# Microdomains of High Calcium Are Not Required for Exocytosis in RBL-2H3 Mucosal Mast Cells

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***Abstract.*** We have previously shown that store-associated microdomains of high $Ca^{2+}$ are not essential for exocytosis in RBL-2H3 mucosal mast cells. We have now examined whether $Ca^{2+}$ microdomains near the plasma membrane are required, by comparing the secretory responses seen when $Ca^{2+}$ influx was elicited by two very different mechanisms. In the first, antigen was used to activate the $Ca^{2+}$ release–activated $Ca^{2+}$ (CRAC) current ($I_{CRAC}$) through CRAC channels. In the second, a $Ca^{2+}$ ionophore was used to transport $Ca^{2+}$ randomly across the plasma membrane. Since store depletion by $Ca^{2+}$ ionophore will also activate $I_{CRAC}$, different means of inhibiting $I_{CRAC}$ before ionophore addition were used. $Ca^{2+}$ responses and secretion in individual cells were compared using simultaneous indo-1 microfluorometry and constant potential amperometry. Secretion still takes place when the increase in intracellular $Ca^{2+}$ occurs diffusely via the $Ca^{2+}$ ionophore, and at an average intracellular $Ca^{2+}$ concentration that is no greater than that observed when $Ca^{2+}$ entry via CRAC channels triggers secretion. Our results suggest that microdomains of high $Ca^{2+}$ near the plasma membrane, or associated with mitochondria or $Ca^{2+}$ stores, are not required for secretion. Therefore, we conclude that modest global increases in intracellular $Ca^{2+}$ are sufficient for exocytosis in these nonexcitable cells.

Key words: exocytosis • calcium signaling • mast cells • indo-1 • serotonin

## *Introduction*

The intracellular free ionized calcium concentration ($[Ca^{2+}]_i$)[1] is a key physiological signal in stimulus–secretion coupling in both excitable and nonexcitable cells. Theoretical models of $Ca^{2+}$ diffusion within the cytoplasm of excitable cells predict that $[Ca^{2+}]_i$ at release sites can reach hundreds of micromolar due to $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (Chad and Eckert, 1984; Neher, 1986; Yamada and Zucker, 1992). $[Ca^{2+}]_i$ reported by diffusible $Ca^{2+}$ indicators represents the mean average $[Ca^{2+}]_i$ and does not reflect $[Ca^{2+}]_i$ levels beneath the plasma membrane or near $Ca^{2+}$ channels. Indeed, these microdomains of high $Ca^{2+}$ are usually beyond the resolution of currently available methods of $Ca^{2+}$ measurement (Neher, 1998).

However, diverse indirect approaches have yielded insight into the behavior of $[Ca^{2+}]_i$ near $Ca^{2+}$ channels and its effect on the secretory machinery of excitable cells. Some of these approaches have supported theoretical predictions that $[Ca^{2+}]_i$ at exocytotic sites can substantially exceed the spatially averaged $[Ca^{2+}]_i$ signal measured during depolarization (Augustine and Neher, 1992; Llinás et al., 1992; Zucker, 1993). Furthermore, localized increases in $[Ca^{2+}]_i$ and smaller increases in average $[Ca^{2+}]_i$ can be used as two different modes of intracellular signalling, allowing differential activation of $Ca^{2+}$-dependent physiological processes even in the same cellular compartment (Swandulla et al., 1991; Verhage et al., 1991). In nerve terminals, different modes of $Ca^{2+}$ signalling allow coupling to secretion of different transmitter classes. For example, a local rise in $Ca^{2+}$ may trigger secretion of amino acid neurotransmitters, whereas an increase in average $[Ca^{2+}]_i$ stimulates secretion of neuropeptides (Verhage et al., 1991). Similarly, in human neutrophils different $Ca^{2+}$ concentrations are required for the release of different types of granule (Lew et al., 1986; Nüsse et al., 1998).

We have investigated whether microdomains of high $Ca^{2+}$ play a role in exocytosis in RBL-2H3 mucosal mast cells. In these nonexcitable cells, aggregation of immunoglobulin E receptors on the cell surface by multivalent antigen results in the generation of inositol 1,4,5-trisphosphate, which causes release of $Ca^{2+}$ from intracellular stores and $Ca^{2+}$ influx through calcium release–activated $Ca^{2+}$ (CRAC) channels in the plasma membrane. Therefore, microdomains of high $Ca^{2+}$ may be generated either by the local release of $Ca^{2+}$ from intracellular stores or by $Ca^{2+}$

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[1]*Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free ionized calcium concentration; CRAC, calcium release–activated $Ca^{2+}$; $I_{CRAC}$, $Ca^{2+}$ release–activated $Ca^{2+}$ current.

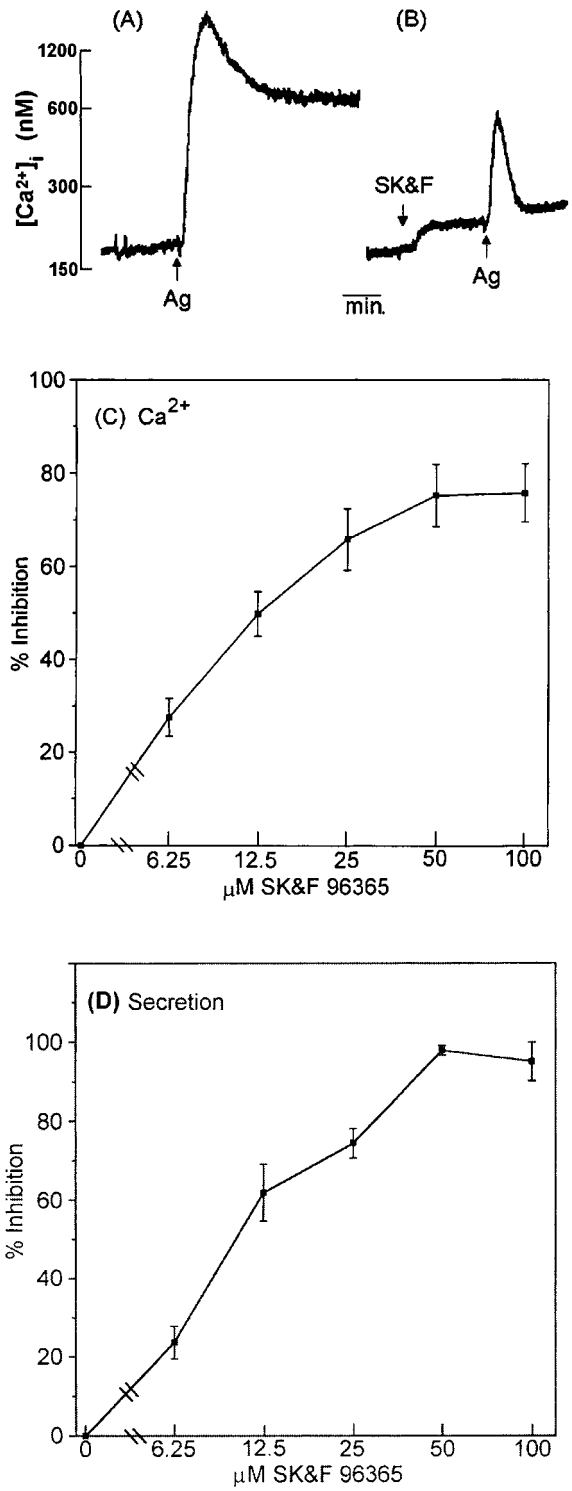


*Figure 1.* SK&F 96365 inhibits antigen-stimulated $Ca^{2+}$ influx and secretion in mucosal mast cells. (A) Typical $Ca^{2+}$ response of a suspension of indo-1–loaded RBL-2H3 cells stimulated with 1 μg/ml antigen (Ag). (B) SK&F 96365 (50 μM) was added to the cells 2 min before antigen. A transient increase in $[Ca^{2+}]_i$ due to release from stores was still seen, but the sustained increase due to $Ca^{2+}$ influx was inhibited. (C) SK&F 96365 inhibited $Ca^{2+}$ influx in a dose-dependent manner with a maximum inhibition at 50 μM. Indo-1–loaded RBL-2H3 cells were stimulated with antigen (1 μg/ml) as shown in A, and SK&F 96365 (6.25–100 μM) was added 3 min later. The decrease in $[Ca^{2+}]_i$ was expressed as a percentage of the plateau $[Ca^{2+}]_i$ immediately before SK&F 96365 addition, after deducting the resting $[Ca^{2+}]_i$ before antigen addition. (D) The inhibition of antigen-stimulated (1 μg/ml) β-hexosaminidase secretion of RBL-2H3 cells measured in the presence of the indicated concentrations of SK&F 96365 is expressed as a percentage of secretion in the absence of the drug (39.1 ± 15.9% [mean ± SD]). Spontaneous secretion (10.9 ± 3.9%) was deducted in each case. Data in C and D represent the means and standard errors of three experiments.

