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 during the midpoints of the release reaction, despite histamine release averaging <50%. However, to a limited extent, an all-or-nothing response could be invoked as an interpretation of the electron micrographic studies as well, provided degranulation was a relatively rapid event that occurred asynchronously. The possibility of such asynchrony has found some support in single-cell cytosolic free ionized calcium ([Ca++]!) studies of rat basophilic leukemia cells (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 prepared by methods previously described (11, 13). N-ε-Benzylpenicilloyl-L-alanine (BPO)-ε-aminocaproic acid was prepared by the technique described by Levine (10). Goat anti-human IgE was prepared as described previously (1); the antibody used for these studies represented the IgG fraction of goat serum prepared by DE-52 chromatography. BPO-HSA and DNP-HSA were prepared as described previously (13). Fura-2 AM was obtained from Calbiochem-Behring Corp. (La Jolla, CA) and fura-2 (potassium salt) was obtained from Molecular Probes Inc. (Junction City, OR).

Cell Preparation

Mast cells were prepared and purified from human lung parenchymal tissue as previously described by this laboratory (13, 19). Briefly, tissue was minced and treated with four proteolytic enzymes—pronase/chymopapain and elastase/collagenase (10). 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), 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).

Mast cells were labeled with 1 μM fura-2 AM (Calbiochem-Behring Corp.) for 20 min at 37°C in RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 0.32 mM EDTA and 2% FCS (3-500,000 mast cells/200 μl). After washing once with 200 μl PAG buffer, the cells were resuspended in 200 μl PAG buffer for loading into the microscope observation chamber. For each kinetic experiment, 15 μl of suspended cells (25,000 cells) were placed in the center of a siliconized (SigmaCote) coverslip which made up the bottom of the observation chamber. After a 3-5 min settling time, the cell drop was overlaid with 1 ml of 37°C PAGCM buffer and the chamber was placed on the microscope scanning stage. The temperature, measured by a probe placed next to the settled cells, was brought to a steady 37°C.

A modified Dvorak-Stotler chamber was used to observe the cells under the microscope. The modification entailed replacing the two-coverslip stainless steel spacer with an 18-mm-diam, 4-mm-thick Durcon spacer, which provided an ~2-ml volume for cell challenges. The upper surface of the buffer was left exposed to the air and the objective was directly immersed for observation. The upper coverslip, normally present in the standard Dvorak-Stotler chamber, was replaced with a 20-mm-diam, 80-μm-pore-size circular mesh Nitex sheet with a 5-mm-diam center hole for the objective and a 2-mm-diam off-center hole for the temperature probe (BASF: Sensortek, Clifton, NJ). The Nitex mesh provided very effective mechanical buffering for the addition of the stimulus. The chamber was covered with a 2.5-inch plastic hemisphere to prevent excessive evaporation. The entire setup was heated to 37°C with an air stream stage incubator (Nicholson Precision Instruments, Gaithersburg, MD).

Measurements of [Ca²⁺] were made before and after the addition of the stimulus. Because the image files created by the experiments were memory intensive, the number of measurements was kept to a minimum by spacing measurements according the anticipated rate of change of [Ca²⁺]'. Thus, a typical experiment included four measurements spaced 30 s apart before the addition of a stimulus; this was followed by 10-15 frames spaced 10-15 s apart, 10 frames spaced 20-30 s apart, 10 frames spaced 60 s apart, and, for longer experiments, 5-10 frames spaced 120 s apart. Stimulation of the cells involved the rapid injection of 1 ml stimulus in buffer through the Nitex screen barrier. Generally, there was little or no cell movement as a consequence of the injection (the Nitex reduced most of the streaming turbulence and the cells were somewhat adherent); tests with a fluorescent dye demonstrated that the addition resulted in homogeneous distribution of the stimulus in <10 s.

Each kinetic experiment generally required 15-30 min, and there were 5-10 such experiments per mast cell preparation. We found that the trapped fura-2 did not leak from the cells over the course of 2-6 h of experimentation and that the cell responses from beginning to end were nearly identical. The amount of light exposure during an experiment was not sufficient to cause noticeable photobleaching. We also did not observe any gross compartmentalization of the fura-2 during the 2-6 h of experimentation.

Histamine release for each challenge condition was directly determined by removing 1 ml of the supernatant from the microscope chamber after the observation period was complete. The total histamine content was obtained by treating 7.5 μl of cells with 200 μl of 8% PCA and bringing the volume to 1 ml with PAGCM buffer.

Standard histamine release measurements were made in the test tube as described previously. Histamine was determined by the automated method of Siraganian (20), and histamine release was calculated as the ratio of released histamine to total histamine after subtracting spontaneous release from each value. Spontaneous release was usually <5%.

