**Cell Preparation**

Mast cells were prepared and purified from human lung parenchymal tissue as previously described by this laboratory. Briefly, tissue was minced and treated with four proteolytic enzymes—pronase/chymopapain and elastase/collagenase (19). Dispersed cells were collected, washed, and subjected to countercurrent elutriation (19). The majority of the mast cells at relatively high purity were further purified on a Percoll step gradient (13), subjected to countercurrent elutriation (19). The majority of the mast cells at relatively high purity were further purified on a Percoll step gradient (19), resulting in mast cells for 23 experiments with an average purity of 92% (range 89-100%). For some experiments the cells were sensitized with either penicillin-specific human IgE (4 μg/ml in RPMI-1640, 1 mM EDTA for 20 min at 37°C) or DNP-specific mouse monoclonal IgE (10 μg/ml in RPMI-1640 in overnight culture).

**Instrumentation**

Ca⁺⁺⁺⁺ measurements were made under a microscope (Universal; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence, phase contrast, and a 200-Hz scanning stage. Fura-2 excitation was made with a 400-nm cutoff dichroic mirror (Carl Zeiss, Inc.) and a 410-nm longpass filter in the epifluorescence assembly. Excitation filters, 352 and 380 nm, and 1-inch bandpass (10-nm) interference filters, were obtained (Parts 52597 and 5420, respectively; Oriel Corp., Stamford, CT). These two filters were mounted on an in-house-constructed filter changer that used two fast rotary solenoids to selectively bring either filter in line with an HBO 100W/2-generated light beam. The filter changer was isolated from the microscope table, but located behind the epifluorescence assembly. All filter changing operations were controlled through an interface controller (IEE488; Carl Zeiss, Inc.) which, in turn, was under computer control (to be described below). The cells were observed with a 40×-long focus immersion phase objective (5236895; Carl Zeiss, Inc.).

The images were directed through the head (MPM03; Carl Zeiss, Inc.) of the microscope to a low-light video camera (Venus T2M; Carl Zeiss, Inc.) equipped with autoHVPS, autobal, and autofit switches set to manual (no automatic light level adjustments). The RS-170 video signal was digitized by a video processor (DS-50; Quantex, Sunnyvale, CA) connected, via an interface bus (IEE488; Carl Zeiss, Inc.) to a microcomputer (Macintosh II; Apple Computer Corp., Cupertino, CA) equipped with an NB-DMA-8/IEE488 card (National Instruments, Austin, TX).

The video camera was kept in manual mode with HVPS gain set to maximum. Under these conditions, the response time of the camera was ~400 ms. Initially the 380-nm image was acquired, followed by a 352-nm image. The software was set to expose the cells and camera faceplate for 400 ms before the acquisition of an average image (running average method for six frames with an averaging parameter of 1/2 = additional 180 ms), and the 352- and 380-nm images were spaced by 600 ms. Thus, the total acquisition time for a ratio image was ~2 s. Alternating the sequence of the filters between ratio samples did produce a ±7% oscillation in the measured Ca⁺⁺⁺⁺ concentrations. However, most experiments were performed without filter alternation and, since an increase in [Ca⁺⁺⁺⁺], resulted in low intensities using excitation at 380 nm, this image was acquired first.

The spatial response of the camera, in conjunction with the light source spatial inhomogeneity, was determined by measuring the fluorescent intensity of a monolayer of fura-2 in solution across the field of view. To minimize edge discrepancies and to ease the real-time computational load on the computer, it was decided that only the central 256×256-pixel section of the 512×512×8-bit-pixel field would be acquired and saved to the hard disk. The computer could acquire this image in ~20 ms. In the center field of view the intensity varied by a maximum of 10% across one diagonal and 5% from side to side. With a ratio image the measured [Ca⁺⁺⁺⁺], concentration would vary ±5% across the acquired image. Camera linearity was ex-

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2. The strong mercury emission line at 362 nm causes the average excitation peak to be between 360 and 361 nm for the 352-nm filter, slightly on the longer wavelength side of the isobestic point for fura-2. The 380-nm filter actually had a center of 381 nm, but with the correction for the mercury arc spectrum it was recentered on 380 nm.
amined several ways. The intensity profile of a fluorescent sphere was compared to the theoretical profile. The intensity of a fluorescent monolayer was measured with different combinations of neutral density filters between the light source and the objective or the objective and the camera. Finally, the intensity of calibrated fluorescent beads (Flow Cytometry Standards Corp., Research Triangle Park, NC) were measured. In all the above situations the camera was found to have a linear response at intensities <128 (out of 256-camera gain at maximum) arbitrary units. Intensity values >150 were suspect because the camera became relatively saturated. All the fura-2 measurements were made on images where the incident light was reduced with neutral density filters to bring the average maximum fluorescent intensity of single cells to <60 arbitrary units above backgrounds of 5-15 arbitrary units (noise <2 units).