entry through CRAC channels in the plasma membrane. Although the conductances of inositol trisphosphate receptor channels are very high (~50 pS) (Bezprozvanny and Ehrlich, 1994), which may allow $Ca^{2+}$ microdomains to be generated near $Ca^{2+}$ stores, our previous studies demonstrated that store-associated $Ca^{2+}$ microdomains are not required for exocytosis in RBL-2H3 cells (Kim et al., 1997). The extremely low conductance of the CRAC channel estimated by fluctuation analysis (Zweifach and Lewis, 1993) (on the order of fS as opposed to pS conductances for the voltage-gated $Ca^{2+}$ channels of excitable cells) suggested that very large gradients of $Ca^{2+}$ were unlikely to be generated close to the plasma membrane. More recently, extrapolation from single-channel recordings of $Na^+$ currents through CRAC channels yielded an estimate of 1.6 pS for the $Ca^{2+}$ conductance of the CRAC channel in 20 mM $Ca^{2+}$ (Kerschbaum and Cahalan, 1999). If this higher estimate is correct, it may be possible to generate significant microdomains of high $Ca^{2+}$ near CRAC channels in mast cells.

In this study, we have examined whether $Ca^{2+}$ microdomains near the plasma membrane are required for exocytosis in RBL-2H3 mucosal mast cells. Our approach has been to compare exocytosis coupled to $Ca^{2+}$ influx through CRAC channels with that evoked by a uniform increase in intracellular free ionized calcium concentration ($[Ca^{2+}]_i$) using a $Ca^{2+}$ ionophore (Verhage et al., 1991; Fagan et al., 1996). Since store depletion by $Ca^{2+}$ ionophore will also activate $Ca^{2+}$ release–activated $Ca^{2+}$ current ($I_{CRAC}$) (Hoth and Penner, 1992; Huang and Putney, 1998), different means of inhibiting $I_{CRAC}$ before the addition of the ionophore were used. The ability of these two different pathways of $Ca^{2+}$ influx to elicit $Ca^{2+}$ responses and secretion was compared in individual RBL-2H3 cells.

## Materials and Methods

### $[Ca^{2+}]_i$ Measurements in RBL-2H3 Cell Suspensions

RBL-2H3 mucosal mast cells (Barsumian et al., 1981) were suspended at $10^6$ cells/ml in a modified Tyrode's solution containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 10 mM Na-Hepes adjusted to pH 7.4 and supplemented with 250 μM sulphinpyrazone and 0.1% BSA. The suspended cells were sensitized with 1 μg/ml dinitrophenyl-specific immunoglobulin E (provided by Drs. Juan Rivera, National Institutes of Health, Bethesda, MD, and David Holowka, Cornell University, Ithaca, NY) and loaded with 0.5 μM indo-1 acetoxymethyl ester for 45 min at 37°C. The cells were then washed and resuspended in modified Tyrode's solution containing 250 μM sulphinpyrazone and 0.05% gelatin instead of BSA. 3 ml of cell suspension ($10^6$ cells/ml) were added to acrylic cuvettes, maintained at 37°C, and constantly stirred. Fluorescence was monitored at 405 nm with a Perkin-Elmer LS-5 fluorescence spectrophotometer (excitation at 365 nm). The

$[Ca^{2+}]_i$ was calculated as described previously, after correcting for dye leakage during the experiment (Mohr and Fewtrell, 1987). The calcium-insensitive indo-1 fluorescence was determined in each experiment, and a $K_d$ of 250 nM for indo-1 was used (Grynkiewicz et al., 1985).

### β-Hexosaminidase Secretion

β-Hexosaminidase secretion was carried out in 24-well plates in which sensitized cells had been plated overnight in culture medium. After washing the cells with modified Tyrode's solution containing 0.05% gelatin, secretion was initiated by adding 1 μg/ml antigen (bovine γ-globulin to which 10–20 dinitrophenyl groups per molecule had been coupled; Eisen et al., 1959). $I_{CRAC}$ was inhibited by exposing the cells to various concentrations of SK&F 96365 ~3 min before antigen addition. Secretion was terminated after 30 min by quenching the wells with ice-cold modified Tyrode's solution. An aliquot from each supernatant was assayed fluorometrically for β-hexosaminidase using 4-methylumbelliferyl-*N*-acetyl β-D-glucosaminide as the substrate.

### Simultaneous Measurements of $[Ca^{2+}]_i$ and Serotonin Secretion in Individual RBL-2H3 Mucosal Mast Cells

RBL-2H3 mucosal mast cells plated at low density on glass coverslips were sensitized with antigen-specific immunoglobulin E and incubated overnight at 37°C in culture medium containing 100 μM L-5-hydroxytryptophan, a serotonin precursor (Smith et al., 1995). On the day of the experiment, cells were loaded with 0.5 μM indo-1 acetoxymethyl ester for 30–45 min at 37°C in modified Tyrode's solution containing 0.1% bovine serum albumin and 250 μM sulphinpyrazone (Millard et al., 1989). The coverslip was then mounted in a Teflon chamber on the microscope stage. The cell monolayer was washed and bathed in modified Tyrode's solution containing 0.05% gelatin and 250 μM sulphinpyrazone. The temperature was maintained at 35–37°C throughout an experiment using an open perfusion microincubator and a bipolar temperature controller (Medical Systems Corp.). Cells were stimulated by perfusing the sample chamber with warm solutions of antigen followed by inhibitor and then the $Ca^{2+}$ ionophore, 4-Br A23187. Indo-1 fluorescence was excited using light from a Xenon lamp filtered at 365 nm and monitored using a Nikon CF 40×/0.85 NA Fluor DL phase objective on a Nikon Diaphot inverted microscope (MVI, Inc.). Emitted light from a single cell was split using a 455-nm dichroic mirror and directed through bandpass filters at 405 and 485 nm to a pair of photomultiplier tubes. Data were acquired and stored using Felix software (PTI, Inc.). The indo-1 ratio ($R$) was calculated after subtraction of background fluorescence. The $[Ca^{2+}]_i$ was determined as described (Kao, 1994) according to the following equation, assuming a $K_d$ of 250 nM for indo-1 (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]\beta.$$

$R$ = F405/F485, $R_{max}$ = F405/F485 (with saturating $Ca^{2+}$), $R_{min}$ = F405/F485 (no $Ca^{2+}$), β = F485 (no $Ca^{2+}$)/F485 (with saturating $Ca^{2+}$). F405 indicates fluorescence intensity at 405 nm, and F485 indicates fluorescence intensity at 485 nm. $R_{max}$ was determined after adding 5 μM ionomycin to the cell, and $R_{min}$ was measured after adding ≥5 mM EGTA.

Constant potential amperometry (Travis and Wightman, 1998) was used to detect secretory events from a single cell using a carbon fiber microelectrode. The microelectrode was prepared as described previously (Kim et al., 1997) by inserting an 8 μm diameter carbon fiber into a glass capillary and pulling the glass in two stages using a patch pipette puller. The carbon fiber was sealed to the glass with cyanoacrylate glue and allowed to dry overnight. It was then trimmed as close as possible to the pipette tip, and the capillary was back filled with 3 M KCl. An Ag/AgCl wire was inserted in the capillary to allow electrical connection to the headstage of a GeneClamp 500 amplifier (Axon Instruments, Inc.). The electrode potential was held at 650 mV, and the fiber tip was placed as close as possible (~5 μm) to the cell surface. Amperometric responses were filtered at 500 Hz, digitized, and stored on videotape for later analysis. Amperometric data were then transferred to Felix at a digitization rate of 25 Hz. Both amperometric and fluorescence data were plotted using StatMost software (DataMost Corp.). Addition artifacts were often observed in the amperometric recordings; these were deleted and replaced with straight lines in the traces.

