The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading

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The Ca concentration ($[Ca^{2+}]_i$) in single rat peritoneal mast cells was measured by means of the new fluorescent Ca-indicator dye fura-2. Dye-loaded cells were made to degranulate with either antigen or compound 48/80. In cells loaded with extracellularly applied, membrane-permeant fura-2 ester, degranulation was accompanied by a permanent loss of 40–60% of the fluorescence, but comparison of fluorescence at different wavelengths indicated no or only small changes in $[Ca^{2+}]_i$. When cells were loaded by microinjection of the impermeant potassium salt of the dye, degranulation resulted in no permanent loss of fluorescence, but instead was preceded by transient fluorescence changes that indicate a rapid, large and transient increase in $[Ca^{2+}]_i$. We suggest that ester-loaded fura-2 accumulates to a significant degree in the secretory granules and is lost from the cell during exocytosis.

Secretion *Mast cell* *Exocytosis* *Fura-2* *Patch clamp* *Antigen*

1. INTRODUCTION

An increase in the concentration of intracellular calcium ($[Ca]_i$) is an essential step in the secretory process [1,2]. The introduction of quin2, a Ca-indicator dye that can be loaded into cells in the form of a membrane permeable ester [3], has allowed direct observation of increases in $[Ca]_i$ in many preparations (review [4]), including mast cells and related secretory cells [5,6]. Although microfluorimetric measurements with quin2 on single cells have been reported [7], the fluorescence signals obtained were too weak for routine measurement. Consequently, most quin2 measurements have been performed on cell suspensions. This necessarily implied a reduction in time resolution, since it cannot be expected that a heterogeneous population of cells reacts to an external stimulus in complete synchrony. A newer dye, fura-2 [8], gives a 30-times larger fluorescence signal than quin2, and hence allows $[Ca]_i$ to be measured readily even in small single cells (10 μm diameter). We report that such measurements in mast cells reveal Ca transients with rise times of 1 s or less. We also have strong indications that fura-2 accumulates in mast cell granules when loaded as membrane permeable ester, and this may lead to erroneous results in secretion studies.

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Abbreviations: fura-2-AM, fura-2 acetoxymethyl ester; PS, dioleoylphosphatidylserine; DNP_{43}-BSA, 2,4-dinitrophenyl bound to bovine serum albumin; IgE, immunoglobulin E

2. MATERIALS AND METHODS

Rat peritoneal mast cells were prepared by peritoneal lavage, plated into glass bottom culture chambers and stored at 37°C until use. The medium for this incubation at 5% CO_2 contained (in mM) 140 NaCl, 2.0 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 5 glucose, 45 $NaHCO_3$, 0.4 KH_2PO_4 and antibiotics. It was adjusted to pH 7.2 with NaOH. Experiments were performed at 24–26°C in an

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Mg-rich saline of the following composition (in mM): 140 NaCl; 2.5 KCl; 2 $CaCl_2$; 5 $MgCl_2$; 5 glucose; 10 Hepes, pH 7.2. For experiments with antigen stimulation [9], cells were preincubated for 1 h at 37°C with this latter solution supplemented with 0.5 μg/ml monoclonal IgE against DNP_{43}-BSA. In some experiments with antigen, the external saline was supplemented by 25 μM PS (Sigma or synthetic from H. Eibl, Göttingen). Cells were stimulated either with compound 48/80 (Sigma) at 10–20 μg/ml or with DNP_{43}-BSA at 20 μg/ml. DNP_{43}-BSA and IgE were kindly supplied by H. Metzger, NIH.

Cells were loaded with fura-2 either by incubation for 30 min at 37°C with saline containing 3.3 μg/ml fura-2-AM, or through a patch pipette in whole-cell configuration [10], as described in section 3. Fura-2-AM as well as the potassium salt of fura-2, referred to simply as fura-2, were both obtained from Molecular Probes, Junction City, OR.

