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Roles for Ca^{2+} Stores Release and Two Ca^{2+} Influx Pathways in the FcεR1-activated Ca^{2+} Responses of RBL-2H3 Mast Cells

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Cross-linking the high affinity IgE receptor, FcεR1, with multivalent antigen induces inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-dependent release of intracellular Ca^{2+} stores, Ca^{2+} influx, and secretion of inflammatory mediators from RBL-2H3 mast cells. Here, fluorescence ratio imaging microscopy was used to characterize the antigen-induced Ca^{2+} responses of single fura-2–loaded RBL-2H3 cells in the presence and absence of extracellular Ca^{2+} (Ca_o^{2+}). As antigen concentration increases toward the optimum for secretion, more cells show a Ca^{2+} spike or an abrupt increase in $[Ca^{2+}]_i$ and the lag time to onset of the response decreases both in the presence and the absence of Ca_o^{2+}. When Ca_o^{2+} is absent, fewer cells respond to low antigen and the lag times to response are longer than those measured in the presence of Ca_o^{2+}, indicating that Ca_o^{2+} contributes to Ca^{2+} stores release. Ins(1,4,5)P_3 production is not impaired by the removal of Ca_o^{2+}, suggesting that extracellular Ca^{2+} influences Ca^{2+} stores release via an effect on the Ins(1,4,5)P_3 receptor. Stimulation with low concentrations of antigen can lead, only in the presence of Ca_o^{2+}, to a small, gradual increase in $[Ca^{2+}]_i$ before the abrupt spike response that indicates stores release. We propose that this small, initial $[Ca^{2+}]_i$ increase is due to receptor-activated Ca^{2+} influx that precedes and may facilitate Ca^{2+} stores release. A mechanism for capacitative Ca^{2+} entry also exists in RBL-2H3 cells. Our data suggest that a previously undescribed response to FcεR1 cross-linking, inhibition of Ca^{2+} stores refilling, may be involved in activating capacitative Ca^{2+} entry in antigen-stimulated RBL-2H3 cells, thus providing the elevated $[Ca^{2+}]_i$ required for optimal secretion. The existence of both capacitative entry and Ca^{2+} influx that can precede Ca^{2+} release from intracellular stores suggests that at least two mechanisms of stimulated Ca^{2+} influx are present in RBL-2H3 cells.

INTRODUCTION

In RBL-2H3 rat mucosal mast cells, cross-linking the high affinity IgE receptor (FcεR1)[1] stimulates a sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that results from the release of intracellular, stored Ca^{2+} and from Ca^{2+} influx from the medium (Pribluda and Metzger, 1987; Stump *et al.*, 1987; Millard *et al.*, 1989). Both phases of the Ca^{2+} response are necessary for the release of inflammatory mediators by degranulation (Fewtrell and Metzger, 1981; Stump *et al.*, 1987).

RBL-2H3 cells contain receptors for inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and they respond to FcεR1 cross-linking by increased phosphatidylinositol-specific phospholipase C activity and Ins(1,4,5)P_3 synthe-

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[1] Abbreviations used: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Ca_o^{2+}, extracellular Ca^{2+}; DNP, dinitrophenol; FcεR1, the high affinity receptor for IgE; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; MEM, minimal essential medium; RBL-2H3, the 2H3 secreting subline of rat basophilic leukemia (RBL) cells; SERCA, the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase.

sis (Cunha-Melo *et al.*, 1987; Pribluda and Metzger, 1987; Meyer *et al.*, 1988; Deanin *et al.*, 1991b). It is therefore likely that the antigen-stimulated release of stored Ca^{2+} occurs, at least in part, from Ins(1,4,5)P_3-sensitive Ca^{2+} stores. As is the case in other nonexcitable cells, stimulated Ca^{2+} influx in RBL-2H3 cells is not well understood at present. There is experimental evidence for the dependence of antigen-stimulated Ca^{2+} influx on G protein activity (Narasimhan *et al.*, 1988; Wilson *et al.*, 1989) and a negative membrane potential (Kanner and Metzger, 1984; Mohr and Fewtrell, 1987; Millard *et al.*, 1989). Inhibition of Ca^{2+} influx has been shown after treatment with metal ions (La^{3+}, Zn^{2+}, and Mn^{2+}), phorbol esters (TPA and PMA), and tenidap, an anti-inflammatory compound (Sagi-Eisenberg *et al.*, 1985; Cunha-Melo *et al.*, 1989; Hide and Beaven, 1991; Cleveland *et al.*, 1993). However, the membrane protein (or proteins) responsible for antigen-stimulated Ca^{2+} influx remains elusive.

Capacitative Ca^{2+} entry, Ca^{2+} influx that occurs in response to depletion of intracellular Ca^{2+} stores (Putney, 1986), has been described in a number of cell types (Takemura *et al.*, 1989; Hoth and Penner, 1992; Lee *et al.*, 1993). Recent studies suggest that this pathway exists in RBL-2H3 cells. Thus, thapsigargin, an inhibitor of the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} into intracellular stores (Inesi and Sagara, 1992), causes sustained increases in $[Ca^{2+}]_i$ by activating capacitative Ca^{2+} entry in suspensions of RBL-2H3 cells (Wong *et al.*, 1991; Cleveland *et al.*, 1993). In addition, Fasolato *et al.* (1993) demonstrated a Ca^{2+} release-activated Ca^{2+} current in patch-clamped RBL-2H3 cells and provided evidence that a G protein is involved in the coupling of empty Ca^{2+} stores to Ca^{2+} influx.

The first examination of antigen-stimulated $[Ca^{2+}]_i$ changes in individual RBL-2H3 cells by Millard *et al.* (1988) showed that responses in single cells can be quite different from the average Ca^{2+} responses measured in cell populations. Lag times from antigen addition to Ca^{2+} response were found to vary widely from cell to cell and it was noted that the increase in $[Ca^{2+}]_i$ in a single cell could occur much more rapidly than was apparent in population responses. In a subsequent study in which ratioed images were acquired in less than 1 s, compared with the 8–10 s time resolution of the previous work, Millard *et al.* (1989) were able to demonstrate that antigen stimulation of RBL-2H3 cells can cause oscillations in $[Ca^{2+}]_i$ in some cells. These oscillations were not abolished by membrane depolarization or removal of Ca_o^{2+}, and thus appeared to be due to periodic Ca^{2+} release from intracellular stores.

To better characterize the antigen-stimulated Ca^{2+} response, particularly at low concentrations of antigen, we have examined the antigen concentration dependence of the stimulated Ca^{2+} response in single RBL-2H3 cells incubated in both the presence and the absence of Ca_o^{2+}. Our data confirm that Ca^{2+} stores release occurs at the beginning of the sustained Ca^{2+} response. They also reveal several new aspects of Ca^{2+} regulation in activated RBL-2H3 cells, including evidence that extracellular Ca^{2+} influences Ca^{2+} stores release and evidence that Ca^{2+} influx may be mediated by both receptor-activated and capacitative Ca^{2+} influx pathways in RBL-2H3 cells. Our results provide the first evidence that Ca^{2+} uptake into stores may be regulated by receptor-activated pathways.

Results of these studies have previously been published in abstract form (Lee and Oliver, 1993a; Lee and Oliver, 1993b).

MATERIALS AND METHODS

Materials

Fura-2AM, Pluronic F-127, and DNP_{25}-conjugated bovine serum albumin (DNP-BSA) were purchased from Molecular Probes (Eugene, OR). Minimal essential medium (MEM) and streptolysin O were purchased from Life Technologies (Grand Island, NY). Dinitrophenyl (DNP)-specific IgE was purified from mouse ascites containing the H1-DNP-ε-26–82 hybridoma (Liu *et al.*, 1980). Thapsigargin and all other reagents were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture, Activation, and Fura-2–loading

RBL-2H3 cells were grown on bacterial grade plates in MEM supplemented with 15% fetal calf serum, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 2 mM L-glutamine. Plates were incubated at 37°C in an atmosphere of 5% CO_2. Cells were primed with 1–2 μg/ml monoclonal DNP-specific IgE (anti-DNP IgE) at least 2 h before an experiment and were activated during experiments by the addition of DNP-BSA. The acetoxy-methylester form of fura-2 (fura-2AM) was dissolved in a 20% weight/weight solution of pluronic acid (Pluronic F-127) in Me_2SO, added to cells in MEM to a final dye concentration of 2 μM, and the mixture was incubated at room temperature (RT) for 25–30 min. These loading conditions led to a uniform cytoplasmic fluorescence with less than 12% of fura-2 fluorescence present in intracellular compartments, as determined by assays of fluorochrome release after streptolysin O permeabilization. For both dye-loading and experiments, cells were incubated in a Leiden coverslip dish/microincubator (Medical Systems, Greenvale, NY) in an atmosphere of 5% CO_2/95% air. Experiments were run at 35°C in Hanks'-BSA solution modified from Becker (25) and containing (in mM): NaCl, 125; KCl, 5; Na_2HPO_4, 0.7; KH_2PO_4, 0.7; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 10; $NaHCO_3$, 15; glucose, 5.5; $MgSO_4$, 0.75; $CaCl_2$, 1.8; and 0.05% BSA. In nominally Ca^{2+}-free Hanks'-BSA, $CaCl_2$ was omitted.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in individual, fura-2AM–loaded RBL-2H3 cells using fluorescence ratio imaging microscopy. Cells were observed with a Zeiss IM35 inverted fluorescence microscope equipped with computer-controlled filter wheels and shutters (CRG Precision Electronics, Houston, TX). Excitation light from a Hg arc lamp was alternately passed through one of two 10-nm bandpass filters centered at 360 and 385 nm. Emitted fluorescence was collected with a 510 nm WB 40 filter (all filters, Omega Optical, Brattleboro, VT). Fluorescence images were acquired with a Photometrics Series 200 CCD array camera (Tucson, AZ) using camera exposure

times of 0.2–0.5 s. A Microvax II computer controlled the filter wheel movement and image acquisition during experiments.

Solutions were exchanged using a two-syringe device that simultaneously added and removed medium. Complete exchange of the 5 ml volume in the Leiden coverslip dish required 45 ml of medium and took approximately 30 s. Rates of solution exchange were determined for each solution change and used to determine the time point at which the solution being added had displaced the dead volume and entered the coverslip dish (generally 2 s after the start of the solution exchange). This time point was used as the time of solution exchange or stimulus addition. Culture medium and extracellular fura-2 were exchanged for Hanks'-BSA solution just before beginning an experiment.