The [Ca++]i signal was calibrated by measuring the 352/380-nm intensities of a monolayer of fura-2 salt in 100 mM KCl, 10 mM Hepes, 10 mM EGTA, with and without calcium, pH 7.2. The data were applied to the general formula provided by Grynkiewicz et al. (7):

$$[\text{Ca}^++]_i = K_b (R - R_{\text{min}}) / (R_{\text{max}} - R) \beta$$

$R_{\text{min}}$ is the 352/380 ratio under calcium-free conditions; $R_{\text{max}}$ is the 352/380 ratio under calcium-saturating conditions; $\beta$ is the 380-[Ca]/380+[Ca] ratio and $K_b$ is taken to be 223 nM. For comparative purposes mast cells were first washed in the absence of calcium and then in the presence of 1 mM Ca++ and 2.5 mM ionomycin to verify that the $R_{\text{min}}$ and $R_{\text{max}}$ ratios, respectively, were approximately correct (this assumes that the intensity resulting from excitation at 380 nm is very close to its maximum for $R_{\text{min}}$ and close to its maximum for $R_{\text{max}}$). Note that in all experiments where ionomycin was used as a stimulus, all cells showed nearly complete ablation of the 380-nm excitation intensity (off-scale [Ca++]i levels), implying that the trapped fura-2 was essentially completely deesterified and responsive to [Ca++]i. $R_{\text{min}}$ was found to be 0.34, $R_{\text{max}}$ was 25, and $\beta$ was 45. Since the emission intensity using 380-nm light was near background levels, $\beta$ was determined by averaging the entire 256×256-pixel field.

Previous rat peritoneal mast cell studies (2) indicated that deesterified fura-2 could be trapped in the granules. This granule-associated fura-2 was unresponsive to cytoplasmic changes in [Ca++]i and could be secreted by the cell during exocytosis. Both situations would grossly complicate the analysis and interpretation of the image data. However, human lung mast cells do not appear to trap fura-2 in granules. The treatment of labeled human lung mast cells with ionomycin lead to a nearly complete loss of 352-nm emission intensity (implying that there was little esterified fura-2AM within the cells and that the deesterified fura-2 was responsive to [Ca++]i changes) with a stable (see below) 352-nm emission intensity. Addition of EDTA to ionomycin-treated cells caused the [Ca++]i, levels to return to sub-100 nM levels and the 380-nm emission intensity to return to its original level (this cycle could be repeated in the same cells). The 352-nm emission was always found to be stable in the experiments below, showing only a slight shift in intensity expected from the monolayer calibration using this optical arrangement. In two experiments, 5 × 10⁶ human lung mast cells at >95% purity were labeled with 1 μM fura-2AM as described above, divided into 2 pots, and stimulated with buffer or 5 μg/ml anti-IgE antibody. The supernatants and Triton X-100-lysed pellets were assayed for fura-2. The kinetic curves were subjected to several forms of analysis, with the general analysis fitting the kinetic curves to the following heuristic equation:

$$C(t) = \delta \left( T_0 C_{\text{max}} \left[1 - e^{-k_1 t}\right] \right) [1 - e^{-k_2 t}]$$