Besides varying amounts of fura-2, the solution for filling patch pipettes contained (in mM): 155 K-glutamate; 4 $MgCl_2$; 10 Hepes neutralized with NaOH to pH 7.2; 0.2 ATP; 2.5 inosine triphosphate. Appropriate mixtures of Ca and EGTA were added as required.

Experiments were carried out on a Zeiss IM-35 inverted microscope equipped for epifluorescence and photometry (Zeiss, photomultiplier system SF). For fluorescence excitation, the light from a xenon arc lamp (Osram XB075 W/2) passed through UV interference filters (see below), a UV-transmitting glass filter (Schott UG1) and a diaphragm, and was then deflected by a dichroic mirror (Zeiss FT 425) into the microscope objective (Zeiss Neofluar 63 x oil immersion; n.a. 1.25) and hence onto the preparation. Fluorescence from the cell was observed through a Schott GG 495 glass barrier filter, and in addition reached the photometer by passing through a broad-band (40 nm) interference filter with center wavelength 500 nm (Ditric Optics). This filter allowed us to view the cell through dim, red (Schott OG 590) transmitted light while fluorescence measurements were in progress. The red light increased the background light level to 2–5% of the fluorescence typically collected from a cell after dye loading; the background was routinely subtracted from the measured fluorescence signal.

To perform excitation at 2 wavelengths [8] a rotating filter wheel was fitted to a slot in the illumination pathway (which is provided in the IM-35 to accept additional excitation filters). The wheel contained interference filters (Oriel) centered at 450 and 490 nm, respectively, which provided light flashes at 2 wavelengths. These flashes of excitation light were detected by a photodiode placed behind the dichroic mirror, the latter signal served to direct the sampling of the photomultiplier signal by a PDP 11-23 digital computer. The computer averaged light intensity over the duration of the excitation periods at each of the 2 wavelengths and calculated ratios of fluorescence from successive illumination periods, which were eventually plotted as a function of mean time of occurrence of the 2 flashes. The speed of rotation of the filter wheel was adjusted to give 0.5–2 ratio measurements per s. A pinhole was placed in the image plane in front of the photomultiplier tube to limit the region from which fluorescence was collected to a circular area of 25 μm diameter in the center of the field of view.

Calibration of the fluorescence signals in terms of Ca concentration was performed as described [8]. We measured, on the microscope, the fluorescence of pipette filling solutions (100 μM fura-2) entrapped in 100 μm diameter glass capillaries. Adding 10 mM EGTA gave a limiting fluorescence ratio (short/long wavelengths) for low Ca concentration, R_0, of between 0.17 and 0.19. Adding instead 10 mM $CaCl_2$ gave the limiting fluorescence ratio for high $[Ca^{2+}]$, R_1, of between 8.5 and 10. At 390 nm, the fluorescence intensity with 10 mM EGTA divided by that with 10 mM $CaCl_2$ (F_0/F_s) was determined to be $\approx$ 30. With these parameters the apparent concentration of free Ca can be estimated according to [8],

$$[Ca] = K_D F_0/F_s \cdot (R - R_0)/(R_1 - R) \quad (1)$$

where K_D is the apparent dissociation constant of fura-2 and R is the fluorescence ratio (short/long wavelength).

Subsequent measurements on dye-loaded cells, however, indicated that the above calibration constants were not quite appropriate for intracellular measurements. Repeatedly, we encountered cells which, according to the above constants, had negative apparent Ca concentrations. Also, when cells had been loaded through a patch pipette, the

apparent concentrations inside the cells were always lower by at least a factor of 2 than the apparent concentrations in the pipettes. This was true even if the pipette contained Ca-EGTA buffers at a total concentration of 10 mM EGTA, and was in contact and diffusional equilibrium with the cell while the measurement was made. The differences between extra- and intracellular calibrations may be due to effects of the cytoplasm on the fluorescence properties of the dye. In addition, the 2 calibrations were obtained with slightly different optical geometries. Cells adhere tightly to the coverslip through which they are excited and observed, and do not extend far beyond the focal plane. When focussing on capillaries and patch pipettes, on the other hand, the plane of focus is necessarily moved some 100 μm into the aqueous phase, and this may slightly degrade the chromatic correction of the objective. Also, patch pipettes and capillaries extend further beyond the focal plane than do mast cells.