After experiments, background images acquired at 360 and 385 nm excitation were subtracted from fluorescence images taken during an experiment and 360/385 ratio images were calculated on a pixel-by-pixel basis. Time resolution of the ratioed images was 2–5 s. Average ratio values for each cell in a field were calculated for a user-defined area within each cell. All software used for image acquisition and analysis was written in our laboratory. Average ratio values were converted to $[Ca^{2+}]$ as described in Grynkiewicz *et al.* (1985) using ratio values measured on calibration solutions containing maximal Ca^{2+} (2 mM) and minimal Ca^{2+} (2 mM EGTA), and a K_d for fura-2 and Ca^{2+} of 155 nM (determined using the Ca^{2+} Calibration Buffer Kit, Molecular Probes). Results are reported as $[Ca^{2+}]_i$ versus time. For determining the percentage of responding cells and lag times to response (Tables 1 and 2), an individual cell was considered to have given a Ca^{2+} spike response if the rate of increase in $[Ca^{2+}]_i$ was 9 nM/s or more and the $[Ca^{2+}]_i$ increased to at least 1.8 times its initial level.

Measurement of Secretion

RBL-2H3 cells were plated into 24-well culture dishes at a density of 2×10^5 cells/well. Sixteen to twenty-four hours before an experiment, 0.4 μCi/ml [^{3}H]serotonin (5-hydroxytryptamine, DuPont-NEN, Boston, MA) was added to each well and cells were primed with 1 μg/ml anti-DNP IgE. To assay secretion, medium was aspirated, wells were washed twice with Ca^{2+}-containing Hanks'-BSA, and 205 μl of warmed Hanks'-BSA containing either no additions (for measurement of spontaneous secretion) or the indicated concentrations of DNP-BSA, was added to each well. Plates were incubated at 37°C under 5% CO_2 for 20 min. To halt secretion, plates were chilled in a tray of ice and 0.2 ml of cold phosphate-buffered saline (PBS) was added to each well. [^{3}H]serotonin released into the supernatant was measured by liquid scintillation counting of 0.3 ml aliquots of the supernatants from each well. Percent serotonin release was calculated based upon duplicate wells lysed with 1% Triton X-100 and was corrected for blanks taken before the 20-min incubation. Measurements were performed in duplicate.

Measurement of Ins(1,4,5)P_3

IgE-primed cells (20×10^6 cells/ml) were washed once and resuspended in either Ca^{2+}-containing or nominally Ca^{2+}-free Hanks'-BSA solution and 0.4 ml aliquots were activated by the addition of 100 ng/ml DNP-BSA. Ins(1,4,5)P_3 levels were determined using the competitive binding assay described by Deanin *et al.* (1991a).

RESULTS

The Antigen Concentration Dependence of Ca^{2+} Responses Measured in the Presence of Ca_o^{2+}

Figure 1 illustrates the typical Ca^{2+} responses of individual, fura-2–loaded RBL-2H3 cells stimulated with increasing concentrations of DNP-BSA in the presence of Ca_o^{2+} (Protocol I). The average lag time from antigen addition to Ca^{2+} response and the number of cells used to determine lag time at each concentration of antigen are documented in Table 1 (Protocol I). The percent responding cells and the total numbers of cells observed at low antigen concentrations are given in Table 2 (Protocol I). As described in MATERIALS AND METHODS, the appearance of a Ca^{2+} spike or an abrupt increase in $[Ca^{2+}]_i$ was taken as evidence that a cell had responded to stimulation, and the onset of the spike was used to calculate a lag time for the response. Later, we will discuss the small elevation in $[Ca^{2+}]_i$ that precedes the Ca^{2+} spike response in some cells.

There is marked heterogeneity in the appearance and magnitude of the response, and in the lag time to response, particularly at the lowest antigen concentrations (0.01 and 0.1 ng/ml DNP-BSA). Despite this heterogeneity, the magnitude of the Ca^{2+} responses generally increased (Figure 1) whereas the average lag times decreased (Table 1), with increasing concentrations of antigen. The standard deviations of the average lag times also decreased with increasing antigen concentration, indicating less cell-to-cell heterogeneity at higher doses of antigen. Millard *et al.* (1988) reported a similar decrease in lag time and in the standard deviation of the lag time in RBL-2H3 cells stimulated with increasing concentrations of antigen in the presence of Ca_o^{2+}.

Addition of the lowest antigen concentrations (0.01 and 0.1 ng/ml DNP-BSA) activated Ca^{2+} responses in only a fraction of the observed cells (Table 2, Protocol I) and these responses frequently consisted of Ca^{2+} spikes superimposed on only a small increase in baseline $[Ca^{2+}]_i$ (Figure 1, A and B). At antigen concentrations of 1 ng/ml DNP-BSA and higher, Ca^{2+} responses occurred in all cells (Table 2). Ca^{2+} responses to stimulation with 1 ng/ml DNP-BSA typically consisted of Ca^{2+} oscillations superimposed on a significantly elevated baseline (Figure 1C) whereas responses to activation with concentrations of 10 ng/ml DNP-BSA and higher were characterized by an abrupt elevation in $[Ca^{2+}]_i$ that was sustained or decreased slowly over time (Figure 1, D–F).

Secretory responses in adherent populations of RBL-2H3 cells parallelled single-cell Ca^{2+} responses, with antigen concentrations of 10–100 ng/ml DNP-BSA eliciting the largest Ca^{2+} responses (Figure 1, D and E) and maximal secretion (Figure 2; Oliver *et al.*, 1988; Seagrave and Oliver, 1990).

The Antigen Concentration Dependence of Ca^{2+} Responses Measured in the Absence of Ca_o^{2+}

Figure 3 illustrates typical single-cell Ca^{2+} responses in experiments using a protocol patterned after the Ca^{2+}-free/Ca^{2+}-reintroduction protocol described by Grohovaz *et al.* (1991). Baseline $[Ca^{2+}]_i$ was estab-

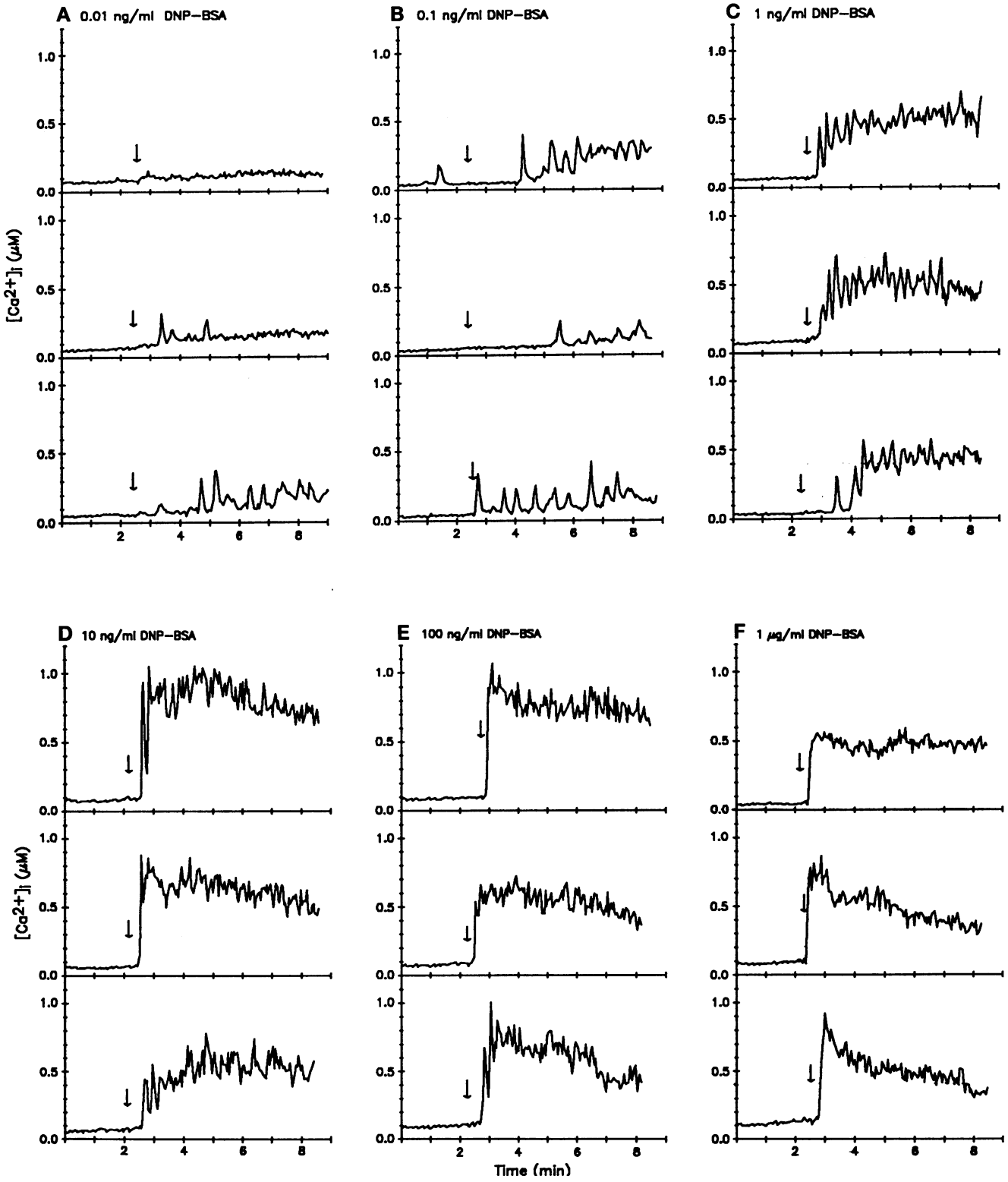

Figure 1. The antigen concentration dependence of Ca^{2+} responses in RBL-2H3 cells stimulated in the presence of Ca_o^{2+} (Protocol I). $[Ca^{2+}]_i$ was measured in individual cells as described in MATERIALS AND METHODS. Cells were activated (arrows) with the indicated concentrations of antigen (DNP-BSA, A–F). Cells were also activated with 5 μg/ml DNP-BSA and responses similar to those illustrated in F were observed. Antigen was present for the duration of the experiment.

Table 1. Comparison of average lag times to Ca^{2+} response measured in the presence (Protocol I) and absence (Protocol II) of Ca_o^{2+}

	Protocol I		Protocol II	
Antigen (ng/ml)	n	Lag time s (SD) + Ca_o^{2+}	n	Lag time s (SD) ± Ca_o^{2+}
0.01	12	106 (125)	10	130 (88)
0.1	18	109 (94)	20	171 (122)
1	23	48 (47)	27	103 (82)
10	29	28 (17)	25	53 (33)
100	48	25 (12)	30	30 (13)
1000	22	15 (5)	12	24 (8)
5000	24	17 (8)	13	13 (5)

Average lag times, in seconds, from antigen (DNP-BSA) addition to Ca^{2+} spike response were determined for each concentration of antigen and protocol. The number of cells used to calculate each average is given by n. The standard deviation of each average lag time is indicated in parentheses.