## Results

### Experimental Approach

The following protocol has been used to compare secretion that is elicited in response to $Ca^{2+}$ influx through CRAC channels ($I_{CRAC}$) with that seen when $Ca^{2+}$ is carried into the cell by a $Ca^{2+}$ ionophore. Since the $Ca^{2+}$ and exocytotic responses of individual cells are quite heterogeneous, we used real-time measurements of both $[Ca^{2+}]_i$ and secretion at the single-cell level using indo-1 fluorescence microscopy and constant potential amperometry (Kim et al., 1997). Each cell was first stimulated with antigen, allowing the generation of inositol 1,4,5-trisphosphate, which depletes stores of $Ca^{2+}$ and activates $I_{CRAC}$, and diacylglycerol, which activates protein kinase C. The exocytotic response resulting from the increase in intracellular $Ca^{2+}$, including any microdomains of high $Ca^{2+}$ close to the CRAC channel pores, and the concomitant activation of protein kinase C, was recorded for several minutes. A CRAC channel inhibitor was then added to block channels that had been activated in response to antigen and to prevent $Ca^{2+}$ influx via additional CRAC channels that might be activated when intracellular stores were further depleted in response to the $Ca^{2+}$ ionophore. Since highly specific CRAC channel inhibitors have yet to be identified, we used three different methods to prevent $Ca^{2+}$ influx via CRAC channels. The nonfluorescent $Ca^{2+}$ ionophore 4-Br A23187 (Debono et al., 1981; Deber et al., 1985) was then added to transport $Ca^{2+}$ randomly across the plasma membrane into the cytosol and generate a diffuse increase in $[Ca^{2+}]_i$ (Verhage et al., 1991; Chiono et al., 1995; Fagan et al., 1996, 1998). The secretory response elicited by this uniform increase in intracellular $Ca^{2+}$ and the $Ca^{2+}$ concentration at which it occurred were compared with those seen when CRAC channels were activated by antigen in the same cell.

### Effect of SK&F 96365 on Antigen-stimulated $[Ca^{2+}]_i$ Responses in Indo-1–loaded RBL-2H3 Cells

The imidazole compound SK&F 96365 is known to inhibit capaciative $Ca^{2+}$ entry (Merritt et al., 1990). Since SK&F 96365 has not been used before in RBL-2H3 cells, we first investigated its effects on $Ca^{2+}$ responses and secretion in these cells. Fig. 1 shows typical $Ca^{2+}$ responses of suspensions of indo-1–loaded RBL-2H3 cells stimulated with an optimum concentration of antigen in the presence or absence of SK&F 96365. In the absence of SK&F 96365, antigen induced a rapid increase in $[Ca^{2+}]_i$, which declined to a stable elevated level due to $Ca^{2+}$ influx through CRAC channels (Fig. 1 A). When SK&F 96365 was added to RBL-2H3 cells before antigen stimulation, a slow increase in basal $[Ca^{2+}]_i$ was observed (Fig. 1 B). This is probably because of some release of $Ca^{2+}$ from stores by SK&F 96365, as is commonly seen with CRAC channel inhibitors (Cleveland et al., 1993). Antigen stimulation after the addition of SK&F 96365 caused a large but transient $Ca^{2+}$ response that declined to the somewhat elevated basal level seen after SK&F 96365 addition. This transient increase in $[Ca^{2+}]_i$ is due to release from stores. The absence of the elevated sustained component is attributed to inhibition of $Ca^{2+}$ influx via CRAC channels. To asses the ability of

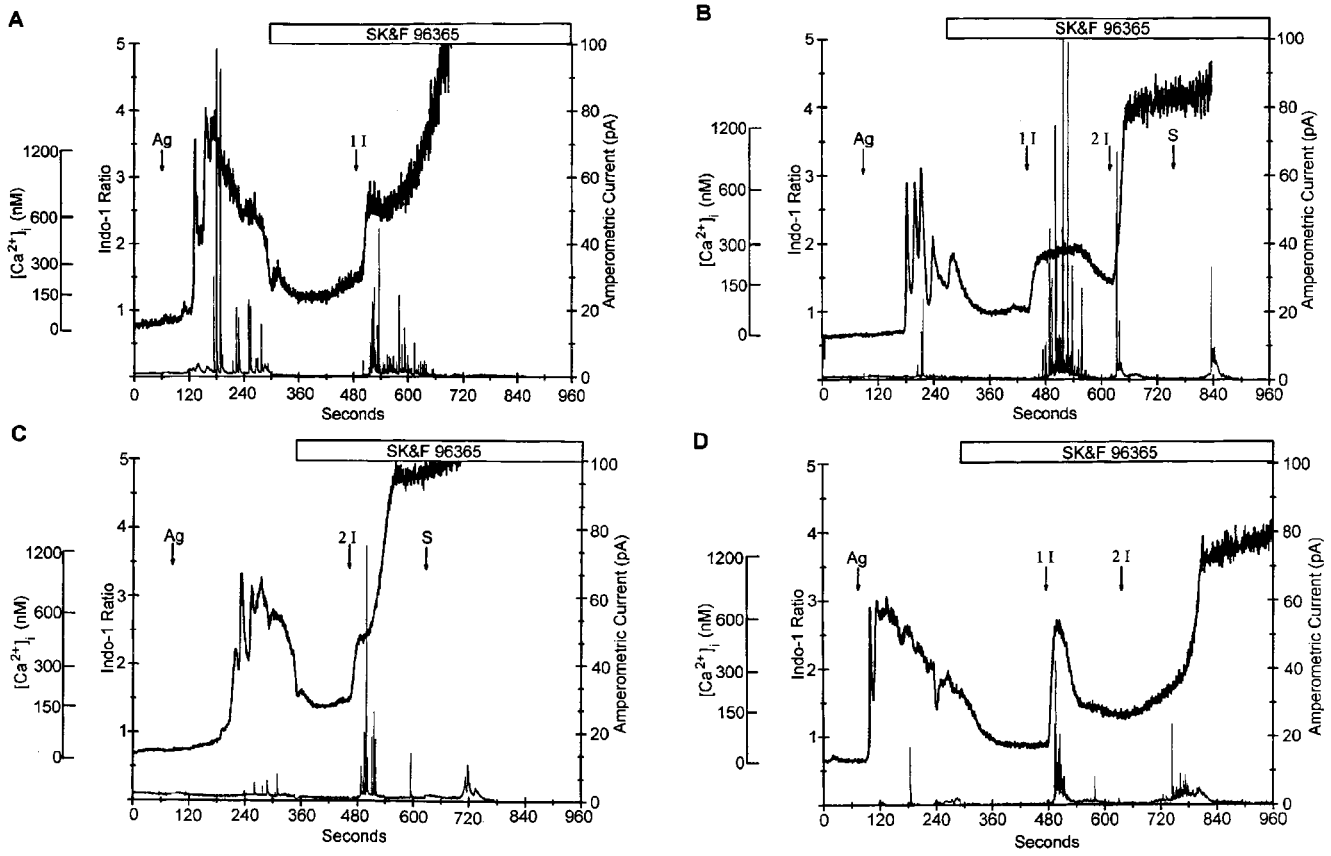


*Figure 2.* The $Ca^{2+}$ ionophore 4-Br A23187 is effective in increasing $[Ca^{2+}]_i$ and eliciting secretion when $I_{CRAC}$ is inhibited by SK&F 96365. Antigen (Ag) (1 μg/ml) stimulation of individual RBL-2H3 cells elicited an increase in intracellular $Ca^{2+}$ (upper trace) as well as a burst of amperometric events (lower trace) corresponding to exocytosis of secretory granules. Four typical single-cell responses are shown. When $Ca^{2+}$ influx was inhibited by adding 50 μM SK&F 96365, intracellular $Ca^{2+}$ dropped, and secretion was immediately halted. Subsequent addition of 1 or 2 μM 4-Br A23187 (1 I or 2 I, respectively) caused an increase in intracellular $Ca^{2+}$ to levels similar to those seen in response to antigen and a resumption of secretion. When $[Ca^{2+}]_i$ rose to higher levels, a burst of secretory events could often be seen on the rising phase (B and D). However, once the $[Ca^{2+}]_i$ reached maximum levels (>1,200 nM), secretion was inhibited. Lysis of the cell with 0.05% saponin (S) released the remaining intracellular serotonin (B and C). The 4 cells shown are representative of 11 cells in which secretion was seen; all 11 cells secreted in response to both antigen and ionophore.