The value of $T_0$ was found to be the best estimate of the noncellular background intensity. A continuation of this iteration would allow the $T$ value to converge on the lowest intensity pixel in the image, which, because of noise, was not the best estimate of the background intensity. A similar procedure was used to estimate the threshold intensity used to produce a binary image in which cells were reasonably (>2 pixels) separated from their neighbors—a condition necessary for the application of the contour search algorithm. Generally, this decision threshold was determined by the following formula:

$$T_{\text{th}} = \sum P_i \left( P_i > T_{\text{th}} \right) / \sum P_i$$

In practice, the number of cells in the image and the signal-to-noise ratio determined the usefulness of this procedure. A different choice of “T”-iteration could usually compensate for experimental differences; cells could also be separated manually by the operator drawing a line of separation between cells. For a given kinetic analysis, the number of T-iterations was fixed and further cell separation was accomplished manually. The decision threshold was used to generate a binary image with pixels being turned on if the corresponding image pixel values were greater than the decision threshold ($T_{\text{th}}$). After further manual cell separation, this binary image was used to determine if a pixel was to be evaluated for a ratio; pixels in the original gray-level images were evaluated if their corresponding pixels were “on” in the binary image; I called these $P_{\text{in}}$. This decision was made on the basis of the 352-nm image since it was relatively stable throughout the experiment. The true intensity of each $P_{\text{in}}$, $P_{\text{in}}$(352) and $P_{\text{in}}$(380), was determined by subtracting the background, $T_0$ determined for each emission image, $T_0$(352) and $T_0$(380). In 68020 CPU machine language, each $P_{\text{in}}$(352) byte was multiplied by 32 (register shift left × 5) and divided by $P_{\text{in}}$(380) (68020 CPU integer divide). Thus, a ratio of 1 has an integer equivalent of 32. To keep display values to 1 byte, ratios >8 were set to 255. However, for whole cell calculations the full long-word (32-bit) values were retained. The contour search algorithm was a machine language-optimized table-lookup derivative of an algorithm described by Pavlidis (17).
The general process described by this equation is an exponential rise with a single constant, $k_r$, convolved with an exponential decay with a single constant, $k_d$ (all of the data for $k_d$ and $k_r$ are expressed as reciprocals - $t_{1/2} = 0.693/k_r$ or $k_d$). The whole process also allowed for the possibility of a lagtime between stimulus addition and the beginning of a transition where $\delta(T_s)$ is a step function whose value was zero for times $<T_s$ and one for times $\geq T_s$. For cells challenged with an optimal concentration of anti-IgE this lagtime was within the limited time resolution (generally any time $<0.40$ s; see below for special cases) of these experiments and was therefore considered to be zero. In addition, the maximum $[Ca^{++}]_i$ value, $C_{max}$, was also allowed to float for these calculations. Note that $C_{max}$ is not the peak $[Ca^{++}]_i$ change observed; if there were no second exponential describing the return to baseline, $C_{max}$ would be equal to the peak $[Ca^{++}]_i$ change. Instead, the observed peak $[Ca^{++}]_i$, change is the point where the first derivative of this equation is zero. Thus, a search of a four-parameter space was made for each kinetic curve, determining $C_{max}$, $T_s$, $k_r$, and $k_d$. As noted above, the peak $[Ca^{++}]_i$ (not $C_{max}$) could be calculated from the derivative of the above equation and was the parameter most frequently used in the studies below. However, the average $[Ca^{++}]_i$ change was also calculated by determining the area under the actual kinetic curves divided by the length of the experiment. For comparative purposes, between experimental conditions, the length of time was fixed to some value. The average data was also used to determine whether a particular kinetic curve should be fit with the above equation. If a cell response oscillated about the baseline, it could not reasonably be fit by the above equation and the average calculation would predetermine this situation. Average values of $<5$ (which were determined by examining the single-cell kinetic curves of "buffer-stimulated" cells) were assigned a peak $[Ca^{++}]_i$ value of 0 for the studies below. As will be noted below, the average and peak $[Ca^{++}]_i$ changes yielded essentially identical general results.

To obtain the average frequency histograms shown in Figs. 9 and 10, where the mean $[Ca^{++}]_i$ change differed among experiments, the data for each experiment were first transformed into distributions about the mean $[Ca^{++}]_i$ change for the cells challenged optimally. For each experiment, the mean net $[Ca^{++}]_i$ change for the cells challenged with 5 $\mu$g/ml anti-IgE was calculated; let this be

$$\frac{\delta C_{opt}}{C_{opt}} = \text{mean}, \quad x_{opt} = \frac{\delta C_{opt}}{C_{opt}}.$$ 

For each cell in the experiment, the ratio ($x_i$) of its net $[Ca^{++}]_i$ change to the mean for the same experiment was calculated. The mean frequency histogram, for an optimum challenge, plots the distribution of these derived values multiplied by the average net $[Ca^{++}]_i$ change for all the experiments (Figs. 9 and 10, n = 5 and 3, respectively). The results for cells challenged under suboptimal circumstances were subjected to a similar calculation, except that the net change for each cell was calculated as the ratio of its net $[Ca^{++}]_i$ change to the mean net change for the cells challenged optimally (paired to the same cell preparation):

$$x_{subopt} = \frac{\delta C_{subopt}}{C_{opt}}.$$ 

This suboptimal challenge distribution was therefore calculated relative to its matched optimal challenge distribution.