Table 2. Comparison of percent responding cells in the presence (Protocol I) and absence (Protocol II) of Ca_o^{2+}

	Protocol I		Protocol II	
Antigen (ng/ml)	n	% Responding cells + Ca_o^{2+}	n	% Responding cells $-Ca_o^{2+}$; $\pm Ca_o^{2+}$
0.01	29	41%	24	25%; 42%
0.1	22	82%	26	54%; 92%
1	24	100%	36	83%; 100%

The percentages of cells giving Ca^{2+} spike responses after the addition of 0.01, 0.1, and 1 ng/ml DNP-BSA are listed for protocols I and II. n indicates the number of cells observed under each condition. The first percentage listed under Protocol II is the fraction of cells responding in the absence of Ca_o^{2+}; the second, the fraction responding at any time during a Protocol II experiment.

lished first in the presence, and then the absence of Ca_o^{2+}. Antigen was added in the absence of Ca_o^{2+}, and stimulation was continued after the re-addition of Ca_o^{2+}. The number of cells examined at each antigen concentration, percent responding cells, and average lag times to response are given in Tables 1 and 2 (Protocol II). The appearance of a Ca^{2+} spike was again taken as evidence that a cell had responded to antigen stimulation.

When cells were activated in nominally Ca^{2+}-free medium, the average lag times to response and their standard deviations decreased (Table 1; Protocol II) and the percentage of cells showing Ca^{2+} spike responses increased (Table 2; Protocol II, first %) with increasing concentrations of antigen. All cells stimulated with DNP-BSA concentrations of 10 ng/ml or higher showed Ca^{2+} spike responses in the absence of Ca_o^{2+}.

In the absence of Ca_o^{2+}, the antigen-stimulated increase in $[Ca^{2+}]_i$ was due only to release of intracellular stores and was transient, returning within 2 min to a level that, after stimulation with higher concentrations of antigen (1 ng/ml DNP-BSA and greater), was approximately 20–50 nM higher than the prestimulation baseline (Figure 3, C–F). As was observed in the presence of Ca_o^{2+}, multiple oscillations in $[Ca^{2+}]_i$ were frequently seen after stimulation with low concentrations of antigen. In general, the oscillations observed in the absence of Ca_o^{2+} were characterized by more rapid rates of $[Ca^{2+}]_i$ increase and decrease and appeared to be more uniform than those seen in the presence of Ca_o^{2+}. As antigen concentration increased, the frequency of the oscillations increased while the number of oscillations decreased (Figure 3, C–F). At the highest concentrations of antigen, a single transient elevation in $[Ca^{2+}]_i$ was the typical response observed in the absence of Ca_o^{2+}. The multiple oscillations activated by low antigen (Figure 3, B–D) were characterized by rapid increases and decreases in $[Ca^{2+}]_i$. The rapid, rising phase of the Ca^{2+} spike is consistent with the positive feedback effect of released Ca^{2+} on the further release of Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1992; Parys *et al.*, 1992). The rapid decline presumably reflects the combined activities of the Ca^{2+} pumps responsible for removing Ca^{2+} from the cytosol by efflux and by re-uptake into Ca^{2+} stores (Tepikin *et al.*, 1992a,b). Although the Ca^{2+} transients stimulated by high concentrations of antigen showed the same rapid rate of increase as those stimulated by low antigen, the rate of $[Ca^{2+}]_i$ decrease from peak to baseline was slower after stimulation with high antigen (compare results in Figure 3, C and D with E and F). Later oscillations at a single antigen concentration also declined more slowly than the initial oscillations (Figure 3D).

In the absence of Ca_o^{2+}, oscillations eventually ceased. However, in the final phase of Protocol II experiments, the re-addition of Ca_o^{2+} always resulted in an immediate increase in $[Ca^{2+}]_i$ whose magnitude increased along with increasing antigen concentration. In some cells, returning Ca_o^{2+} to the medium also resulted in the resumption of oscillations (Figure 3C).

Figure 1 cont. $[Ca^{2+}]_i$ (μM) is plotted as a function of time (min) for three typical cells from at least two separate experiments at each concentration of antigen; 20–30 cells were observed at each concentration. The top cell in panel A did not show a Ca^{2+} spike response meeting the criteria defined in MATERIALS AND METHODS. The top cell in panel B showed a spontaneous oscillation in $[Ca^{2+}]_i$ before the addition of antigen. Spontaneous oscillations were observed in 10–15% of unstimulated cells. Because some spontaneous oscillations were observed in the absence of Ca_o^{2+} (see Figure 3, A, C, and D), they appeared to be due to Ca^{2+} release from and re-uptake into intracellular stores.

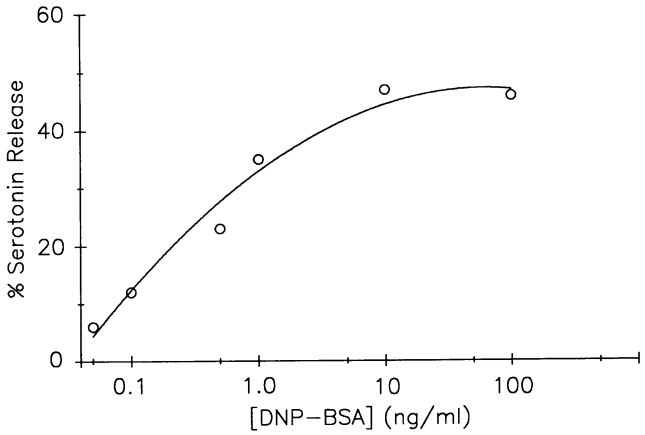

Figure 2. The antigen concentration dependence of secretion in RBL-2H3 cells. Secretion was assayed using adherent cells as described in MATERIALS AND METHODS and is reported as % release of total cellular [^{3}H]serotonin in 20 min at 37°C. Results are corrected for spontaneous secretion measured in parallel aliquots of unstimulated cells. Antigen concentrations are indicated. Data are averages of four separate experiments, each performed in duplicate.

Based on $[Ca^{2+}]_i$ measurements made in cell populations, previous investigators have concluded that the release of intracellular Ca^{2+} stores occurs at the beginning of the sustained Ca^{2+} response measured in the presence of Ca_o^{2+} (Albert and Tashjian, 1984). Our single cell measurements support this conclusion. The transient Ca^{2+} spike responses of cells stimulated in the absence of Ca_o^{2+} result from Ca^{2+} release from intracellular stores (Figure 3). Very similar Ca^{2+} spikes are the first response of many cells to stimulation with low antigen in the presence of Ca_o^{2+} (Figure 1, A and B). At higher antigen concentrations, the sustained Ca^{2+} response typically begins with one or two rapid oscillations that most likely result from the release of intracellular Ca^{2+} stores (Figure 1, C–E).

Extracellular Ca^{2+} Is Involved in Triggering Intracellular Ca^{2+} Responses in Antigen-stimulated RBL-2H3 Cells

Comparison of the percent responding cells and the average lag times to Ca^{2+} response of individual RBL-2H3 cells stimulated in the presence and absence of Ca_o^{2+} (Tables 1 and 2) revealed that extracellular Ca^{2+} is important in activating Ca^{2+} spike responses.

At the lowest concentrations of antigen, more cells gave Ca^{2+} responses in the presence of Ca_o^{2+} (Table 2, Protocol I) than in its absence (Table 2, Protocol II, first %). However, the percentage of cells showing Ca^{2+} responses at any time ± Ca_o^{2+}) during Protocol II experiments, the second percentage listed under Protocol II (Table 2), is comparable to the percentage of cells showing Ca^{2+} responses in the presence of Ca_o^{2+} (Protocol I) for a given antigen concentration. Thus, some cells that did not respond in the absence of Ca_o^{2+} did show Ca^{2+} spike responses once Ca_o^{2+} was returned to the medium. Three cells demonstrating this pattern of response are plotted in Figure 3, A and B. Two additional examples are given in Figure 4. In approximately 50% of cells (10 of 19) exhibiting stimulated Ca^{2+} responses after the re-addition of Ca_o^{2+}, the onset of the stimulated response was well correlated with the re-addition of Ca_o^{2+}, as illustrated in Figure 4. The cells plotted in Figure 4, A and B showed increases in $[Ca^{2+}]_i$ at 10 s and 4 s, respectively, after the re-addition of Ca_o^{2+}. These results suggest that Ca_o^{2+} is involved in triggering Ca^{2+} spike responses.

Similar to the effect of removing Ca_o^{2+}, the addition of Ni^{2+}, a Ca^{2+} influx blocker (Hide and Beaven, 1991), also reduced the percentage of cells showing Ca^{2+} spike responses after stimulation with a low concentration of antigen. Spike responses were observed in 44% of cells (15/34) treated with 5 mM $NiCl_2$ approximately 1 min before stimulation with 0.1 ng/ml DNP-BSA, whereas 82% of control cells stimulated with the same concentration of antigen gave Ca^{2+} spike responses (Table 2). These experiments were done in the presence of Ca_o^{2+}. Ca^{2+} spike responses in Ni^{2+}-containing medium were similar in appearance to spikes observed in the absence of Ca_o^{2+} after stimulation with low concentrations of antigen (Figure 3, A–C).

The average lag times measured in the presence of Ca_o^{2+} are shorter than those in its absence after stimulation with low concentrations of antigen (Table 1). Using the Wilcoxon Rank Sum Test, these differences were found to be significant at $p < 0.074$ for stimulation with 0.1 ng/ml, $p < 0.006$ at 1 ng/ml, and $p < 0.004$ at 10 ng/ml DNP-BSA. Although the average lag time after stimulation with 0.01 ng/ml DNP-BSA was longer in the absence of Ca_o^{2+} than in its presence, this difference was not significant, probably because cells were not observed for a long enough period of time to get a good estimate of the true average lag time at this low concentration of antigen. Because of uncertainties in the time of antigen addition and the initial Ca^{2+} response, it was not possible to conclude that differ-

Figure 3 (facing page). The antigen concentration dependence of Ca^{2+} responses in RBL-2H3 cells stimulated first in the absence, and then in the presence of Ca_o^{2+} (Protocol II). $[Ca^{2+}]_i$ was measured as described in MATERIALS AND METHODS. The indicated concentrations of antigen (A–F) were added at the arrows and were present for the duration of each experiment. Cells were also activated with 1 μg/ml DNP-BSA and responses similar to those illustrated in E and F were observed. Extracellular Ca^{2+} was removed from and returned to the medium by solution exchange at the times indicated by the bars beneath each graph.