SK&F 96365 to inhibit $I_{CRAC}$, the drug was added 3–4 min after antigen stimulation to ensure that $Ca^{2+}$ stores had released their contents and $I_{CRAC}$ was fully activated. Thus, the drop in the $[Ca^{2+}]_i$ signal after the addition of SK&F 96365 is due to inhibition of $I_{CRAC}$. Fig. 1 C shows that SK&F 96365 inhibited $I_{CRAC}$ in a dose-dependent manner with a maximum inhibition at 50 μM.

### *Antigen-induced Secretion Is Inhibited by SK&F 96365*

It is well established that inhibition of $Ca^{2+}$ influx prevents secretion in RBL-2H3 cells (Mohr and Fewtrell, 1987; Fewtrell et al., 1989). Having shown that SK&F 96365 can inhibit $I_{CRAC}$ in RBL-2H3 cells, we examined its effect on antigen-induced secretion of β-hexosaminidase in adherent RBL-2H3 cells. The acid hydrolase β-hexosaminidase is known to be present in mast cell granules (Schwartz et al., 1979), together with histamine and serotonin, and its release is widely used as an assay for exocytosis (Mohr and Fewtrell, 1987; Beaven and Ozawa, 1996). As expected, SK&F 96365 inhibited antigen-stimulated secretion in a dose-dependent manner (Fig. 1 D). The inhibition of β-hexosaminidase secretion correlated well with the inhibition of $I_{CRAC}$, with complete inhibition of secretion at 50 μM SK&F 96365. Therefore, it seems likely that inhibition of secretion by SK&F 96365 is due to blockade of $Ca^{2+}$ entry through CRAC channels.

### *The $Ca^{2+}$ Ionophore 4-Br A23187 Is Effective in Increasing $[Ca^{2+}]_i$ and Eliciting Secretion when $I_{CRAC}$ Is Inhibited by SK&F 96365*

Antigen stimulation of individual RBL-2H3 cells elicited an increase in intracellular $Ca^{2+}$ as well as a burst of secretory events (Fig. 2). Four typical cell responses are presented. As we have previously shown (Kim et al., 1997), secretion occurred when $[Ca^{2+}]_i$ was elevated and, when $[Ca^{2+}]_i$ oscillated, secretory events were clustered around the peaks of oscillations. When $Ca^{2+}$ influx was inhibited by adding 50 μM SK&F 96365, intracellular $[Ca^{2+}]_i$ dropped and secretion was immediately halted. When the ionophore 4-Br A23187 was subsequently added, an increase in intracellular $Ca^{2+}$ and resumption of secretory events were observed. A concentration of 1–2 μM 4-Br A23187 was needed in order

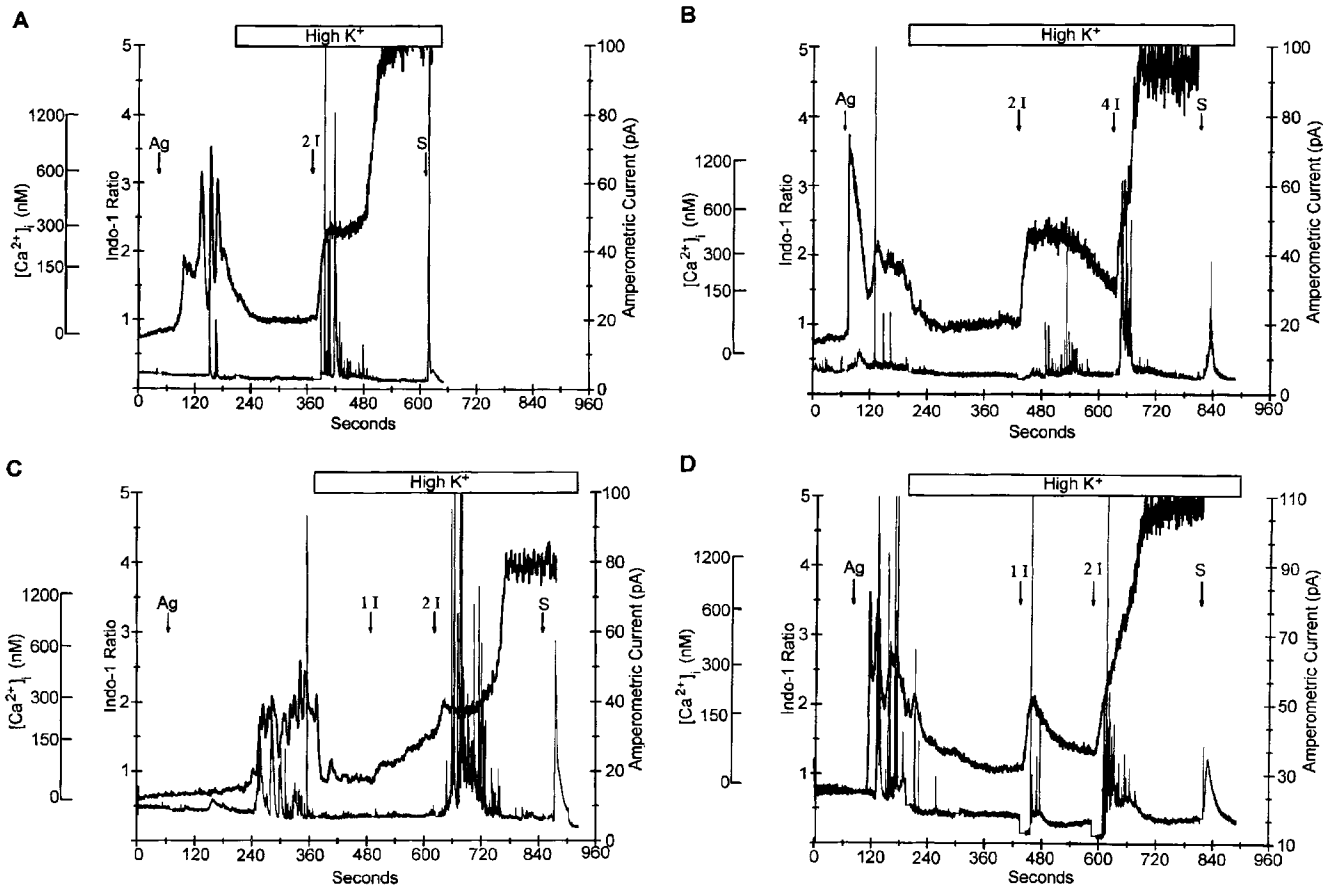


*Figure 3.* 4-Br A23187 also triggers exocytosis when $I_{CRAC}$ is inhibited by depolarizing the cells in high $K^+$. The increase in $[Ca^{2+}]_i$ and exocytosis induced by 1 μg/ml antigen (Ag) were inhibited in individual RBL-2H3 cells upon exposure to high potassium saline (140 mM). Addition of 1 or 2 μM 4-Br A23187 (1 I or 2 I) was effective in triggering additional secretory events at $[Ca^{2+}]_i$ levels similar to those induced by antigen. Again, no secretory events were observed when $[Ca^{2+}]_i$ reached very high levels in response to 4-Br A23187. Individual cell responses showed that depolarization in high $K^+$ did not entirely inhibit the $Ca^{2+}$ response, whereas exocytotic events were completely abolished. Addition of 0.05% saponin (S) lysed the cells and released the remaining serotonin. The 4 cells shown are representative of 12 cells in which secretion was seen. 10 cells responded to both antigen and ionophore, one responded to antigen but not ionophore, whereas one cell failed to secrete in response to antigen but did so when ionophore was added.

to achieve a significant increase in the mean $[Ca^{2+}]_i$ after inhibition of $I_{CRAC}$. These concentrations were not cytotoxic to the cells, as assessed by the retention of intracellular indo-1. In different responsive cells, the average $[Ca^{2+}]_i$ that was effective in triggering secretion in response to 4-Br A23187 was in the range of 300–600 nM. This is equal to or less than the effective $[Ca^{2+}]_i$ observed in response to antigen at the beginning of the experiment, which served as an internal control. However, when $[Ca^{2+}]_i$ reached very high levels (>1,200 nM) during exposure to 4-Br A23187, secretion was never observed. Therefore, secretion is inhibited at extremely high concentrations of $Ca^{2+}$, which do not usually occur in response to physiological stimuli. One notable feature, however, is that when $[Ca^{2+}]_i$ rises rapidly to maximum levels, a burst of secretory events can often be seen on the rising phase (Fig. 2, B and D). This supports the idea that, although an increase in $[Ca^{2+}]_i$ is needed for secretion, once $[Ca^{2+}]_i$ reaches unphysiologically high levels, the cells stop secreting. Under these conditions, the cells remain viable since there is no loss of intracellular indo-1. At the end of each experiment, saponin lysis released the remaining intracellular serotonin as well as the fluorescent dye indo-1.