**Results**

The effect of labeling human mast cells with fura-2 was first assessed by examining the amount of histamine release resulting from the challenge of mast cells with an optimal concentration of goat anti-IgE (5 $\mu$g/ml) prelabeled for 20 min at 37°C with 0.1, 0.5, 1, 2.5, and 5 $\mu$M fura-2AM. In addition, the cells were examined for measurable fluorescence under the microscope-camera configuration discussed above. These experiments indicated only a slight (6%), but inconsistent, inhibitory effect of fura-2 on histamine release in cells prelabeled with 1-5 $\mu$M fura-2AM. However, while labeling cells with 5 $\mu$M fura-2AM produced cells two- to threefold more fluorescent than cells labeled with 1 $\mu$M fura-2AM, there was no difference in inhibition between 1 and 5 $\mu$M. This suggested that the slight inhibition was not related to the presence of fura-2. Optimizing the labeling for fluorescent intensity and minimizing the possibility of an inhibitory effect led to the routine prelabeling of mast cells with 1 $\mu$M fura-2. Using this labeling procedure, the mast cells were then examined more closely for changes in the

![Figure 1.](https://jcb.rupress.org)
dose–response curve, the kinetics of release, and desensitization due to labeling with fura-2AM. The results of these experiments are shown in Fig. 1. For these six experiments, the average labeled cell histamine release was 89% of the control (DMSO-labeled) cells. However, as can be noted, all the curves were virtually superimposable. We anticipated that if the chelation effect of fura-2 were responsible for the slight inhibition observed in labeled cells, either the kinetics of release at early time points or the amount of histamine release at suboptimal concentrations of anti-IgE would be preferentially inhibited. None of these effects occurred. An additional test for a fura-2 effect will be discussed below. In two experiments (not shown) we also found that prostaglandin D2 and leukotriene C4 release were not effected by prelabeling with 1 μM fura-2 (labeled cells released 103 and 106% of the controls, respectively).

Histamine release from mast cells is usually measured with the cells in suspension. Under the microscope, the mast cells were partially surface bound (there was no clear cellular spreading or adhesion, but the cells were bound tightly enough not to be moved by the addition of stimulus). To facilitate a comparison between the experiments discussed in these studies, kinetic studies of mast cell degranulation, the kinetics of release in the test tube and the microscope chamber was compared. A multichamber version of the microscope stage was constructed to allow kinetic studies, since sampling from a single chamber was found to resuspend the cells. The multichamber was kept at 37°C on a microscope slide warming plate. These experiments revealed no difference in the characteristics of histamine release.

Using the multichamber described above for determining the kinetics of histamine release, four experiments examined the temporal relationship between histamine release and changes in [Ca++]. Histamine release was examined in the multichamber in parallel with a single challenge under the microscope for [Ca++], measurements (the final histamine release under the microscope was also measured and found to be within ±2% of the release measured in the multichamber 20 min after stimulation). Fig. 2 shows the results of these experiments and indicates that [Ca++] concentrations change (t1/2 = 35 s) before histamine release occurs (t1/2 = 110 s). Control histamine release was <5%, and control (buffer) challenges under the microscope caused no net changes in [Ca++], (these buffer challenges were also used to determine the average [Ca++] variability in unstimulated single cells, which was used to decide if a peak [Ca++], change could be calculated for stimulated cells; see Materials and Methods). Fig. 3 demonstrates that there was a significant correlation (R = 0.67, n = 23 lung preparations) between histamine release and the net [Ca++] change obtained after stimulation of purified (mean = 92%, range 89-100%) mast cells with an optimal concentration (5 μg/ml) of anti-IgE antibody (average histamine release was 26 ± 4%). For these values, the net changes for all the cells in the field of view were averaged. The average net change in [Ca++], for these 23 experiments was 190 nM for a 5-μg/ml anti-IgE stimulus with an average baseline of 85 nM. For a single mast cell preparation, [Ca++], measurements were usually performed singly. However, in several experiments, duplicate runs indicated that the average net change varied <10%.