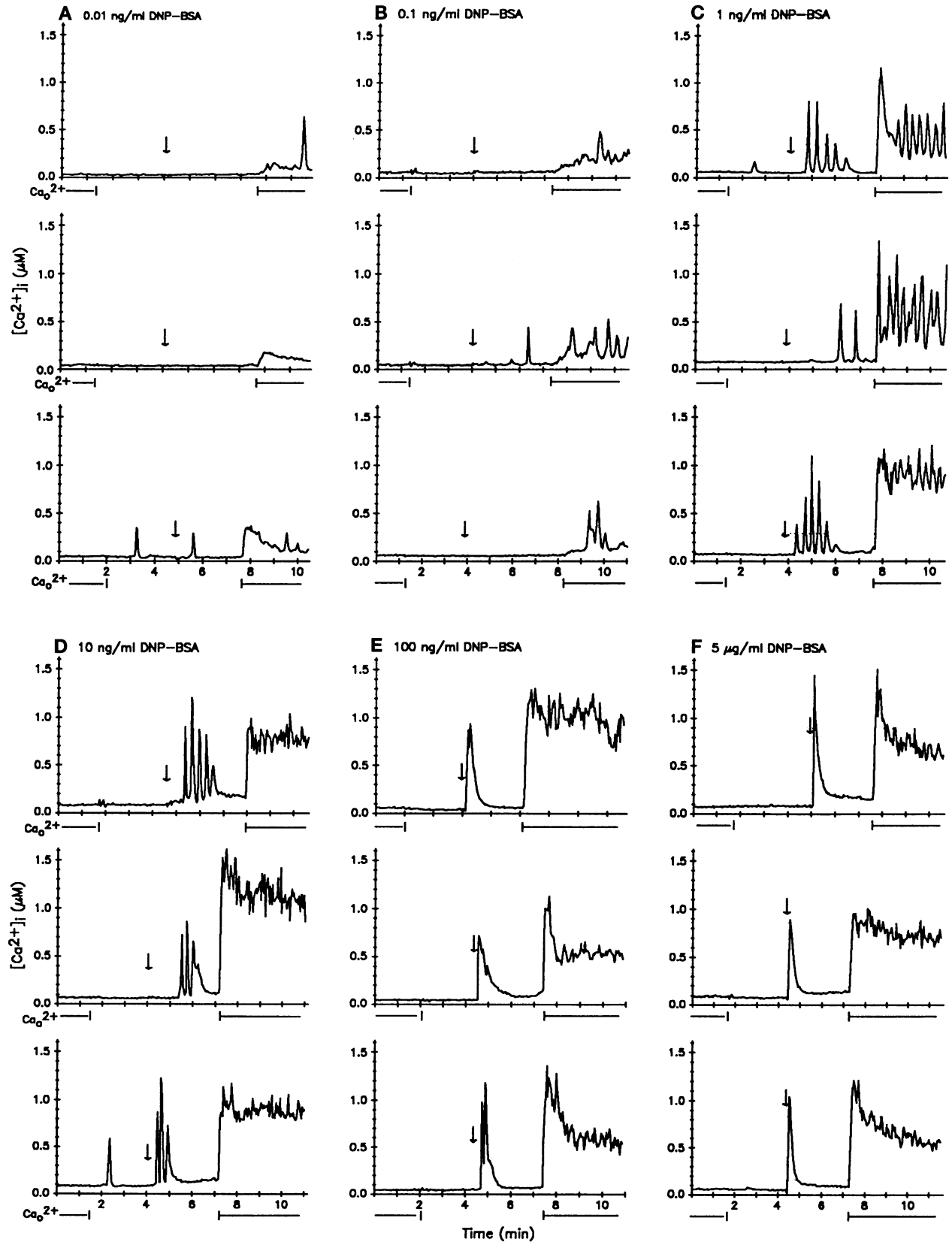
A 0.01 ng/ml DNP–BSA
B 0.1 ng/ml DNP–BSA
C 1 ng/ml DNP–BSA
D 10 ng/ml DNP–BSA
E 100 ng/ml DNP–BSA
F 5 μg/ml DNP–BSA
[Ca2+]i (μM)
Cao2+
Time (min)

Figure 3.

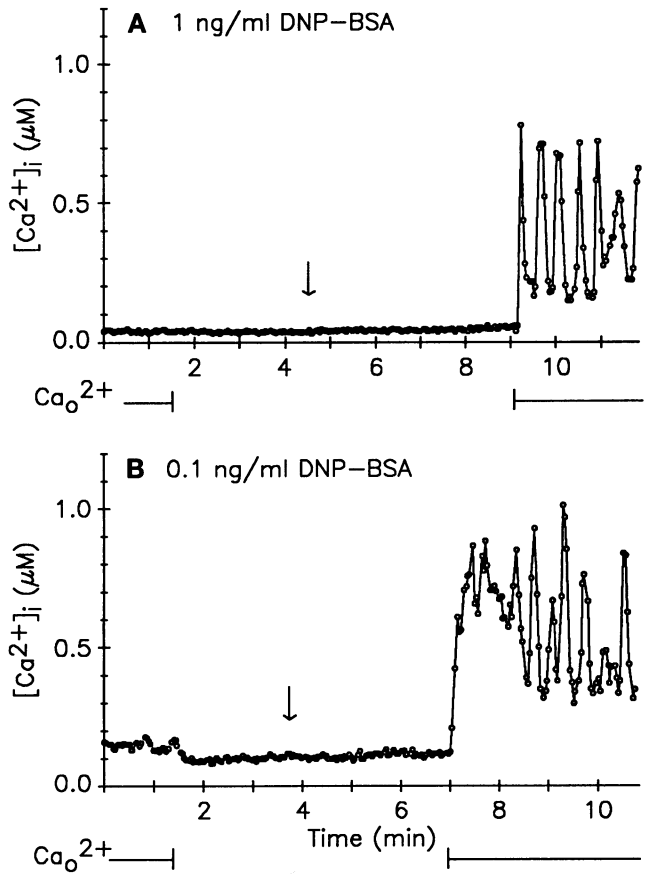

Figure 4. Re-addition of Ca_o^{2+} triggers Ca^{2+} responses in some cells that did not respond to stimulation in the absence of Ca_o^{2+}. Antigen, at the indicated concentrations, was added at the arrows and was present for the duration of the experiment. Extracellular Ca^{2+} was present as indicated below each graph. The cell plotted in B shows an unusually large decrease in $[Ca^{2+}]_i$ upon the removal of Ca_o^{2+}.

ences in average lag times between the two protocols were significant when those differences were smaller than 8–10 s (Table 1, compare lag times at 100 ng/ml, 1 μg/ml, and 5 μg/ml DNP-BSA).

Extracellular Ca^{2+} Is Not Required for Antigen-stimulated Production of Ins(1,4,5)P_3

One possible explanation for the effect of Ca_o^{2+} on the lag times to Ca^{2+} response and the percentage of responding cells is that Ins(1,4,5)P_3 production might be reduced in the absence of Ca_o^{2+}. If less Ins(1,4,5)P_3 were produced, lag times to release of Ins(1,4,5)P_3-sensitive Ca^{2+} stores would be longer, and the fraction of cells that could give a Ca^{2+} response to a suboptimal concentration of antigen might be reduced. Figure 5 shows that antigen-stimulated Ins(1,4,5)P_3 production is not inhibited in the absence of Ca_o^{2+}. Although this time course of Ins(1,4,5)P_3 production shows that the amount of Ins(1,4,5)P_3 produced in the absence of Ca_o^{2+} (dashed line) is elevated compared with control (solid line) at the early time points, this was true in only three of five experiments. In the other two experiments, Ins(1,4,5)P_3 levels were approximately the same in the presence and absence of Ca_o^{2+} for the first 5 min of stimulation.

Stimulation with Low Concentrations of Antigen Reveals a Ca^{2+} Influx that Precedes Ca^{2+} Stores Release in some Cells

In the presence of Ca_o^{2+}, the Ca^{2+} response of some cells to low antigen had two components, the spike response discussed above that we attribute to Ca^{2+} release from and uptake into intracellular stores, and a more gradual elevation in $[Ca^{2+}]_i$ that is not observed in the absence of Ca_o^{2+} and is due, therefore, to Ca^{2+} influx. This response is illustrated in Figure 6. The cells plotted in Figure 6, A and B, show Ca^{2+} spikes superimposed on a gradual elevation in $[Ca^{2+}]_i$. This type of response was observed in 73% of cells stimulated with 0.01, 0.1, and 1 ng/ml DNP-BSA. In 19% of the same cells, a small sustained increase in $[Ca^{2+}]_i$ that began shortly after antigen addition was the only Ca^{2+} response observed (solid line, Figure 6C). In unstimulated control cells (n = 14), baseline $[Ca^{2+}]_i$ was generally steady until 5–7 min after the start of the experiment, at which point it increased at a rate of 0.1 nM/s or less for the remainder of the experiment (dashed line, Figure 6C). There was intercellular heterogeneity in both the time of onset of the increase and its rate. In unstimulated controls, the increase in

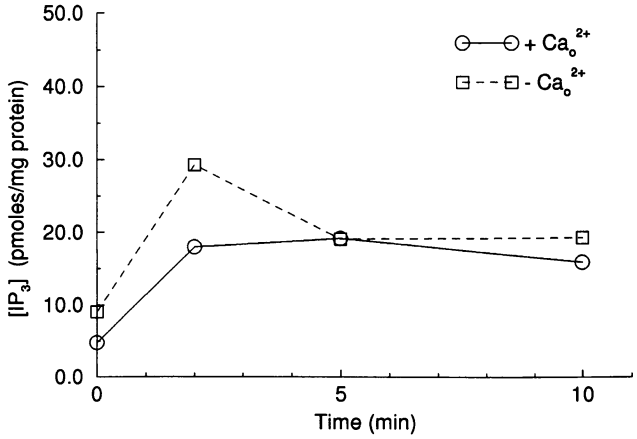

Figure 5. Ins(1,4,5)P_3 production in the presence and absence of Ca_o^{2+}. RBL-2H3 cells were stimulated at t = 0 with 100 ng/ml DNP-BSA and the amount of Ins(1,4,5)P_3 produced at the indicated times in either the presence (circles, solid line) or the absence (squares, dashed line) of Ca_o^{2+} was measured as described in MATERIALS AND METHODS. Each data point is the average of duplicate samples. Four replicate experiments gave similar results (see text).