### *4-Br A23187 also Triggers Exocytosis When $I_{CRAC}$ is Inhibited by Depolarizing the Cells in High $K^+$*

A residual $Ca^{2+}$ signal was usually observed after $I_{CRAC}$ inhibition by SK&F 96365 (Fig. 2). Although this might simply be due to an elevation in the resting level of $Ca^{2+}$, as described earlier (Fig. 1 B), it could also result from incomplete inhibition of $I_{CRAC}$. Other means of inhibiting $Ca^{2+}$ influx via CRAC channels were therefore explored. Depolarizing the cells in high potassium is known to inhibit $Ca^{2+}$ influx (Mohr and Fewtrell, 1987), and we confirmed that both the increase in $[Ca^{2+}]_i$ and secretory events induced by antigen are inhibited in single RBL-2H3 cells upon depolarization in a high potassium (140 mM) saline solution (Fig. 3). Subsequent addition of 4-Br A23187 increased $[Ca^{2+}]_i$ and was effective in triggering additional secretory events. Individual RBL-2H3 cell responses show that depolarization in high $K^+$ does not completely inhibit the $Ca^{2+}$ response at the physiological $Ca^{2+}$ concentration used (1.8 mM), whereas exocytotic events were completely abolished (Fig. 3).

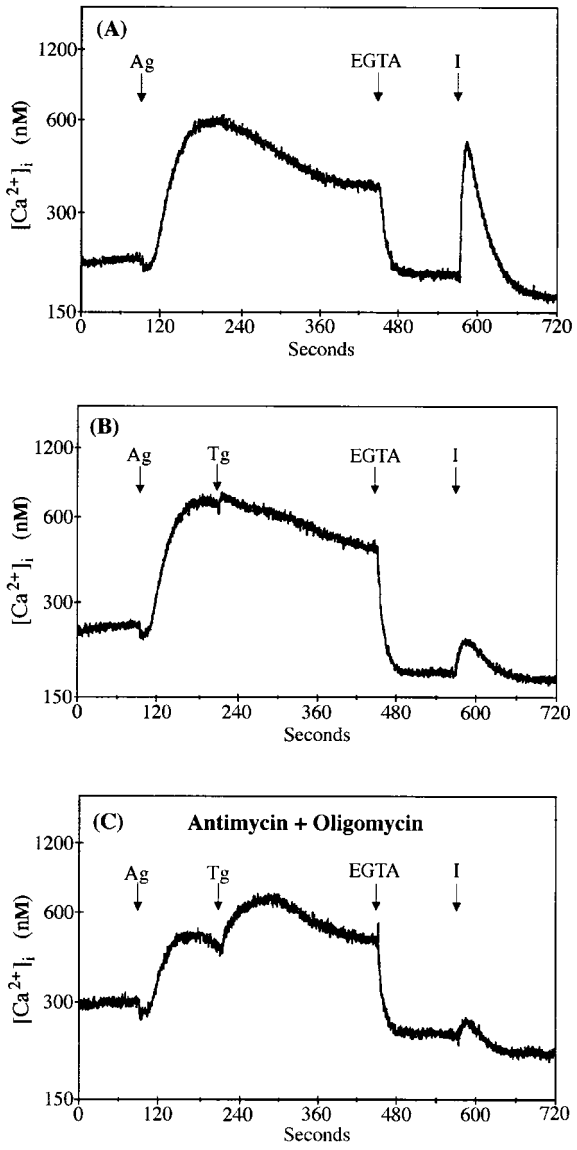


*Figure 4.* 4-Br A23187 releases additional intracellular $Ca^{2+}$ from antigen-stimulated mucosal mast cells. (A) RBL-2H3 cells in suspension were stimulated with antigen (1 μg/ml); extracellular $Ca^{2+}$ was then chelated with 2 mM EGTA. Subsequent addition of 2 μM 4-Br A23187 (I) induced a transient $Ca^{2+}$ response due to incomplete depletion of antigen-sensitive stores or release from a different pool. (B) Addition of thapsigargin (1 μM) after antigen almost completely abolished the subsequent response to 4-Br A23187 in the absence of extracellular $Ca^{2+}$. (C) Cells were exposed to the mitochondrial inhibitors, antimycin A (0.1 μM) and oligomycin B (1.2 μM), for ~40 min (in the presence of glucose) before stimulating with antigen and thapsigargin. After EGTA addition, a very small $Ca^{2+}$ response was still observed in response to 4-Br A23187. Similar results were obtained in four separate experiments.

### *Is the $Ca^{2+}$ Response to 4-Br A23187 Entirely Due to $Ca^{2+}$ Influx Via the Ionophore?*

The residual $Ca^{2+}$ signal seen in both depolarized and SK&F 96365–treated RBL-2H3 cells could result from incomplete inhibition of $I_{CRAC}$. This is of concern since subsequent addition of the ionophore 4-Br A23187 might further deplete stores and activate additional CRAC channels. This would then argue against our assumption that the increase in $[Ca^{2+}]_i$ is due solely to ionophore-mediated $Ca^{2+}$ entry across the plasma membrane. In order to test this hypothesis, $[Ca^{2+}]_i$ measurements in RBL-2H3 cell populations were performed in the absence of extracellular $Ca^{2+}$. Fig. 4 A confirms that, after antigen addition, chelation of extracellular $Ca^{2+}$ with EGTA completely abolishes the sustained increase in $Ca^{2+}$, which is due to $Ca^{2+}$ influx. Subsequent addition of 4-Br A23187 elicited a transient increase in $[Ca^{2+}]_i$, which could be due to incomplete $Ca^{2+}$ release from stores in response to antigen. Indeed, when the $Ca^{2+}$–ATPase inhibitor thapsigargin was added to antigen-stimulated cells, addition of 4-Br A23187 in the presence of EGTA elicited a much smaller $Ca^{2+}$ response (Fig. 4 B). Prior exposure of the cells to the mitochondrial inhibitors, antimycin and oligomycin, caused a slight further reduction in the $Ca^{2+}$ response to 4-Br A23187 after antigen and thapsigargin (Fig. 4 C). This suggests that only a very small part of the $Ca^{2+}$ response to 4-Br A23187 could be due to release of $Ca^{2+}$ from mitochondria. Since mitochondria are known to accumulate $Ca^{2+}$ when the cells are stimulated with antigen (Mohr and Fewtrell, 1990) or thapsigargin (Ali et al., 1994), this contribution might be greater if the $Ca^{2+}$ ionophore was added while intracellular $[Ca^{2+}]_i$ was still high, and the mitochondria were loaded with calcium. However, under the conditions used in our experiments (Figs. 2–5), intracellular $[Ca^{2+}]_i$ had returned close to resting levels before the ionophore was added. The mitochondria should therefore have released most of their calcium. The tiny residual response to 4-Br A23187 seen in Fig. 4 C presumably originates from a different population of stores (Pizzo et al., 1997; Huang and Putney, 1998).

The transient $Ca^{2+}$ signal due to release from intracellular stores is not normally sufficient to support secretion in the absence of $Ca^{2+}$ influx (Mohr and Fewtrell, 1987; Kim et al., 1997). Furthermore, we confirmed that in the absence of extracellular $Ca^{2+}$, 2 μM 4-Br A23187 was not able to evoke exocytosis in cells that had been activated by antigen (data not shown). Although the additional release from stores (Fig. 4) will contribute to the global increase in $[Ca^{2+}]_i$, store-associated microdomains of high $Ca^{2+}$ should not be generated in response to the $Ca^{2+}$ ionophore. Furthermore, intracellular calcium release by the $Ca^{2+}$ ionophore is complete within 1 min (Fig. 4), so it cannot contribute to the secretory events seen several minutes after the addition of 4-Br A23187 in Figs. 2, 3, and 5.