To circumvent this difficulty, intracellular calibration measurements were made. Cells were injected with solutions containing, besides fura-2, either 10 mM EGTA or 10 mM $CaCl_2$ (see section 3 for details on the loading method) and the limiting ratios R_0 and R_1, defined as above, were measured directly in the cell. The quantity $K^* = K_D F_0/F_s$ was calculated from a third measurement of fluorescence ratio with a solution containing 6 mM Ca-EGTA and 3 mM free EGTA assuming an apparent K_D for EGTA at pH 7.2 of 151 nM [8]. [Ca] was then calculated for all intracellular measurements according to

$$[Ca] = K^*(R - R_0)/(R_1 - R) \quad (2)$$

with $K^* = 9.98$ μM, $R_0 = 0.144$ and $R_1 = 4.264$. It should be pointed out that these constants pertain only to our particular light source, set of filters, objective, etc.

3. RESULTS

In fig.1A, fura-2 was loaded into the cell in the form of the membrane-permeant ester. A few seconds after compound 48/80 was applied (arrow), the cell was seen to degranulate under the microscope; at the same time, there occurred a pronounced and maintained decrease in fluorescence both at short (350–360 nm, trace a) and long excitation wavelengths (390 nm, trace b). When traces a and b were analyzed according to eqn 1, they indicated only very small changes in $[Ca^{2+}]$ (trace c). Similar results were obtained when cells were stimulated with antigen (not shown). In 25 experiments of this kind, $[Ca^{2+}]$ before stimulation had an apparent value of 250 ± 170 nM (mean ± SD), and fluorescence at 350–360 nm declined to 49 ± 11% during degranulation.

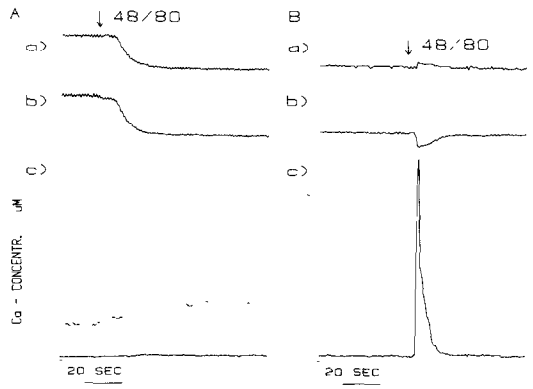

Fig.1. Comparison of different methods of loading cells with fluorescent dye; response to stimulation by compound 48/80 (20 μg/ml). (A) Cells were loaded by incubation in fura-2-AM (membrane-permeable ester) at 3.3 μM and stimulated at the time marked by an arrow. Fluorescence of a single cell was measured at 2 excitation wavelengths. (a) Fluorescence at 350–360 nm; (b) fluorescence at 390 nm, scaled down by a factor of 4 with respect to trace (a); (c) apparent free Ca concentration as calculated from eqn 2. The dotted line is a 10-fold expanded version of the continuous curve (full range for the dotted line: 1 μM). (B) Analogous measurement with a single cell that had been loaded by injection of fura-2 potassium salt to a final concentration of approx. 50 μM.

In fig.1B, fura-2 was loaded by allowing the membrane-impermeant potassium salt of the dye to diffuse into the cytoplasm through the orifice of a patch pipette, as detailed below. Once again, observation through the microscope showed that the cell degranulated completely after stimulation (arrow). However, while there were large transient changes in fluorescence (trace b), no permanent loss in fluorescence occurred; indeed, fluorescence excited near the isosbestic point (trace a) stayed nearly constant. The $[Ca^{2+}]$ calculated by eqn 2 went through a sharp peak.