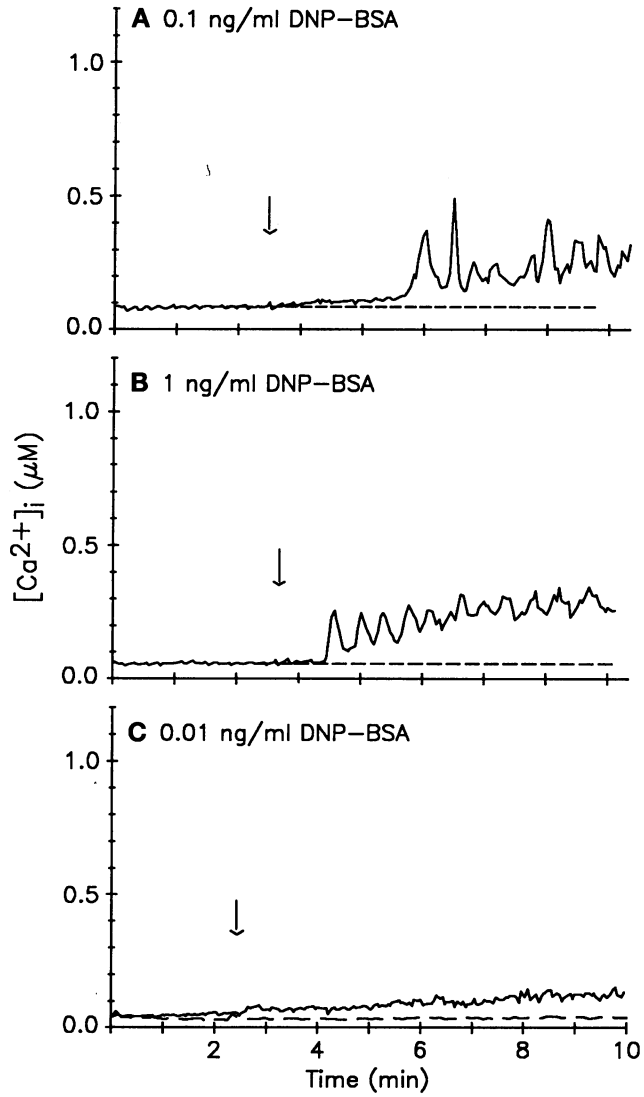

Figure 6. Ca^{2+} responses to activation with low concentrations of antigen in the presence of Ca_o^{2+}. Antigen, at the indicated concentrations, was added at the arrows and maintained in the medium for the duration of each experiment. (A and B) The dashed line extends the pre-stimulation Ca^{2+} baseline into the period after antigen addition. (C) The dashed line is a plot of $[Ca^{2+}]_i$ over time for an unstimulated control cell. The Ca^{2+} response illustrated in panel A, consisting of a small increase in $[Ca^{2+}]_i$ that precedes the first Ca^{2+} spike, was observed in 30% of cells stimulated in the presence of Ca_o^{2+} with 0.01, 0.1, or 1 ng/ml DNP-BSA. In 43% of these cells, no baseline increase in $[Ca^{2+}]_i$ was detected until after the first Ca^{2+} spike (B). In 19% of the cells, a small increase in the $[Ca^{2+}]_i$ baseline, whose onset was well-correlated with antigen addition, was the only Ca^{2+} response observed (C, solid line).

$[Ca^{2+}]_i$ over baseline during 10 min of observation ranged from 5–25 nM. In the experiments represented by the remaining plots in Figure 6, antigen addition occurred at approximately 2.5 min. In the 19% of cells that showed the small, gradual increase response (solid line, Figure 6C), the average rate of increase was 0.3 nM/s and its onset was well-correlated with antigen addition, becoming apparent within 30–60 s. In the remaining 8% of cells stimulated with the three lowest concentrations of antigen, no stimulated Ca^{2+} response, either a Ca^{2+} spike or a gradual increase different from unstimulated controls, was apparent.

The 73% of total cells demonstrating Ca^{2+} spikes superimposed on a gradual elevation in $[Ca^{2+}]_i$ could be further subdivided into two groups. In 30% of total cells, a gradual increase in $[Ca^{2+}]_i$ preceded the first spike response. The cell in Figure 6A illustrates this response, showing an increase in $[Ca^{2+}]_i$ that is sustained for 140 s before the first Ca^{2+} spike and that reached approximately 60 nM above baseline concentration just before the spike. For all cells in which a small increase in $[Ca^{2+}]_i$ was apparent before the first Ca^{2+} spike, the average $[Ca^{2+}]_i$ increase just before the spike was 45 nM, and the average lag time between antigen addition and the first spike response was 115 s. In 43% of total cells, the gradual $[Ca^{2+}]_i$ increase was not apparent until after the first Ca^{2+} spike. The cell in Figure 6B illustrates this response. Because a gradual elevation in $[Ca^{2+}]_i$ that preceded the Ca^{2+} spike response was not observed in the absence of Ca_o^{2+}, this elevation was apparently due to Ca^{2+} influx and suggests that antigen-stimulated Ca^{2+} influx can occur before release of intracellular Ca^{2+} stores. We hypothesize that this small influx of Ca_o^{2+} is directly activated by antigen. Its function may be to facilitate Ca^{2+} stores release, at least when antigen concentrations are low.

Capacitative Ca^{2+} Entry Occurs in RBL-2H3 Cells

The existence of a mechanism for capacitative Ca^{2+} entry in RBL-2H3 cells was confirmed using thapsigargin, an inhibitor of SERCA, the Ca^{2+}-ATPase responsible for Ca^{2+} uptake into intracellular stores (Takemura *et al.*, 1989; Inesi and Sagara, 1992). Under resting conditions, Ca^{2+} continually "leaks" from intracellular stores and is returned to the stores by SERCA activity. Inhibition of SERCA by thapsigargin gradually empties intracellular Ca^{2+} stores as Ca^{2+} leaks from them and is not pumped back in. Capacitative Ca^{2+} entry occurs in response to the depletion of Ca^{2+} stores. In RBL-2H3 cells incubated according to Protocol II, thapsigargin addition in the absence of Ca_o^{2+} led to a slow increase and decrease in $[Ca^{2+}]_i$. After the re-addition of Ca_o^{2+}, $[Ca^{2+}]_i$ increased rapidly due to capacitative Ca^{2+} influx (Figure 7A).

Further evidence for a mechanism of capacitative Ca^{2+} entry in RBL-2H3 cells is given in Figure 7, B and C. Here, cells were simply incubated in the absence of Ca_o^{2+} and not stimulated in any way. Removal of Ca_o^{2+} did not cause a dramatic change in $[Ca^{2+}]_i$, although decreases of 10–20 nM were common. Re-addition of Ca_o^{2+}, however, led to an immediate increase in $[Ca^{2+}]_i$

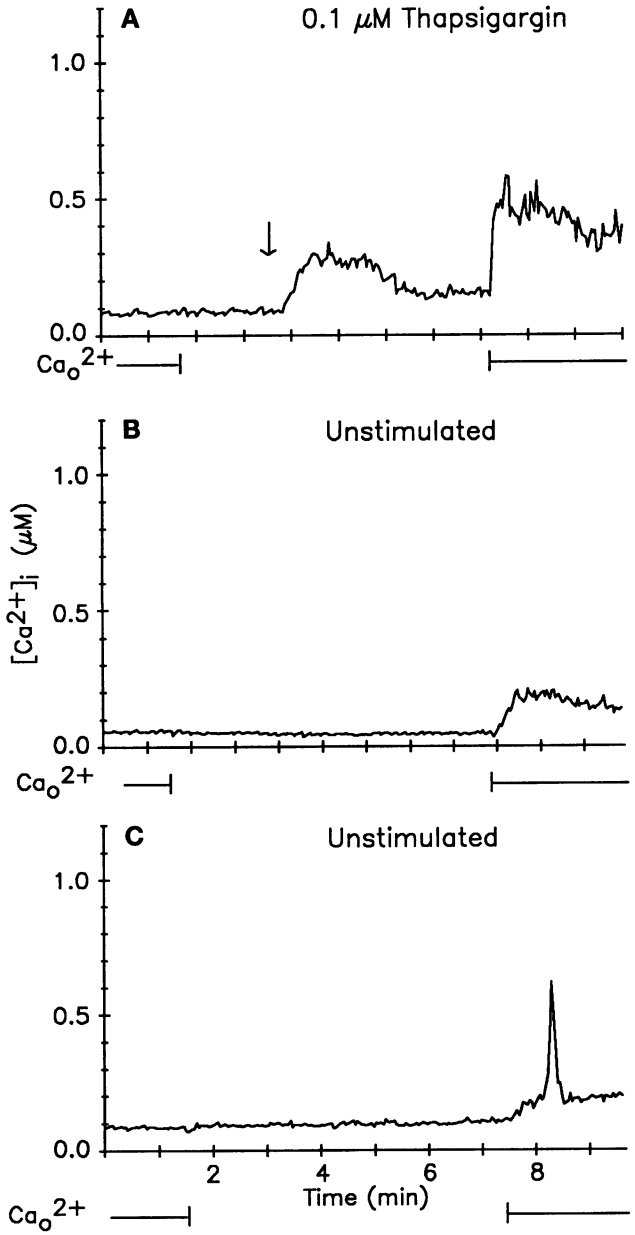

Figure 7. Capacitative Ca^{2+} entry occurs in RBL-2H3 cells. Cells were incubated with 0.1 μM thapsigargin (A) or with no addition (B and C) according to Protocol II; Ca_o^{2+} was present as indicated under each graph. Thapsigargin addition (arrow, A) led to a transient elevation in $[Ca^{2+}]_i$ in the absence of Ca_o^{2+}, and a sustained elevation in $[Ca^{2+}]_i$ after the re-introduction of Ca_o^{2+}. Simply returning Ca_o^{2+} to the medium after a 5–7 min incubation in its absence (B and C) was sufficient to trigger an elevation in $[Ca^{2+}]_i$ in 95% of cells. This Ca^{2+} elevation led to a Ca^{2+} spike response in approximately 25% of cells (C).

in 95% of the cells (40 of 42 cells). The magnitude of this increase varied from cell to cell, but was generally smaller than the increases that were observed after re-addition of Ca_o^{2+} to stimulated cells (Figure 3, A–F). In approximately 25% of the cells, a Ca^{2+} spike response was observed after the re-addition of Ca_o^{2+} (Figure 7C). We suppose that cells incubated in the absence of Ca_o^{2+} use stored Ca^{2+} to help maintain a steady $[Ca^{2+}]_i$, depleting their Ca^{2+} stores in the process. Once Ca_o^{2+} is returned to the medium, the partially depleted stores are able to activate Ca^{2+} influx via a capacitative mechanism.