### *Lanthanum Inhibition of $I_{CRAC}$ Does Not Prevent 4-Br A23187 from Inducing Exocytosis*

Since we were unable to demonstrate unequivocally that $Ca^{2+}$ ionophore-induced secretion in the presence of SK&F 96365 or in $K^+$-depolarized cells was not due to $Ca^{2+}$ influx through a few remaining CRAC channels, a third method of inhibiting $I_{CRAC}$ that completely inhibited $Ca^{2+}$ influx was needed. It is well established that the lanthanide metal ion $La^{3+}$ blocks $Ca^{2+}$ influx (Millard et al., 1989) through CRAC channels in mast cells (Hoth and Penner, 1993). Fig. 5 shows that addition of 10 μM $La^{3+}$ completely abolished the $Ca^{2+}$ response induced by antigen and interrupted secretion. Subsequent addition of the

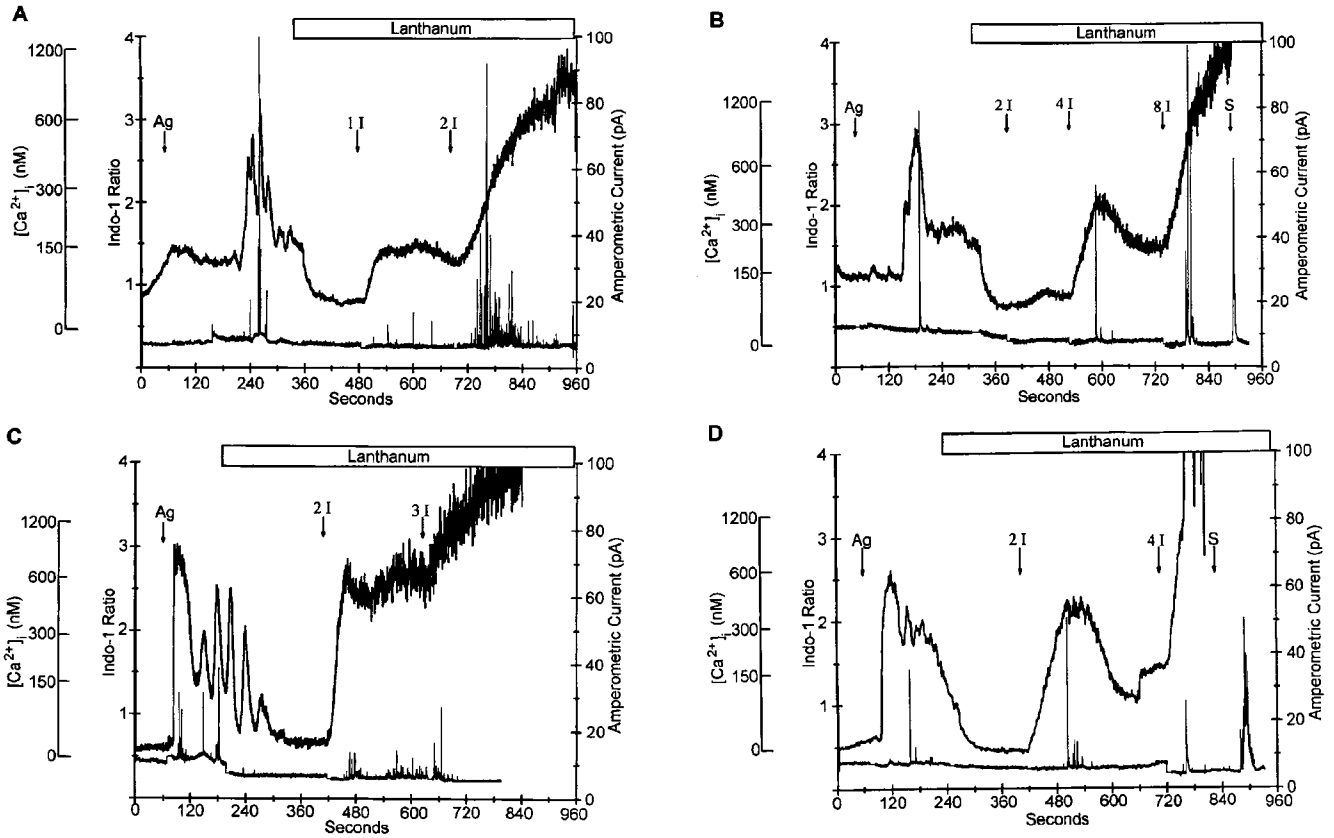


*Figure 5.* Lanthanum inhibition of $I_{CRAC}$ does not prevent 4-Br A23187 from inducing exocytosis in individual RBL-2H3 cells. The addition of 10 μM $La^{3+}$ was completely effective in abolishing the $Ca^{2+}$ response and secretion induced by 1 μg/ml antigen (Ag). Subsequent addition of 4-Br A23187 (I) generated an increase in $[Ca^{2+}]_i$ comparable to that induced by antigen. As a result, another train of secretory events occurred. Secretion was then halted when $[Ca^{2+}]_i$ reached very high levels. Saponin lysis (S) released the remaining serotonin (B and D). The four cells shown are representative of seven cells in which secretion was seen in response to both antigen and ionophore. Five additional cells failed to secrete in response to antigen but did so when ionophore was added.

$Ca^{2+}$ ionophore 4-Br A23187 generated an increase in $[Ca^{2+}]_i$ comparable to or less than that induced by antigen. As a result, another train of secretory events occurred. Secretion was then halted at very high $[Ca^{2+}]_i$ levels that exceeded 1,200 nM. These results are consistent with those observed using SK&F 96365 and depolarization in high $K^+$, which showed that the mean $[Ca^{2+}]_i$ levels at which secretion occurs in response to the diffuse $Ca^{2+}$ signal generated by the $Ca^{2+}$ ionophore are no greater than those measured during exocytosis in response to CRAC channel activation.

### *Inhibiting Mitochondria Has Little Effect on Secretion*

The possibility that mitochondrial $Ca^{2+}$ sequestration and/or release could affect secretion (Montero et al., 2000) in response to 4-Br A23187 was also explored. Fig. 6 shows the secretory responses of control cells and of cells that had been exposed to antimycin and oligomycin in the continued presence of glucose. Under the latter conditions, ATP levels are maintained by glycolysis, but mitochondrial function is inhibited and mitochondria are no longer able to sequester $Ca^{2+}$ or synthesize ATP (Mohr and Fewtrell, 1990). As expected, inhibition of antigen-induced $Ca^{2+}$ influx with lanthanum completely abolished β-hexosaminidase secretion from control cells, unless the $Ca^{2+}$ ionophore 4-Br A23187 was used to carry $Ca^{2+}$ into the cells (Fig. 6, white bars). Inhibiting mitochondrial function (Fig. 6, black bars) only slightly reduced secretion in response to antigen, which is consistent with the somewhat lower $Ca^{2+}$ response seen under these conditions (Fig. 4 C). Again, blocking CRAC channels with lanthanum completely inhibited antigen-induced secretion. Furthermore, the $Ca^{2+}$ ionophore was still able to restore the secretory response in cells whose mitochondria were inhibited and therefore unable to sequester $Ca^{2+}$ (Fig. 6, black bars). This clearly confirms that secretion in response to 4-Br A23187 is not due to release of $Ca^{2+}$ from mitochondria.

### *$Ca^{2+}$ Dependence of Exocytosis*

The $Ca^{2+}$ dependence of secretion in response to activation of $I_{CRAC}$ by antigen and when $Ca^{2+}$ influx occurs via the ionophore 4-Br A23187 is shown in Fig. 7. It is clear that the mean $[Ca^{2+}]_i$ levels at which secretory events are detected are quite similar for the two pathways of $Ca^{2+}$ influx. When CRAC channels were activated, the frequency of secretory events peaked between 0.6 and 0.8 μM $Ca^{2+}$. When influx occurred via the $Ca^{2+}$ ionophore 4-Br A23187, the frequency of secretion was somewhat greater at all $Ca^{2+}$ concentrations, and it peaked between 0.4 and 0.6 μM $Ca^{2+}$. We have previously shown that secretion is