Instrumentation

[Ca²⁺] 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 54200, 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 was located behind the epifluorescence assembly. All filter changing operations were controlled through an interface controller (IEEE488; 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.) with autoHVPS, autoblank, and autotarget 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 (IEEE488; Carl Zeiss, Inc.) to a microcomputer (Macintosh II; Apple Computer Corp., Cupertino, CA) equipped with an NB-DMA-8/IEEE488 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. Routinely, the 380-nm image was acquired, followed by the 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 [Ca²⁺] concentrations. However, most experiments were performed without filter alternation and, since an increase in [Ca²⁺], 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 homogeneity, was determined by measuring the fluorescent intensity of a monolayer of fura-2 in solution across the field of view. To minimize edge differences and to ease the real-time computational load on the computer, it was decided that only the center 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 ~0.120 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 [Ca²⁺] concentration would vary ~±5% across the acquired image. Camera linearity was ex...
examined 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 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++] 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):

\[
[Ca] = K_0 \left( \frac{R-R_{min}}{R_{max}-R} \right) \beta.
\]

R_{min} is the 352/380 ratio under calcium-free conditions; R_{max} is the 352/380 ratio under calcium-saturating conditions. \( \beta \) is the slope of 380-[Ca]/380-[Ca+] ratio and then the K_0 is taken to be 224 nM. For comparative purposes most cells where also examined in the absence of neutral calcium and in the presence of 1 mM Ca++ and 2.5 \( \mu \)M ionomycin to verify that the R_{max} and R_{min} ratios, respectively, were approximately correct (this assumes that the intensity resulting from excitation at 380 nm is very close to its maximum for R_{max} and close to its minimum for R_{min}). Note that in all experiments where ionomycin was used as stimulus, all cells showed nearly complete ablation of the 380-nm excitation intensity (off-scale [Ca++], levels), implying that the trapped fura-2 was essentially completely deesterified and responsive to [Ca++]. R_{max} was found to be 0.34, R_{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 256x256-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++], 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 380-nm emission intensity (implying that there was little esterified fura-2AM within the cells and that the deesterified fura-2 was responsive to [Ca++] changes) with a stable (see below) 352-nm emission intensity. Addition of EDTA to ionomycin-treated cells caused the [Ca++], 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 the slight shift in intensity expected from the monolayer calibration using this optical arrangement. In two experiments, 5 \( \times \) 10^6 human lung mast cells at >95% purity were labeled with 1 \( \mu \)M fura-2AM as described above, divided into 2 pools, and stimulated with buffer or anti-IgE antibody. The supernatants and Triton X-100 lysed pellets were assayed for fura-2. In 68020 CPU machine language, each Px,y [352] byte was multiplied by 32 (register shift left x 5) and divided by Px,y [380] (68020 CPU integer divide). Thus, loading human lung mast cells with fura-2-AM does not appear to involve the granules, and the evidence suggests that resulting calculations of [Ca++] should be relatively accurate at all time points. This data also indicates that the labeling conditions resulted in cytosolic fura-2 concentrations of 50 \( \mu \)M.

### Data Analysis

Generally, a cell suspension of 3-500,000 cells in 200 ml was loaded as described above resulted in 30-80 cells in the 256x256-pixel field that was acquired and saved during the experiment. The experiments were subsequently analyzed by software, which could generally automatically separate the cell images, although some user interaction was required. Automatic separation was most easily accomplished by an automatic threshold algorithm (which is only useful if the light intensity is uniform across the field of view, as it was in these cases). The threshold algorithm could be set to eliminate the outer 20% of the cell diameter from the analysis since that is where the low intensity emissions would normally have contributed the most error to the measurements. A good approximation of the background could also be automatically determined with this algorithm; the background for each image was determined before calculating the ratio. Each cell generally consisted of 400-600 pixels, and the single-cell ratio was calculated as the average of all the single-pixel ratios for the cell. A contour search algorithm automatically isolated each cell image for analysis, and the single-cell files were automatically collated to obtain single-cell kinetics. The software could track slowly drifting (moving?) cells, although >90-100% of the cells showed no movement during the 20-s experiment. Relatively rapid cell movement (>5 pixels/s) resulted in extremely asymmetric (high [Ca++] on one side and low [Ca++] on the other) single-cell ratio images; this data was excluded from the analysis.

The kinetic curves were subjected to several forms of analysis, with the general analysis fitting the kinetic curves to the following heuristic equation:

\[
C(t) = \delta (T_0) C_{max} \left[ e^{-k_{1}t} - e^{-k_{2}t} \right].
\]

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:

\[
T_{min} = \frac{\sum P_i \cdot (P_i < T_0)}{n \cdot (P_i < T_0)}, \quad \text{and} \quad T_{max} = \frac{\sum P_i \cdot (P_i > T_0)}{n \cdot (P_i > T_0)}.
\]

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-). 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 grey-level images were evaluated if their corresponding pixels were "on" in the binary image; I called these P_{x,y}. 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_{x,y}, P_{x,y} [352] and P_{x,y} [380], was determined by subtracting the background, T_{b} (determined for each emission image, T_3[352] and T_3[380]). In 68020 CPU machine language, each P_{x,y} [352] byte was multiplied by 32 (register shift left x 5) and divided by P_{x,y} [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, $k_1$, convoluted with an exponential decay with a single constant, $k_2$ (all of the data for $k_2$ and $k_1$ are expressed as reciprocals $1_{12} = 0.693/k_2$ or $k_1$). The whole process also allowed for the possibility of a lagtime between stimulus addition and the beginning of a transition where $\delta(T_l)$ is a step function whose value was zero for times $<T_l$ and one for times $\geq T_l$. For cells challenged with an optimal concentration of anti-IgE this lagtime was within the limited time resolution (generally any time $<10$ 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 $C_{max}$, $T_l$, $k_1$, and $k_2$. 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 net change in $[Ca^{++}]_i$. First, 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