Low Antigen plus Thapsigargin Induces Ca^{2+} and Secretory Responses Resembling those Activated by High Antigen

We showed in Figure 3, C and D that cells in nominally Ca^{2+}-free medium respond to low antigen concentrations with multiple Ca^{2+} oscillations characterized by rapid rates of increase and decrease. Activation with higher concentrations of antigen caused an increase in the frequency and reduction in the number of oscillations observed (Figure 3E). Stimulation with the highest concentrations of antigen produced single Ca^{2+} transients that showed the same rapid increase in $[Ca^{2+}]_i$ as those activated by lower antigen, but a slower rate of $[Ca^{2+}]_i$ decrease (Figure 3F). To explain the transition from multiple oscillations to single Ca^{2+} transients, we hypothesized that high antigen concentrations may inhibit the refilling of intracellular Ca^{2+} stores. Inhibition of Ca^{2+} store refilling would inhibit Ca^{2+} oscillations by blocking repeated cycles of release and re-uptake and would also reduce the rate of $[Ca^{2+}]_i$ decrease from a spike response by eliminating one pathway for Ca^{2+} removal from the cytoplasm.

To test this hypothesis, cells were activated simultaneously with thapsigargin and 1 ng/ml DNP-BSA in the absence of Ca_o^{2+} (Figure 8). No Ca^{2+} oscillations were observed in 15 of 15 cells, confirming that the multiple oscillations usually observed at this antigen concentration (Figure 3C) require Ca^{2+} reuptake into intracellular stores. Instead, approximately 40% of cells gave a Ca^{2+} response that consisted of a single Ca^{2+} spike (Figure 8A) comparable to Ca^{2+} responses after high concentrations of antigen (Figure 3, E and F). In these cells, antigen stimulation was apparently able to elicit a substantial release of stored Ca^{2+} before it had been depleted by thapsigargin, but the presence of thapsigargin prevented Ca^{2+} re-uptake necessary for a second Ca^{2+} spike. Another 25% of cells showed a Ca^{2+} response consisting of an initial slow increase followed by a spike response, suggesting that thapsigargin was able to cause some release of stored Ca^{2+} before antigen took effect (Figure 8B). The remaining 35% of cells gave just a slow increase and decrease in $[Ca^{2+}]_i$ (Figure 8C), resembling the response to thapsigargin alone (Figure 7A). These

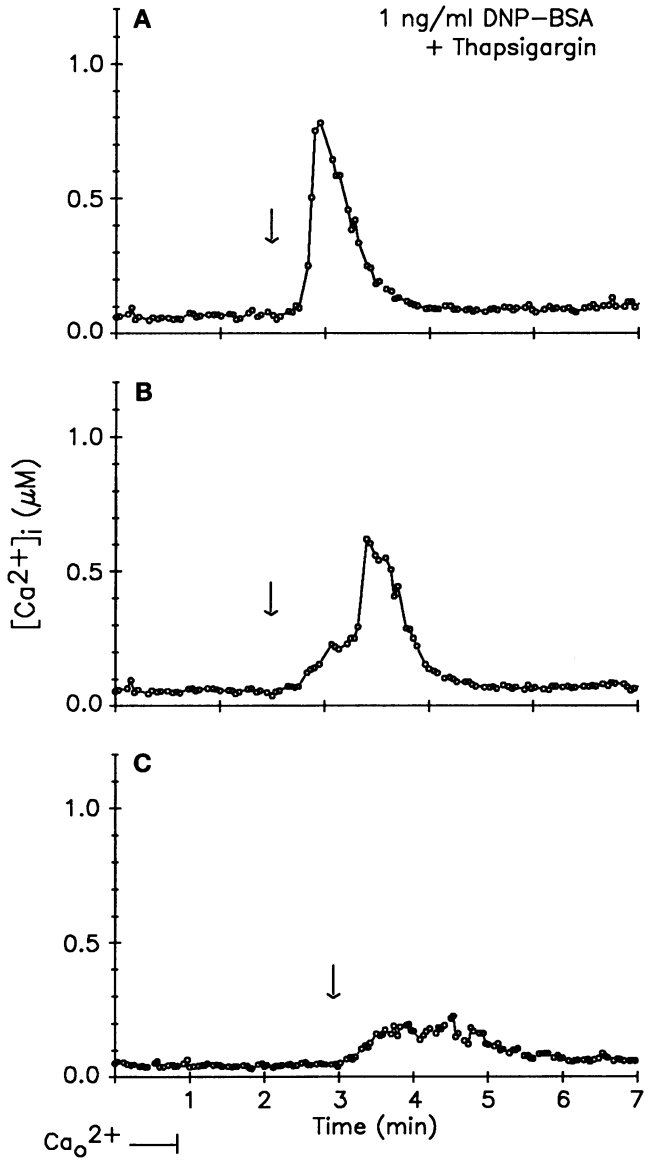

Figure 8. The addition of thapsigargin to cells stimulated with low antigen elicits Ca^{2+} responses comparable to those activated by high concentrations of antigen. The cells plotted in panels A–C demonstrate the types of Ca^{2+} response observed when RBL-2H3 cells were activated with low antigen (1 ng/ml DNP-BSA) and thapsigargin (0.1 μM) together, in Ca^{2+}-free medium. The cells plotted in panels A and B were from the same experiment, performed in the absence of Ca_o^{2+}. The cell plotted in panel C was observed initially in the presence of Ca_o^{2+}; solution exchange to Ca^{2+}-free medium was made at the time indicated under the graph.

results establish that thapsigargin, a known inhibitor of SERCA, in combination with a low concentration of antigen, is able in some cells to produce Ca^{2+} responses that are comparable to those activated by high concentrations of antigen alone.

Parallel secretion assays (Figure 9) showed that thapsigargin alone and low antigen alone both induce a small secretory response. By inhibiting Ca^{2+} uptake into intracellular stores, thapsigargin potentiated the sub-optimal secretory response produced by 1 ng/ml DNP-BSA, eliciting the same percentage secretion as was measured in response to 100 ng/ml DNP-BSA. These results suggest that activating capacitative Ca^{2+} influx can provide the high $[Ca^{2+}]_i$ needed for optimum secretion, and support our hypothesis that inhibition of Ca^{2+} uptake into intracellular stores may be a mechanism by which antigen-stimulated cells maintain an elevated $[Ca^{2+}]_i$.

DISCUSSION

Cross-linking the FcεR1 on RBL-2H3 cells leads to a sustained elevation in $[Ca^{2+}]_i$ that is critical for secretion. To dissect the antigen-stimulated Ca^{2+} responses of RBL-2H3 cells, we have examined these responses in single cells activated with a wide range of antigen concentrations in both the presence and absence of Ca_o^{2+}. Observing changes in $[Ca^{2+}]_i$ in single cells allowed us to determine the percentages of responding cells, the lag times to response, and the nature of the responses in individual cells. Our results show that as antigen concentration increases toward the optimum for secretion, the percentage of cells showing Ca^{2+} stores release increases and the delay to the onset of Ca^{2+} responses decreases. They confirm previous evidence that Ca^{2+} stores release occurs at the beginning of the sustained Ca^{2+} response. They reveal several new aspects of Ca^{2+} regulation in activated RBL-2H3 cells, including evidence that extracellular

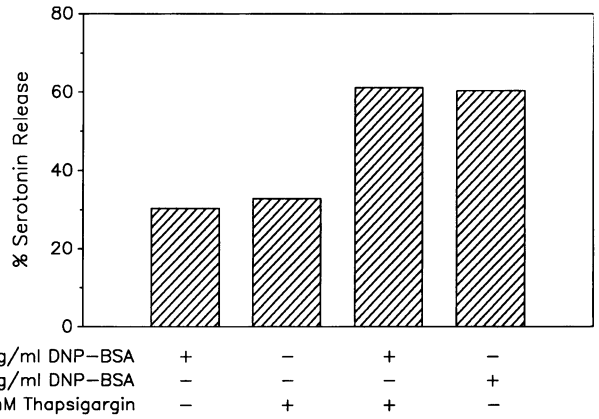

Figure 9. Thapsigargin addition potentiates secretion stimulated by low antigen. Secretion was determined from the % release of [^{3}H]serotonin during a 20 min assay after the addition of the indicated stimuli. Results, corrected for spontaneous secretion, are the average of duplicate (antigen alone and thapsigargin alone) or quadruplicate (antigen and thapsigargin together) measurements from a representative experiment.

Ca^{2+} is involved in triggering the Ca^{2+} stores release response and evidence that Ca^{2+} influx may be mediated by multiple pathways in RBL-2H3 cells.

A role for extracellular Ca^{2+} in activating the release of intracellular Ca^{2+} stores was indicated by the greater percentage of responding cells and the shorter lag times to the Ca^{2+} stores release response measured in cells activated in medium containing Ca^{2+} compared with cells activated in nominally Ca^{2+}-free medium. A reduced percentage of responding cells was also observed in the presence of extracellular Ni^{2+}, and suggests that influx of Ca_o^{2+} is involved in triggering the release of Ca^{2+} stores. Although the secretory response is completely inhibited in Ca^{2+}-free medium, the pathway from receptor cross-linking to F-actin assembly, membrane ruffling, and spreading is intact, demonstrating that removing Ca_o^{2+} does not have a general deleterious effect on transmembrane signalling (Pfeiffer *et al.*, 1985). Although there is substantial evidence that Ca_o^{2+} is involved both in maintaining and setting the frequency of Ca^{2+} oscillations (Harootunian *et al.*, 1988; Jacob *et al.*, 1988; Friel and Tsien, 1992), there is little experimental evidence, particularly in nonexcitable cells, that Ca_o^{2+} is involved in triggering stores release (oscillations) in the first place. Previous evidence for the latter comes from stopped-flow fluorimeter measurements of $[Ca^{2+}]_i$ in populations of platelets. After stimulation with thrombin, vasopressin, platelet-activating factor, and ADP, the lag times from agonist addition to Ca^{2+} response were longer in the absence of Ca_o^{2+} than in its presence (Sage and Rink, 1987). Subsequent studies in ADP-stimulated platelets showed that the initial $[Ca^{2+}]_i$ increase was comprised of Ca^{2+} influx, and suggested that this increase was important in activating the subsequent release of intracellular Ca^{2+} stores (Sage *et al.*, 1990).