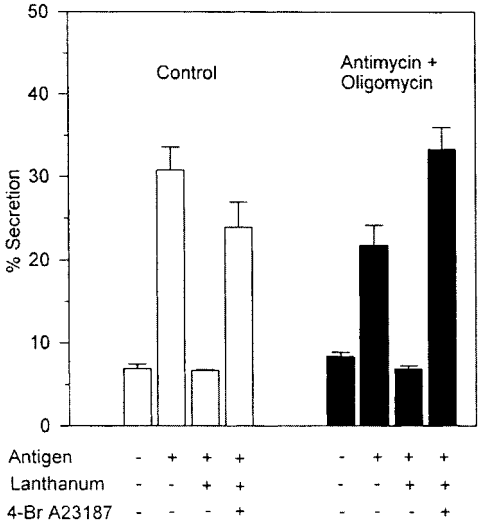


*Figure 6.* Mitochondrial inhibitors have little effect on secretion. White bars show spontaneous and antigen-stimulated β-hexosaminidase secretion, the inhibitory effect of lanthanum and the secretion observed when the $Ca^{2+}$ ionophore 4-Br A23187 was also added to adherent RBL-2H3 mucosal mast cells. Black bars show data obtained from cells that had been exposed to the mitochondrial inhibitors antimycin A (0.1 μM) and oligomycin B (1.2 μM) for 20–30 min (in the presence of glucose) before stimulation. Cells were stimulated with antigen (1 μg/ml), with or without lanthanum (10 μM), and 4-Br A23187 (2 μM) for 10–12 min. Each column shows the mean ± SEM of triplicate determinations from four separate experiments. Stimulated secretion was abolished when cells were incubated with mitochondrial inhibitors in the absence of glucose (not shown), thus confirming that the inhibitors were effective.

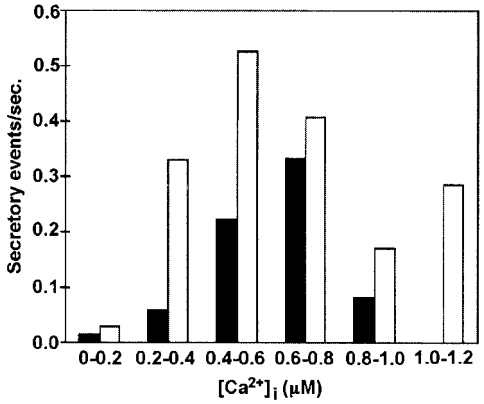


*Figure 7.* Frequency of exocytotic events at different $[Ca^{2+}]_i$. Histogram analysis of the $Ca^{2+}$ dependence of serotonin secretion in response to activation of $I_{CRAC}$ by antigen (black bars) and when $Ca^{2+}$ influx occurred via the ionophore 4-Br A23187 (white bars). The data are from 15 RBL-2H3 mucosal mast cells, including the ones shown in Figs. 2, 3, and 5. Analyses for each cell were conducted from the time of the first secretory event in response to antigen to the point at which the inhibitor was added (for antigen) and from the time between the first and last secretory events after the addition of 4-Br A23187 (for $Ca^{2+}$ ionophore). The number of secretory events between these times for all of the cells were expressed as a function of the aggregate length of time the cells spent at the indicated $Ca^{2+}$ concentrations during each of these time windows.

not seen in the troughs between $Ca^{2+}$ oscillations or in cells where $Ca^{2+}$ oscillates from baseline levels (Kim et al., 1997). It is therefore possible that the enhanced secretion seen with the $Ca^{2+}$ ionophore occurs because intracellular $Ca^{2+}$ levels remain elevated and no longer oscillate, as they usually do in response to antigen. Alternatively, we found that the average delay between the initial increase in intracellular $Ca^{2+}$ and the onset of exocytosis in response to antigen was decreased from 34 to 17 s when protein kinase C was preactivated with phorbol myristate acetate (Kim et al., 1997). If, as these results suggest, protein kinase C is not fully activated during the early part of the $Ca^{2+}$ response to antigen, this might explain why the frequency of secretion is somewhat higher when the $Ca^{2+}$ ionophore is subsequently added.

Although $[Ca^{2+}]_i$ rarely exceeds 0.8 μM under physiological conditions, it is interesting to note that the frequency of exocytosis, in response to both antigen and $Ca^{2+}$ ionophore, apparently decreases at these high $Ca^{2+}$ concentrations (Fig. 7). Furthermore, as noted above and shown in Figs. 2, 3, and 5, exocytosis is completely inhibited at the very high $[Ca^{2+}]_i$ (>1.2 μM) that are sometimes seen in response to the $Ca^{2+}$ ionophore.

## *Discussion*

It is well established that the transient signal due to release of $Ca^{2+}$ from intracellular stores, in the absence of $Ca^{2+}$ influx, is unable to trigger exocytosis in RBL-2H3 mucosal mast cells (Mohr and Fewtrell, 1987; Kim et al., 1997). However, the precise role played by $Ca^{2+}$ influx is not yet understood. One possibility is that it is simply required to sustain the global $Ca^{2+}$ response. Alternatively, it is possible that $Ca^{2+}$ influx is needed to generate domains of high $Ca^{2+}$. Many lines of evidence suggest that $Ca^{2+}$ concentrations of 10–100 μM can be generated transiently near the pores of open voltage-gated $Ca^{2+}$ channels in excitable cells and that these high $Ca^{2+}$ concentrations are essential for exocytosis (Augustine and Neher, 1992; Llinás et al., 1992; Zucker, 1993; Chow et al., 1994; von Gersdorff and Matthews, 1994; Bollmann et al., 2000; Schneggenburger and Neher, 2000). Since the conductance of the inositol trisphosphate receptor channel (53 pS) (Bezprozvanny and Ehrlich, 1994) is much higher than that of the CRAC channel, we initially proposed that $Ca^{2+}$ microdomains associated with stores might be more critical for secretion in mucosal mast cells. However, our results showed that even when refilling and continued release of $Ca^{2+}$ from stores was prevented by the $Ca^{2+}$–ATPase inhibitor thapsigargin, $Ca^{2+}$ influx through CRAC channels could still trigger secretion, suggesting that store-associated microdomains of $Ca^{2+}$ are not required for exocytosis (Kim et al., 1997). Furthermore, we have shown that inhibition of $Ca^{2+}$ uptake and release by mitochondria in stimulated mucosal mast cells (Mohr and Fewtrell, 1990; Ali et al., 1994) appears to have little effect on $Ca^{2+}$ responses (Millard et al., 1989; Fig. 4) and secretion (Mohr and Fewtrell, 1990; Fig. 6).

At a membrane potential of −70 mV, the unitary current amplitude associated with a 1.6 pS CRAC channel (Kerschbaum and Cahalan, 1999) would correspond to a flux of $\sim 3 \times 10^5$ $Ca^{2+}$ ions/s. In contrast, $Ca^{2+}$ ionophores are lipophilic mobile ion carriers that transport $Ca^{2+}$ randomly across the plasma membrane into the cytoplasm

(Pressman, 1976; Debono et al., 1981). The turnover number of ionophore-mediated $Ca^{2+}$ transport was reported to be 45 ions/s for the ionophore A23187 and 22 ions/s for its nonfluorescent 4-bromo derivative (Pfeiffer et al., 1978; Debono et al., 1981). Thus, a $Ca^{2+}$ ionophore will induce a relatively uniform increase in intracellular $Ca^{2+}$ with no microdomains of high $Ca^{2+}$ (Verhage et al., 1991; Fagan et al., 1996). We have therefore compared the exocytotic response elicited as a result of $Ca^{2+}$ influx through CRAC channels with that evoked by a $Ca^{2+}$ ionophore.

Since both the $Ca^{2+}$ responses and secretion in individual mast cells are extremely heterogeneous (Millard et al., 1989; Kim et al., 1997), it was important to carry out these experiments at the single-cell level. This allowed us to compare the secretory response seen when $I_{CRAC}$ is activated by antigen with that seen in the same cell when $Ca^{2+}$ is carried by the ionophore 4-Br A23187. Both an increase in intracellular $Ca^{2+}$ (Mohr and Fewtrell, 1987; Kim et al., 1997) and activation of protein kinase C (Ozawa et al., 1993; Wolfe et al., 1996) are essential for exocytosis in mucosal mast cells, and it has been suggested that other as yet unidentified signals may also be involved (Beaven et al., 1987; Cunha-Melo et al., 1989). Addition of antigen will therefore generate the full physiological response, including activation of $I_{CRAC}$, protein kinase C, and any other necessary signals. Furthermore, when $Ca^{2+}$ influx via CRAC channels is inhibited and the $Ca^{2+}$ ionophore is added, the other signals generated in response to antigen should remain activated. Since there are no highly selective inhibitors of CRAC channels, three different pharmacological approaches were used to inhibit $I_{CRAC}$ in antigen-stimulated mucosal mast cells. All three manipulations, SK&F 96365, potassium depolarization, and lanthanum, blocked the $Ca^{2+}$ influx and secretion seen in response to antigen. However, in all cases diffuse $Ca^{2+}$ entry via the ionophore 4-Br A23187 was effective in restoring exocytosis. These results clearly suggest that secretion is not tightly coupled to $Ca^{2+}$ entry through CRAC channels and that it is the global $Ca^{2+}$ concentration that is being sensed by the exocytotic machinery in mast cells.