The simplest interpretation of the fluorescence decline in fig.1A is that the dye leaves the cell during exocytosis and is then washed away by the stream of extracellular perfusion fluid. Since loss of fluorescence is observed only in cells loaded with the permeant form, and not when access of the impermeant dye is restricted to the cytoplasm, one may suggest that the fura-2 ester becomes trapped not only in the cytoplasm but also in the lumen of the granules. Comparing fluorescence in fig.1A before and after degranulation suggests that at most 30% of the dye was located in the cytoplasm. The larger Ca signal in fig.1B(c) and its lack or much smaller size in fig.1A indicate that only the cytoplasm, and not the lumen of the vesicles, experiences changes in $[Ca^{2+}]$ after stimulation.

To monitor cytoplasmic $[Ca^{2+}]$, it is evidently necessary to load the impermeant dye through a patch pipette, as illustrated in fig.2. In part A, a patch pipette containing 200 μM fura-2 has been sealed onto the surface of a single mast cell (cell-attached configuration [10]) at the beginning of the record. Only very small fluorescence was observed initially originating mostly from the tip of the dye-filled pipette. At the time of the first arrow the patch was broken by a pulse of suction allowing dye to diffuse from the pipette into the cell. This resulted in a marked increase in fluorescence at both wavelengths, which exponentially approached an almost steady-state level (time constant 14 s). 80 s after breaking the patch, the cell was stimulated by compound 48/80. The cell gave a small Ca transient but did not degranulate. The lack of degranulation was verified in this case both visually and by measurement of membrane capacitance [11].

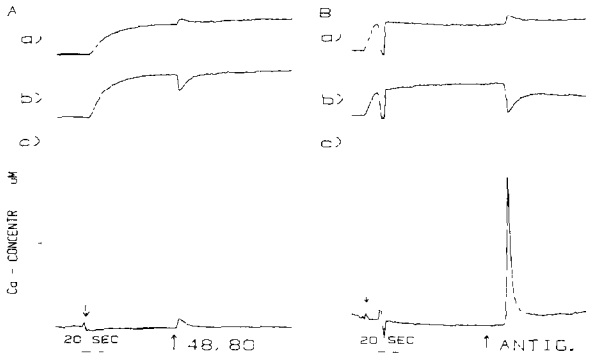

Fig.2. Dye-loading with a patch pipette. (A) The cell was perforated at the time indicated by the first arrow, to allow dye to diffuse from the pipette to the cell interior. The pipette stayed in contact with the cell throughout the recording; the individual traces have the same meaning as those in fig.1. Stimulation by compound 48/80 at the time indicated by the second arrow yielded a small Ca transient but no degranulation; standard pipette filling solution plus 200 μM fura-2, 320 μM EGTA, 160 μM $CaCl_2$. (B) The pipette contained standard pipette solution with 400 μM fura-2, no added EGTA buffer. It was removed from the cell 12 s after perforation. Stimulation by antigen produced a big Ca transient and degranulation.

The failure to degranulate is not surprising in fig.2A, since cells are known to lose their responsiveness after prolonged whole-cell contact with a micropipette, probably due to 'washout' of essential cytoplasmic components [11]. By the time the dye has reached diffusional equilibrium between pipette and cell, any second messengers and other regulators of cell function of comparable size will have had time to diffuse out of the cell, and dilute into the practically infinite volume of the patch pipette. We therefore made an effort to terminate the loading process before it came to equilibrium. For this purpose we filled the pipette with a higher concentration of fura-2 (400 μM) and withdrew the cell from the pipette after a few seconds, when the fluorescence had reached a level which corresponded to that of a 50–100 μM equilibrium loading. This is illustrated in fig.2B, where the dip in fluorescence shortly after the first arrow occurred when the cell was moved out of the measuring area to separate it from the pipette. When the cell was brought back into place a few seconds later, it had a stable level of fluorescence and low intracellular Ca, indicating that removal of the pipette had caused no significant damage to the cell membrane. Stimulation by antigen resulted in a pronounced, spike-like Ca transient followed by degranulation. This method of dye loading preserved the ability to degranulate in response to antigen or compound 48/80 in 28 out of 32 cases tested. It also allowed control readings of autofluorescence (usually negligible) to be taken from an individual cell before loading.