We report here that levels of $Ins(1,4,5)P_3$ are comparable in cells activated in both the presence and absence of Ca_o^{2+}. These measurements, made with an $Ins(1,4,5)P_3$-specific receptor assay, demonstrate that removal of Ca_o^{2+} does not affect the activation of phosphatidylinositol-specific phospholipase Cγ (PtdIns-specific PLCγ), the enzyme implicated in antigen-stimulated $Ins(1,4,5)P_3$ production in RBL-2H3 cells (Park *et al.*, 1991; Li *et al.*, 1992; Beaven and Metzger, 1993). Previous studies indicated that the DNP-BSA-induced production of total inositol phosphates (Cunha-Melo *et al.*, 1987) and $InsP_3$ (containing both the 1,3,4 and the 1,4,5 isomers) (Pribluda and Metzger, 1987) was reduced in cells stimulated in the absence of Ca_o^{2+}. $Ins(1,4,5)P_3$ comprises only a small fraction of the total inositol phosphates released after stimulation of RBL-2H3 cells (Cunha-Melo *et al.*, 1987). Because multiple inositol phosphate species were included in the measurements made in these previous studies, it is not possible to conclude from them that the removal of Ca_o^{2+} inhibits the production of the specific isomer, $Ins(1,4,5)P_3$. It may also be that Ca_o^{2+} removal did inhibit $Ins(1,4,5)P_3$ production in these studies, but that different experimental conditions can explain our apparently contradictory results. The Ca^{2+}-free medium used in both our Ca^{2+} experiments and $Ins(1,4,5)P_3$ assays contained no added Ca^{2+} or EGTA. Solution exchange to Ca^{2+}-free medium either had no effect on $[Ca^{2+}]_i$ or led to a small decrease (Figure 3). Rhee and Choi (1992) have reported that Ca^{2+} is required for the activity of PtdIns-specific PLCγ. It is possible that the presence of EGTA in the Ca^{2+}-free medium used in both of the previous studies lowered $[Ca^{2+}]_i$ sufficiently that PtdIns-specific PLCγ activation and subsequent $Ins(1,4,5)P_3$ production were inhibited. We conclude that, in our experiments, the same amount of $Ins(1,4,5)P_3$ is present after antigen stimulation in the presence and the absence of Ca_o^{2+}. The longer lag times to release of Ca^{2+} stores and the reduced percentage of responding cells observed in the absence of Ca_o^{2+} must be explained by something other than a reduction in stimulated $Ins(1,4,5)P_3$ levels.

We hypothesize that removing Ca_o^{2+} or blocking Ca^{2+} entry impairs Ca^{2+} stores release in part by reducing the open probability of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel. There are at least two ways in which this might occur. A number of studies have demonstrated that Ca^{2+} can modulate the opening of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel. For a fixed concentration of $Ins(1,4,5)P_3$, increases in $[Ca^{2+}]$ to 300–500 nM promote opening of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel, whereas higher Ca^{2+} concentrations inhibit Ca^{2+} release from stores (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Parys *et al.*, 1992). Removing extracellular Ca^{2+} may impair intracellular stores release simply by lowering $[Ca^{2+}]_i$. In approximately 50% of cells observed in Protocol II experiments, removal of Ca_o^{2+} led to a small (10–20 nM) decrease in $[Ca^{2+}]_i$. This decrease may be sufficient to delay or inhibit the opening of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel at a given, low concentration of $Ins(1,4,5)P_3$. The Ca^{2+} content of the intracellular stores has also been shown to affect the open probability of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel, under certain conditions. Specifically, stores that contain low levels of Ca^{2+} show a much greater enhancement of Ca^{2+} release by increases in $[Ca^{2+}]_i$ than do stores that are maximally loaded (Missiaen *et al.*, 1994). Our data suggest that incubation in the absence of Ca_o^{2+}, even for short periods, can partially deplete intracellular Ca^{2+} stores (Figure 7, B and C). A reduced Ca^{2+} content of the stores would decrease the probability that the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel would be open for a given $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ level.

We also propose that extracellular Ca^{2+} may play a more active role in antigen-induced release of Ca^{2+}

stores through activated influx that can precede and facilitate stores release. The existence of this influx pathway was inferred from evidence that approximately 30% of the cells stimulated with low concentrations of antigen in the presence of Ca_o^{2+} show a gradual increase in $[Ca^{2+}]_i$ that precedes the Ca^{2+} spike response. This increase is not observed in Ca^{2+}-free medium, indicating that it results from Ca^{2+} influx. We propose that this influx is directly stimulated by IgE receptor cross-linking, although we cannot rule out the possibility that it may be due to capacitative entry activated by the emptying of a small pool of intracellular Ca^{2+} that was not detected in our measurements. To our knowledge, this is the first time measurements of $[Ca^{2+}]_i$ in single cells have suggested that Ca^{2+} influx can precede release of intracellular stores. As discussed previously, Ca^{2+} influx that precedes release from intracellular stores has been measured in populations of platelets (Sage *et al.*, 1990). In the generalized model for Ca^{2+}-induced Ca^{2+} release from either ryanodine- or $Ins(1,4,5)P_3$-sensitive intracellular stores proposed by Berridge (1993), Ca^{2+} influx that precedes release from intracellular stores is important both in triggering release by binding to the stores release channel and in filling the stores before release. The data presented here are consistent with this model.

Although we do not conclusively demonstrate the cause of the extracellular Ca^{2+} effect on the release of intracellular stores, our data support the idea that the open probability of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel is reduced when Ca_o^{2+} is removed. The $[Ca^{2+}]_i$, the filling state of intracellular Ca^{2+} stores, and the presence or absence of stimulated influx that can precede stores release may all contribute to the activated Ca^{2+} stores release response.

Additional results reported here indicate that capacitative Ca^{2+} entry secondary to antigen-mediated Ca^{2+} stores depletion contributes importantly to the Ca^{2+} influx necessary for secretion. Studies in RBL-2H3 cells depleted of Ca^{2+} stores by thapsigargin treatment and by incubation in nominally Ca^{2+}-free medium established that Ca^{2+} stores depletion results in Ca^{2+} influx by a capacitative pathway. Subsequent analyses suggested that high antigen concentrations are able to prevent the refilling of Ca^{2+} stores after their $Ins(1,4,5)P_3$-mediated release. Thus, cells respond to low antigen concentrations with multiple Ca^{2+} oscillations that are characterized by rapid increases and decreases in $[Ca^{2+}]_i$ and reflect the combined activities of Ca^{2+} stores release, uptake, and efflux pathways. They respond to higher antigen concentrations with fewer, and frequently single, elevations in $[Ca^{2+}]_i$ whose rates of decrease are significantly slower than those activated by lower concentrations of antigen. We interpret these results as evidence that high antigen inhibits Ca^{2+} stores refilling, thereby eliminating one mechanism of Ca^{2+} removal from the cytoplasm, and interrupting the cycle of Ca^{2+} release and re-uptake that produces multiple oscillations. Consistent with this interpretation, it was shown that thapsigargin-induced inhibition of stores refilling in combination with a low concentration of antigen produced transient Ca^{2+} responses that were comparable to those induced by optimal antigen in a significant fraction of cells. Finally, we found that low antigen plus thapsigargin induced the same level of secretion as optimal antigen when cells were stimulated in the presence of Ca_o^{2+}, indicating that capacitative Ca^{2+} entry secondary to Ca^{2+} stores depletion can contribute to the secretory response. Previously, Byron *et al.* (1992) demonstrated in bradykinin-stimulated fibroblasts that intracellular Ca^{2+} stores are emptied and do not refill in the presence of agonist, despite the presence of high levels of both intra- and extracellular Ca^{2+}. The similarity between these results and our current data suggests that the agonist-induced inhibition of Ca^{2+} stores refilling may be a general phenomenon.

The refilling of Ca^{2+} stores could be inhibited if the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel were continuously open in cells treated with optimal antigen. Although some studies suggest that the $Ins(1,4,5)P_3$ receptor is maintained in an open state in the continuous presence of high $Ins(1,4,5)P_3$, there is also evidence that, in the presence of high levels of intracellular Ca^{2+}, this is not necessarily the case. Thus, Nakade *et al.* (1991) have shown in isolated microsomes that the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel does not appear to desensitize to repeated applications of the same or higher concentrations of $Ins(1,4,5)P_3$. This result was confirmed in intact HeLa cells stimulated by increasing concentrations of histamine (Bootman *et al.*, 1994). On the other hand, it has also been demonstrated that concentrations of $[Ca^{2+}]$ above 250 nM reversibly inhibit $Ins(1,4,5)P_3$ receptor/Ca^{2+} channels reconstituted into planar lipid bilayers (Bezprozvanny *et al.*, 1991). The studies demonstrating maintained receptor sensitivity to repeated $Ins(1,4,5)P_3$ challenge were conducted in an vitro system in which Ca^{2+} was maintained at a low level (Nakade *et al.*, 1991), or in intact cells in which the stimulus was removed and $[Ca^{2+}]_i$ was allowed to decrease before the next application of agonist (Bootman *et al.*, 1994). In intact cells in which stimulation in the presence of Ca_o^{2+} leads to a maintained elevation in $[Ca^{2+}]_i$, it seems quite possible that the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel may be inactivated by the high level of Ca^{2+}. Additionally, recent work in permeabilized hepatocytes has shown that, after Ca^{2+} stores release, $Ins(1,4,5)P_3$ itself causes a time-dependent, although not complete, inactivation of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel that is potentiated by increases in $[Ca^{2+}]_i$ (Hajnoczky and Thomas, 1994). At present, it is not clear whether or not

$Ins(1,4,5)P_3$ receptors remain open in intact cells in the presence of high concentrations of both $Ins(1,4,5)P_3$ and Ca^{2+}.

Alternatively, the refilling of Ca^{2+} stores may be blocked in antigen-stimulated RBL-2H3 cells by the antigen dose-dependent inhibition of SERCA, two isotypes of which have recently been shown to be expressed in rat mucosal mast cells (Wuytack *et al.*, 1994). This hypothesis assumes that SERCA inhibition can occur in two ways, as a pharmacological response to thapsigargin and, we are suggesting, as a physiological response to optimal antigen. Support for this hypothesis comes from measurements of Ca^{2+}-activated Cl^- current in pancreatic acinar cells. Petersen *et al.* (1993) demonstrated that inhibition of SERCA with low concentrations of thapsigargin could increase both the spiking frequency and the duration of a single current spike, results that are consistent with our observations that the frequency of Ca^{2+} oscillations and the duration of the Ca^{2+} transient both increased with increasing concentrations of antigen. In addition, glucagon stimulation of rat hepatocytes has been shown to inhibit the activity of a related enzyme, the plasma membrane Ca^{2+}-ATPase, via a G_s-dependent pathway (Lotersztajn *et al.*, 1990; Jouneaux *et al.*, 1993).

In summary, our data confirm that the marked increase in Ca^{2+} levels in antigen-activated RBL-2H3 cells is initiated by Ca^{2+} stores release and maintained by Ca^{2+} influx. They provide substantial evidence that extracellular Ca^{2+} modulates Ca^{2+} stores release in intact cells, perhaps via an antigen-induced influx of Ca_o^{2+}. The immediate onset of antigen-activated Ca^{2+} influx in some cells suggests that this Ca^{2+} entry mechanism is closely coupled to the cross-linked IgE receptor complexes and can be activated before intracellular Ca^{2+} stores release. They suggest that a previously undescribed response of RBL-2H3 cells to FcεR1 cross-linking, the inhibition of Ca^{2+} stores refilling, supports capacitative Ca^{2+} influx by maintaining Ca^{2+} stores in an empty state after activation with high concentrations of antigen. We hypothesize that capacitative Ca^{2+} entry may contribute importantly to the sustained elevation of $[Ca^{2+}]_i$ that is necessary for secretion.