Rat peritoneal mast cells respond to 48/80 or antigen/IgE-mediated challenge with a rapid transient change in [Ca++], that peaks at 3–5 μM (2, 16). The half-width of this transient was found to be 1–5 s. The time intervals generally used in our studies could miss an initial transient of this duration or miss (on an individual cell basis) such transients occurring later in the release process. However, several results indicate that such transients do not occur in human lung mast
cells. First, in the experiments we have performed, ~4,000 cells have been examined kinetically and no high 
Ca²⁺ transients have been observed (no cell ratio that was >1 μM which was preceded and followed by an image which showed much lower concentrations). This was also true in two experiments where the time interval was 5 s for periods of 3-5 min before and after the stimulus. Since >40 cells were observed in each field for each time point, we would expect to observe high transients, which occur >5 s after stimulation in at least 1-5 cells per field per time point in the first 60-120 s after stimulation based on previous data (16). These high transients have never been observed. The addition of the stimulus is always made while observing cells excited with 380-nm light (to observe cell movement), and frequently the first ratio image follows the addition of stimulus by <5 s. Observation at 380 nm has never shown a more rapid decrease in fluorescence than expected from the normal ratio technique, and the series of ratio images after the first has never shown a decrease in [Ca²⁺]. (as if following a large initial transient). Fig. 4 demonstrates this early observational data. The stability of fluorescence at an excitation wavelength of 352 nm during stimulation of mast cells suggested that there was little change in cell shape or size. Therefore, the observation of fluorescence excited with only 380-nm light provided a reasonable measure of [Ca²⁺]. Under these circumstances, the 380-nm illumination could be continuous and the digital images could be acquired and saved every 0.5 s. We have used this technique in two experiments. Generally 4-8 cells were found within the field of view used in these experiments, and for each experiment three challenges were made to acquire information on 15-18 cells. Fig. 4 shows the monotonic decrease in the 380-nm fluorescence of 13 cells (six and seven cells from the two experiments, respectively) to a steady-state level equivalent to ~240 nM [Ca²⁺]. There were also no rapid transients observed in the later stages of the reaction (data not shown). Thus, rapid transients do not appear to be a feature of the human lung mast cell [Ca²⁺] response under these conditions.

Single-cell analysis was used to determine whether stimulation resulted in graded or all-or-nothing changes in [Ca²⁺]. Detailed parameter fitting (see Materials and Methods) of each cell in the field of view was performed to obtain the

![Figure 4. Fluorescent intensity of individual cells monitored continuously with an excitation wavelength of 380 nm before and after the addition of 5 μg/ml anti-IgE antibody (at the time indicated by the arrows). The traces are representative of the data obtained from two lung preparations (A and B). The fluorescent intensity of each cell has been normalized for graphical presentation. The addition of anti-IgE caused some cells to shift position slightly or temporarily move out of focus, leading to the artifactual oscillation seen at the location of the arrows.](image)
Figure 5. Pseudo-color representation of the ratio images obtained from an experiment using >99% mast cells challenged with 5 μg/ml anti-IgE antibody. A demonstrates the low [Ca++] before stimulation and B demonstrates the response (yellow/orange ~300 nM) 96 s after stimulation.

results below. Fig. 5 shows the monitor display obtained during (96 s after the addition of anti-IgE) an experimental run and demonstrates the heterogeneity in response characteristics of the normal mast cell population challenged with 5 μg/ml anti-IgE antibody. This particular image was chosen because (a) the mast cells were >99% pure; (b) lower levels of histamine release better expose underlying heterogeneity; and (c) despite a low histamine release (8%), nearly all cells were found to respond to some extent. The changes in [Ca++] associated with each of these cells are shown in Fig. 6. In general, for 13 experiments (with an average purity of 94% and average histamine release of 28%) an average of 85 ± 4% of the cells were found to respond in varying degrees. Thus, not all cells that respond appear to contribute significantly to the final histamine release. (Poisson statistics indicate that there is only a 2% probability of having >10% non-mast cells in the field of view for an average purity of 94%, suggesting also that some mast cells do not respond.) The correlation between the average net [Ca++] change and subsequent histamine release, shown in Fig. 2, shows a y-intercept of 52 nM, suggesting that a cell could have a [Ca++] response without the release of histamine. The data in Figs. 5 and 6 and the complete single-cell data suggest the same conclusion.