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

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):

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

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. Effect of fura-2 labeling on human lung mast cells' IgE-mediated response.](image-url)  

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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 [Ca++], studies and previous 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

Figure 2. Kinetics of histamine release and [Ca++], changes in four preparations of mast cells (mean purity of 93%) after challenge with 5 μg/ml anti-IgE antibody. Circles demonstrate the control [Ca++], response after the addition of PAGCM buffer. Control histamine was <5%. The average [Ca++], curve represents the average of 137 cells, 25–40 cells in each of the four experiments.

Figure 3. Correlation between final histamine release and net peak [Ca++], change after challenge of mast cells (purity = 89–100%, mean = 92%) with 5 μg/ml anti-IgE antibody in 23 separate lung preparations. Each dot represents the average net peak [Ca++], changes of 30–80 cells.
cells. First, in the experiments we have performed, ~4,000 cells have been examined kinetically and no high [Ca++] 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 [Ca++] (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 [Ca++].. 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 [Ca++].. 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 [Ca++] response under these conditions.

Single-cell analysis was used to determine whether stimulation resulted in graded or all-or-nothing changes in [Ca++].. 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 artificial oscillation seen at the location of the arrows.
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-2AM 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 $[\text{Ca}^{++}]_i$ change (Fig. 8, lower left).

In Fig. 9, the cells were challenged with an optimal concentration of anti-IgE (5 $\mu$g/ml) or a concentration (0.25 $\mu$g/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 $[\text{Ca}^{++}]_i$ mirrored the average histamine release ($p < 0.001$ for histamine release or $[\text{Ca}^{++}]_i$ elevations being different for 5 and 0.25 $\mu$g/ml).

Statistically, in a paired analysis, the ratio of the net peak $[\text{Ca}^{++}]_i$ change at 5 $\mu$g/ml over the net $[\text{Ca}^{++}]_i$ change at 0.25 $\mu$g/ml vs. the net histamine release at 5 $\mu$g/ml over net histamine release at 0.25 $\mu$g/ml were indistinguishable ($p = 0.175$; means different from zero). (A similar analysis of the average change in $[\text{Ca}^{++}]_i$ vs. histamine release indicated the $[\text{Ca}^{++}]_i$ changes determined in this manner also correlated with histamine release.) In other words, net elevations in $[\text{Ca}^{++}]_i$ were precisely reflected in histamine release. The single-cell data for the 5 and 0.25 $\mu$g/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 $\mu$g/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-
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.

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 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.
these cells. Although the data is not shown, the frequency histograms plots for the average net change in [Ca++] resembled the peak [Ca++] data presented in Figs. 9 and 10. The curve fitting procedure extracted several other parameters of the single-cell response from these experiments (Figs. 9 and 10) that were not particularly revealing. The only marked difference between suboptimal and optimal anti-IgE concentrations was noted in the decay constants (expressed as $t_{1/2}$, paired analysis), which averaged 491 ± 73 and 788 ± 94 s for 5 and 0.25 μg/ml, respectively. The decay constants were not different for antigen at 5 μg/ml and anti-IgE at 5 μg/ml. The lagtime for 0.25 μg/ml anti-IgE (56 ± 13 s) was also slightly longer than for 5 μg/ml (23 ± 14 s) while the rise times were similar ($t_{1/2}$, 35 ± 10 s). Lagtimes and rise constants were similar for the antigen vs. anti-IgE experiments (Fig. 10). Some differences in the lag times can be appreciated in Fig. 4 in mast cells challenged with an optimal concentration of anti-IgE. However, until the [Ca++] response is examined with this degree of temporal resolution in a larger number of experiments, the statistical and biological significance of these lagtimes will be unclear.

**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.
results shown in Figs. 9 and 10. These results lead to the conclusion that the magnitude of the [Ca\(^{++}\)] response in mast cells is a simple monotonic function of the signal strength.

These studies have examined the all-or-nothing question from the perspective of changes in [Ca\(^{++}\)]. However, the response curve characteristic of or the transfer function between a given [Ca\(^{++}\)], change and the act of degranulation remains unknown. In this context, it was interesting to note that in some experiments, similar [Ca\(^{++}\)] responses did not always lead to similar histamine release (although, on the whole, the [Ca\(^{++}\)], changes were clearly predictive). Thus, the coupling of [Ca\(^{++}\)] to degranulation may not be a fixed aspect of the cell response. It may be that it is in this coupling that an all-or-nothing response could be found. For example, based on the single-cell distributions in Figs. 9 and 10 and the histamine release in these experiments, the threshold between the presence or absence of degranulation would occur in the range of a 150–250 nM net peak [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 to us that this is unlikely.

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