Several studies have demonstrated the validity of the approach of using a $Ca^{2+}$ ionophore to generate a uniform increase in intracellular $Ca^{2+}$ with no microdomains of high $Ca^{2+}$. The first of these compared the abilities of voltage-sensitive $Ca^{2+}$ channel activation and the $Ca^{2+}$ ionophore, ionomycin, to evoke secretion of synaptic vesicles and dense core vesicles from isolated nerve terminals (Verhage et al., 1991). For a given increase in mean $[Ca^{2+}]_i$, the $Ca^{2+}$ ionophore was much less effective than $Ca^{2+}$ channel activation in releasing amino acids from synaptic vesicles, whereas neuropeptide release occurred at similar global $Ca^{2+}$ concentrations with both stimuli. It was therefore concluded that exocytosis of amino acid–containing synaptic vesicles requires the higher $Ca^{2+}$ concentrations generated in the vicinity of $Ca^{2+}$ channels in the active zone, whereas neuropeptide secretion from large dense core vesicles is triggered by much smaller elevations in $Ca^{2+}$ in the bulk cytoplasm. In other studies (Chiono et al., 1995; Fagan et al., 1996, 1998), it has been shown that $Ca^{2+}$ influx via capacitative $Ca^{2+}$ entry channels is required to regulate the activity of several $Ca^{2+}$-regulated adenylyl cyclases in glioma cells, whereas $Ca^{2+}$ signals generated by a $Ca^{2+}$ ionophore or by release of stored $Ca^{2+}$ were ineffective. It was therefore proposed that $Ca^{2+}$-sensitive adenylyl cyclases are functionally colocalized with capacitative $Ca^{2+}$ entry channels and that their activity is regulated by microdomains of high $Ca^{2+}$ associated with these channels (Cooper et al., 1995).

The localized nature of $Ca^{2+}$ microdomains associated with voltage-gated $Ca^{2+}$ channels in neuronal cells led to the suggestion that these domains of $[Ca^{2+}]_i$ may trigger secretion from synaptic vesicles in close proximity or tightly coupled to these channels (Matthews, 1996; Bennett, 1997). The fast time course of secretion at synapses was also attributed to the tight association between vesicles and $Ca^{2+}$ channels (Simon and Llinas, 1985; Adler et al., 1991; Heidelberger et al., 1994). This is in contrast to the slower time course of secretion in neuroendocrine cells, where vesicles and channels are not strictly colocalized (Chow et al., 1994). By analogy, the very slow time course of secretion in mucosal mast cells, and the ability of diffuse $Ca^{2+}$ entry to elicit secretion in the absence of $I_{CRAC}$, is most likely to reflect spatial separation between the sites of $Ca^{2+}$ entry and exocytosis in mast cells. This is consistent with the fact that mast cell granules are distributed throughout the cytoplasm and are not clustered at the plasma membrane. Although we have shown that microdomains of high $Ca^{2+}$ are not required for exocytosis, this does not rule out the possibility that $Ca^{2+}$ microdomains associated with CRAC channels (Zweifach and Lewis, 1995) are important in regulating other cell functions (Chiono et al., 1995; Fagan et al., 1998) in mast cells.

The $[Ca^{2+}]_i$ at which secretion is greatest in mucosal mast cells is well below 1 μM both when $Ca^{2+}$ enters the cell through CRAC channels and when it enters via the $Ca^{2+}$ ionophore 4-Br A23187 (Fig. 7). Consistent with this finding, it has been shown that similarly low concentrations of $Ca^{2+}$ support secretion in washed permeabilized RBL-2H3 cells, provided that protein kinase C is replenished and activated (Ozawa et al., 1993; Beaven and Ozawa, 1996). These concentrations of $Ca^{2+}$ are remarkably similar to those required for the exocytosis of large dense core vesicles from nerve terminals (Verhage et al., 1991). In neutrophils, the $Ca^{2+}$ dependence of exocytosis depended on the type of granule being secreted and the way in which the $Ca^{2+}$ dependence was measured (Lew et al., 1986; Nüsse et al., 1998). In all cases, half-maximal $Ca^{2+}$ concentrations were significantly higher, ranging from 1 to 100 μM $Ca^{2+}$. However, neutrophils, like mast cells, require other synergistic signals, in addition to $Ca^{2+}$, for optimal secretion (Lew et al., 1986; Nüsse et al., 1997). Since $Ca^{2+}$ was the only signal provided in these studies, the lack of additional signals (Lew et al., 1986) or their loss during microperfusion of the cell (Nüsse et al., 1998) may explain why higher $[Ca^{2+}]_i$ were required for exocytosis in neutrophils.

Current models for exocytosis suggest that synaptic vesicles have developed low-affinity $Ca^{2+}$ sensors that respond to locally high increases in $[Ca^{2+}]_i$ generated near the mouths of $Ca^{2+}$ channels at the active zone of a nerve terminal. Our results, obtained using a $Ca^{2+}$ ionophore with single mucosal mast cells, suggest that granule exocytosis occurs at $Ca^{2+}$ concentrations between 0.3 and 0.8 μM. Furthermore, these $Ca^{2+}$ concentrations are very similar to those at which secretion in response to antigen was seen in the same cells, before ionophore addition, and also in

our earlier studies (Kim et al., 1997). This suggests that the secretory granules of mast cells are spatially well separated from CRAC channels and respond to the global $[Ca^{2+}]_i$ rather than microdomains of high $[Ca^{2+}]_i$ associated with CRAC channels. They would therefore be expected to have a much higher affinity $Ca^{2+}$ sensor than synaptic vesicles.

It has been shown that mast cells express three different isoforms of the $Ca^{2+}$-binding protein synaptotagmin (Baram et al., 1999), which has been proposed as the $Ca^{2+}$ sensor for exocytosis (Südhof and Rizo, 1996). Synaptotagmin II, the most abundant isoform in mast cells, is associated with lysosomes rather than with secretory granules (Baram et al., 1999). The low sensitivity (200 μM) of synaptotagmin II for $Ca^{2+}$ (Südhof and Rizo, 1996) would be consistent with the high $Ca^{2+}$ requirement for lysosomal exocytosis (Rodríguez et al., 1997; Baram et al., 1999). Synaptotagmin III, which has a high sensitivity (<1 μM) for $Ca^{2+}$ (Südhof and Rizo, 1996), is another of the isoforms identified in mast cells. It is not known whether it is associated with the secretory granules, but the relatively low cytoplasmic $Ca^{2+}$ concentrations (0.3–1 μM) at which most granule exocytosis occurs (Kim et al., 1997; this paper) make synaptotagmin III an attractive candidate for the $Ca^{2+}$ sensor for exocytosis in mast cells.

In summary, we have shown that microdomains of high $Ca^{2+}$ associated with CRAC channels are not essential for exocytosis in RBL-2H3 mucosal mast cells. Therefore, both release from stores and influx across the plasma membrane can contribute to the global increase in $[Ca^{2+}]_i$ that leads to exocytosis. Furthermore, it is clear that the optimal $[Ca^{2+}]_i$ for secretion in these cells is <1 μM. This is in contrast to neuronal cells, where microdomains of very high $[Ca^{2+}]_i$ (10–100 μM) associated with voltage-dependent $Ca^{2+}$ channels are required for the rapid release of neurotransmitters at the neuromuscular junction and other synapses. Our findings are consistent with the fact that exocytosis in mast cells occurs relatively slowly. Furthermore, mast cell granules are distributed throughout the cytoplasm, and there is no evidence for active zones of secretion where granules might be in very close proximity to CRAC channels. The relatively low $[Ca^{2+}]_i$ at which secretion occurs in mast cells may also explain why concomitant activation of protein kinase C is also necessary (Ozawa et al., 1993; Wolfe et al., 1996), whereas a large increase in $[Ca^{2+}]_i$ is sufficient for secretion in excitable cells.

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