In the preliminary experiments we examined whether fura-2 labeling altered the cell response. While the data suggested the possibility of a small effect, the results were inconsistent and did not appear to depend on the concentration of fura-2 AM used for labeling. We also examined whether an effect of labeling could be observed in the single-cell studies since this could have been an important determinant of the variability in response between cells. It was found that the fluorescent intensity of the mast cells excited with 352-nm light (before stimulation) was essentially Gaussian with a 1-SD width of ±17% of the mean. This gave a working two-fold range of labeling intensities. The five parameters calculated by the heuristic fit procedure were then each correlated to the intensity of the cell; Fig. 7 shows the correlation values for each of 14 experiments. It can be seen that there was no significant correlation between the intensity of labeling and any parameter, on a single experiment basis, and that the average correlation coefficients were zero for each parameter. This data suggests that within the range of labeling generally used for these experiments fura-2 did not interfere with response of the mast cells. This suggests that the variability in response generally observed was due to some intrinsic parameter of each cell.

The single-cell data for several challenge conditions is expressed as a frequency histogram in Figs. 9 and 10. The procedure to average the net [Ca++] change (obtained from the curve fit procedure) data from separate experiments is described in Materials and Methods. The averaging did introduce some dispersion of the somewhat Gaussian curves but retained the essential features of the individual experiments. Since the lung mast cells were dispersed from heterogeneous tissue, it was expected that there would be some dispersion of the response between cells. However, the dispersion noted was quite large, with some cells failing to respond and others responding twice the mean response. Nevertheless, in itself, this did not determine whether there was a graded or an all-or-nothing response. This was determined by three methods, two of which are presented here and a third which will be in a future manuscript describing the desensitization of mast cells. Suboptimal stimulation matched with optimal stimulation provided the needed test. Fig. 8 shows the expected outcome of ideal experiments for each of the two possible models of the mast cell response. For an all-or-nothing model of release, challenging cells under conditions leading to suboptimal release should increase the number of cells that fall into the region associated with no
response (Fig. 8, lower right). In contrast, in a graded response, the entire distribution might be expected to shift to lower values of net [Ca++] change (Fig. 8, lower left).

In Fig. 9, the cells were challenged with an optimal concentration of anti-IgE (5 μg/ml) or a concentration (0.25 μg/ml) that resulted in 30–50% of the histamine release obtained with an optimal challenge. As can be seen (Fig. 9, top), the average net change in peak [Ca++], mirrored the average histamine release (p < 0.001 for histamine release or [Ca++], elevations being different for 5 and 0.25 μg/ml). Statistically, in a paired analysis, the ratio of the net peak [Ca++], change at 5 μg/ml over the net [Ca++], change at 0.25 μg/ml vs. the net histamine release at 5 μg/ml over net histamine release at 0.25 μg/ml were indistinguishable (p = 0.175; means different from zero). (A similar analysis of the average change in [Ca++], vs. histamine release indicated the [Ca++], changes determined in this manner also correlated with histamine release.) In other words, net elevations in [Ca++] were precisely reflected in histamine release. The single-cell data for the 5 and 0.25 μg/ml challenges are shown in Fig. 9, B and C, respectively. It is clear from these experiments that there was a graded response; >90% of the cells stimulated with 0.25 μg/ml anti-IgE antibody responded less than the mean response found for an optimal challenge.