Cells usually attained a steady level of $[Ca]_i$ within a minute after termination of the loading process. In 33 cells the average resting Ca concen-

tration, determined at that time according to eqn 2, was 0.155 ± 0.09 μM (mean ± SD). The wide distribution of values implies that approx. 1/3 of the cells studied had values of $[Ca]_i$ between 50 and 100 nM and 6 out of 33 cells had resting $[Ca]_i$ above 200 nM.

One to three transients in Ca concentration, like those shown in fig.2B, were usually observed within 10–20 s after stimulation by compound 48/80 or antigen. Transients had rise times of the order of 1 s or less, too short to be properly resolved by our apparatus. The role of Ca transients in the initiation of degranulation and some of their other properties will be the subject of a forthcoming publication.

4. DISCUSSION

Measurement of $[Ca]_i$ on single cells using dye injection reveals, in response to stimulation, an extremely fast transient rise. Ca transients with similarly fast time course have been observed using aequorin in mouse oocytes during fertilisation [12] and in mammalian cardiac muscle [13]. In measurements on cell suspensions, one usually observes only much slower responses [5,6], that are maintained for minutes and probably result from time averaging of the signals from many cells undergoing Ca transients at different times. Indirect evidence for the occurrence of fast transients in $[Ca]_i$ has been obtained through measurements of membrane conductance. In many preparations, including smooth muscle [14], neurones [15] and lacrimal gland cells [16] transient episodes of conductance increase can be observed which reflect the opening of Ca-dependent ion channels.

No similar transient responses were observed when fura-2 had been loaded into cells as the membrane permeant ester. Instead, degranulation was accompanied by a permanent loss of fluorescence at both wavelengths, with 49% of the fluorescence remaining after degranulation had gone to completion. We interpret this effect as representing loss of dye by exocytosis. On this basis, 51% of the dye in unstimulated, ester-loaded cells must be contained in the granules. Its presence there is not unexpected. Being membrane permeant, fura-2 ester will reach all intracellular compartments, and become trapped wherever there are esterases that can cleave the acetoxymethyl groups off the fura-2 moiety. Mast cell granules are known to contain several enzymes with esterase activity, such as arylsulfatase, tryptase (in human pulmonary mast cells) and acid hydrolases [17].

Dye inside the granules would explain why large Ca transients are not seen to precede degranulation in ester-loaded cells. Suppose that half the dye is in the cytoplasm. Then, even if $[Ca^{2+}]$ rose to 10 mM there, total fluorescence at 390 nm would fall only by about one half, indicating a rise in 'average' $[Ca^{2+}]$ by an amount approximately equal to 150 nM, the dissociation constant of the Ca–fura-2 complex.

In $[Ca^{2+}]$ measurements on cell suspensions enclosed in a cuvette, intragranular dye might have the opposite effect. Since exocytosed dye will report the high $[Ca^{2+}]$ usually present in the extracellular medium, cuvette measurements may report an erroneously large and maintained $[Ca^{2+}]$ increase accompanying degranulation.

Finally, it is of interest to consider the intragranular $[Ca^{2+}]$. Since for unstimulated cells, the dye reports a similar $[Ca^{2+}]$ regardless of whether dye is in the cytoplasm only or in both, cytoplasm and granules, the intragranular and cytoplasmic $[Ca^{2+}]$ must be similarly low.

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