Fig. 10 demonstrates essentially the same result obtained by a second method of suboptimal stimulation. Since the release process is dynamic, suboptimal stimulation using a lower and, consequently, slower binding concentration of antigen is distinct from sensitizing the cells with a lower density of IgE and challenging with an optimal concentration of antigen. From previous studies, it was known that the lung mast cell possessed few unoccupied receptors. Passively sensitized mast cells frequently released less histamine after challenge with the appropriate optimal concentration of anti-
gen than was obtained with an optimal concentration of anti-IgE (13). In three experiments presented here, mast cells were sensitized with either 10 μg/ml anti-DNP mouse IgE or anti-BPO human IgE in overnight culture. Subsequent histamine release with antigen was ~29% of the release found for anti-IgE, as shown in Fig. 10 A. Likewise, the net [Ca++] change for antigen challenge was 36% of the change associated with anti-IgE. As found above for suboptimal anti-IgE challenges, the ratio of net changes for the two conditions were not statistically different (p = 0.754, means different from zero). Once again, the single-cell analysis (Fig. 10, cf. B and C) clearly suggests a graded response by

Figure 7. Spearman rank correlation coefficients for 14 experiments for each of five parameters determined by the Heuristic fitting procedure described in Fig. 4. Within each experiment, the fluorescent intensity of each cell (30–80 per experiment), excited with 352-nm light, was plotted against each of the five parameters, and correlation coefficients were obtained. The average correlation coefficient for the 14 experiments is shown as the circle with the standard deviation.

Figure 8. Expected (hypothetical) character of the single-cell frequency histogram plots for the net [Ca++] change after optimal and suboptimal stimulation for the two models of mast cell activation.
Figure 9. Frequency distribution of the single-cell responses (net peak [Ca++] change) of five experiments for mast cells challenged with optimal (5 µg/ml anti-IgE) or suboptimal (0.25 µg/ml) stimulation. A shows the average responses for these five experiments; the histamine release is on the left and the [Ca++] change is on the right. B and C show the frequency plots for the 5- and 0.25-µg/ml stimulations, respectively. The total number of cells in each distribution is shown in the upper right of each panel.

Discussion

The [Ca++] response of mast cells was found to be remarkably heterogeneous. However, the observed heterogeneity was probably not an artifact of the measuring instruments or the differential labeling of mast cells with a compound that chelates Ca++. Labeling studies could not establish a relationship between the amount of fura-2 labeling and the subsequent cell response in the concentration-time range examined. No parameter of the cell response was found to correlate with the intensity of labeling. The spatial response of the camera (and light source) was found to be essentially uniform for the region of the image acquired for analysis so that ratio measurements were essentially homogeneous across the analyzed image.

These studies have verified that [Ca++] elevations preceded histamine release in the human lung mast cell and that the magnitude of the change correlated well with the subsequent amount of histamine release. At the single-cell level, a small fraction of the cells were found to be unresponsive while a similar sized fraction of the cells underwent 10-fold changes in the baseline level of [Ca++]. A certain degree of heterogeneity was expected since the mast cells studied here were derived from whole lung fragments and presumably represent mast cells obtained from many anatomical sites at different stages of maturation. Also, there may be some dispersion in the [Ca++] response because these cells were obtained from enzymatically dispersed lung tissue. For this reason, a single challenge with an optimal concentration of antigen was not sufficient to establish the graded nature of the mast cell response. Each of the different levels of response could have represented the total and maximal response of each particular mast cell. However, the graded nature of the response was clear from the suboptimal antigen challenge experiments. These experiments examined the same question from two slightly different perspectives. In one case, a lower concentration of anti-IgE induced less than optimal release and shifted the frequency distribution to smaller [Ca++] elevations in a proportional manner. In the second case, cells were sensitized so that they were less responsive to antigen because there was a lower antigen-specific IgE density than total IgE density and therefore less possible cross-links. Under these circumstances, [Ca++] kinetics were similar for suboptimal and optimal challenges. In each case, the all-or-nothing model of response would have predicted that more cells would have become nonresponders, as depicted in Fig. 8, lower right (note that for the purposes of the hypothetical model we assumed some dispersion under optimal conditions because of the anticipated heterogeneity of the cells). In contrast, the graded response depicted in Fig. 8, lower left, is reflected in the experimental
The magnitude of the \([\text{Ca}^{++}]\) response in mast cells is a simple monotonic function of the signal strength. These results, shown in Figs. 9 and 10, lead to the conclusion that the presence or absence of degranulation would occur in the range of a 150–250 nM net peak \([\text{Ca}^{++}]\) elevation. Alternatively, other parallel pathways in the signal transduction cascade may have more distinctive threshold characteristics. The activation of protein kinase C is one possibility. If this were the case, the degranulation process could still be all-or-nothing. However, the current studies suggest that this is unlikely.

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