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REFERENCES

Albert, R.A., and Tashjian, A.H., Jr. (1984). Relationship of thyrotropin-releasing hormone-induced spike and plateau phases in cytosolic free Ca^{2+} concentrations to hormone secretion. J. Biol. Chem. *259*, 15350–15363.

Beaven, M.A., and Metzger, H. (1993). Signal transduction by Fc receptors: the FcεR1 case. Immunol. Today *14*, 222–226.

Becker, E.L. (1972). The relationship of the chemotactic behavior of the complement-derived factors C_3A, C_5A and C567, and a bacterial chemotactic factor to their ability to activate the proesterase of rabbit polymorphonuclear leukocytes. J. Exp. Med. *135*, 376–387.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature *361*, 315–325.

Bezprozvanny, I., Watras, J., and Ehrlich, B.E. (1991). Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$ and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751–754.

Bootman, M.D., Cheek, T.R., Moreton, R.B., Bennett, D.L., and Berridge, M.J. (1994). Smoothly graded Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. J. Biol. Chem. *269*, 24783–24791.

Byron, K.L., Babnigg, G., and Villereal, M.L. (1992). Bradykinin-induced Ca^{2+} entry, release and refilling of intracellular Ca^{2+} stores: relationships revealed by image analysis of individual human fibroblasts. J. Biol. Chem. *267*, 108–118.

Cleveland, P.L., Millard, P.J., Showell, H.J., and Fewtrell, C.M.S. (1993). Tenidap: a novel inhibitor of calcium influx in a mast cell line. Cell Calcium *14*, 1–16.

Cunha-Melo, J.R., Dean, N.M., Moyer, J.D., Maeyama, K., and Beaven, M.A. (1987). The kinetics of phosphoinositide hydrolysis in rat basophilic leukemia (RBL-2H3) cells varies with the type of IgE cross-linking agent used. J. Biol. Chem. *262*, 11455–11463.

Cunha-Melo, J.R., Gonzaga, H.M.S., Ali, H., Huang, R.L., Huang, K.-P., and Beaven, M.A. (1989). Studies of protein kinase C in the rat basophilic leukemia (RBL-2H3) cell reveal that antigen-induced signals are not mimicked by the actions of phorbol myristate acetate and Ca^{2+} ionophore. J. Immunol. *143*, 2617–2625.

Deanin, G.G., Cutts, J.L., Pfeiffer, J.R., and Oliver, J.M. (1991a). Role of isoprenoid metabolism in IgE receptor-mediated signal transduction. J. Immunol. *146*, 3528–3535.

Deanin, G.G., Martinez, A.M., Pfeiffer, J.R., Gardner, M.E., and Oliver, J.M. (1991b). Tyrosine kinase-dependent phosphatidylinositol turnover and functional responses in the FcεR1 signalling pathway. Biochem. Biophys. Res. Comm. *179*, 551–557.

Fasolato, C., Hoth, M., and Penner, R. (1993). A GTP-dependent step in the activation mechanism of capacitative calcium influx. J. Biol. Chem. *268*, 20737–20740.

Fewtrell, C., Lagunoff, D., and Metzger, H. (1981). Secretion from rat basophilic leukaemia cells induced by calcium ionophores: effect of pH and metabolic inhibition. Biochem. Biophys. Acta *644*, 363–368.

Finch, E.A., Turner, T.J., and Goldin, S.M. (1991). Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. Science *252*, 443–448.

Friel, D.D., and Tsien, R.W. (1992). Phase-dependent contributions from Ca^{2+} entry and Ca^{2+} release to caffeine-induced $[Ca^{2+}]_i$ oscillations in bullfrog sympathetic neurons. Neuron *8*, 1109–1125.

Grohovaz, F., Zacchetti, D., Clementi, E., Lorenzon, P., Meldolesi, J., and Fumagalli, G. (1991). $[Ca^{2+}]_i$ imaging in PC12 cells: multiple response patterns to receptor activation reveal new aspects of transmembrane signaling. J. Cell Biol. *113*, 1341–1350.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440–3450.

Hajnoczky, G., and Thomas, A.P. (1994). The inositol trisphosphate calcium channel is inactivated by inositol trisphosphate. Nature *340*, 474–477.

Harootunian, A.T., Kao, J.P.Y., and Tsien, R.Y. (1988). Agonist-induced calcium oscillations in depolarized fibroblasts and their manipulation by photoreleased $Ins(1,4,5)P_3$, Ca^{2+}, and Ca^{2+} buffer. Cold Spring Harbor Symp. Quant. Biol. *53*, 935–943.

Hide, M., and Beaven, M.A. (1991). Calcium influx in a rat mast cell (RBL-2H3) line: use of multivalent metal ions to define its characteristics and role in exocytosis. J. Biol. Chem. *266*, 15221–15229.

Hoth, M., and Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature *355*, 353–355.

Inesi, G., and Sagara, Y. (1992). Thapsigargin, a high affinity and global inhibitor of intracellular Ca^{2+} transport ATPases. Arch. Biochem. Biophys. *298*, 313–317.

Jacob, R., Merritt, J.E., Hallam, T.J., and Rink, T.J. (1988). Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. Nature *335*, 40–45.

Jouneaux, D., Audigier, Y., Goldsmith, P., Pecker, F., and Lotersztajn, S. (1993). G_s mediates hormonal inhibition of the calcium pump in liver plasma membranes. J. Biol. Chem. *268*, 2368–2372.

Kanner, B.I., and Metzger, H. (1984). Initial characterization of the calcium channel activated by the cross-linking of the receptors for immunoglobulin E. J. Biol. Chem. *259*, 10188–10193.

Lee, K.-M., Toscas, K., and Villereal, M.L. (1993). Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. J. Biol. Chem. *268*, 9945–9948.

Lee, R.J., and Oliver, J.M. (1993a). Single-cell $[Ca^{2+}]_i$ measurements reveal a complex relationship between Ca^{2+} stores release and influx in activated RBL-2H3 cells. J. Immunol. *150*, 221a.

Lee, R.J., and Oliver, J.M. (1993b). Antigen concentration dependence studies of Ca^{2+} responses in RBL-2H3 cells: new aspects of the relationship between Ca^{2+} influx and release from intracellular stores and effects of stimulation on re-uptake into stores. Mol. Biol. Cell *4*, Supplement 132a.

Li, W., Deanin, G.G., Margolis, B., Schlessinger, J., and Oliver, J.M. (1992). FcεR1-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor $\beta\gamma_2$ complex, in RBL-2H3 rat basophilic leukemia cells. Mol. Cell. Biol. *12*, 3176–3182.

Liu, R.-T., Bohn, J.W., Fenry, E.L., Yanamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R., and Katz, D.H. (1980). Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. J. Immunol. *124*, 2728–2736.

Lotersztajn, S., Pavoine, C., Brechler, V., Roche, B., Dufour, M., Le-Nguyen, D., Bataille, D., and Pecker, F. (1990). Glucagon-(19–29) exerts a biphasic action on the liver plasma membrane Ca^{2+} pump which is mediated by G proteins. J. Biol. Chem. *265*, 9876–9880.

Meyer, T., Holowka, D., and Stryer, L. (1988). Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. Science *240*, 653–656.

Millard, P.J., Gross, D., Webb, W.W., and Fewtrell, C. (1988). Imaging asynchronous changes in intracellular Ca^{2+} in individual stimulated tumor mast cells. Proc. Natl. Acad. Sci. USA *85*, 1854–1858.

Millard, P.J., Ryan, T.A., Webb, W.W., and Fewtrell, C. (1989). Immunoglobulin E receptor cross-linking induces oscillations in intracellular free ionized calcium in individual tumor mast cells. J. Biol. Chem. *264*, 19730–19739.

Missiaen, L., De Smedt, H., Parys, J.B., and Casteels, R. (1994). Co-activation of inositol trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+} is loading-dependent. J. Biol. Chem. *269*, 7238–7242.

Mohr, F.C., and Fewtrell, C. (1987). Depolarization of rat basophilic leukemia cells inhibits calcium uptake and exocytosis. J. Cell Biol. *104*, 783–792.

Nakade, S., Maeda, N., and Mikoshiba, K. (1991). Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. Biochem. J. *277*, 125–131.

Narasimhan, V., Holowka, D., Fewtrell, C., and Baird, B. (1988). Cholera toxin increases the rate of antigen-stimulated calcium influx in rat basophilic leukemia cells. J. Biol. Chem. *263*, 19626–19632.

Oliver, J.M., Seagrave, J.C., Stump, R.F., Pfeiffer, J.R., and Deanin, G.G. (1988). Signal transduction and cellular response in RBL-2H3 mast cells. In: Progress in Allergy, ed. E.L. Becker, Basel, Switzerland: S. Karger, 185–245.

Park, D.J., Min, H.K., and Rhee, S.G. (1991). IgE-induced tyrosine phosphorylation of phospholipase C-γ1 in rat basophilic leukemia cells. J. Biol. Chem. *266*, 24237–24240.

Parys, J.B., Sernett, S.W., Delisle, W., Snyder, P.M., Welsh, M.J., and Campbell, K.P. (1992). Isolation, characterization, and localization of the inositol 1,4,5-trisphosphate receptor protein in *Xenopus laevis* oocytes. J. Biol. Chem. *267*, 18776–18782.

Petersen, C.C.H., Petersen, O.H., and Berridge, M.J. (1993). The role of endoplasmic reticulum calcium pumps during cytosolic calcium spiking in pancreatic acinar cells. J. Biol. Chem. *268*, 22262–22264.

Pfeiffer, J.R., Seagrave, J.C., Davis, B.H., Deanin, G.G., and Oliver, J.M. (1985). Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. J. Cell Biol. *101*, 2145–2155.

Pribluda, V.S., and Metzger, H. (1987). Evidence for an early rise in inositol 1,4,5-trisphosphate which precedes the rise in other inositol phosphates and in cytoplasmic calcium. J. Biol. Chem. *262*, 11449–11454.

Putney, J.W., Jr. (1986). A model for receptor-regulated calcium entry. Cell Calcium *7*, 1–12.

Rhee, S.G., and Choi, K.D. (1992). Studies of inositol phospholipid-specific phospholipase C. J. Biol. Chem. *267*, 12393–12396.

Sage, S.O., Reast, R., and Rink, T.J. (1990). ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets: evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store. Biochem J. *265*, 675–680.

Sage, S.O., and Rink, T.J. (1987). The kinetics of changes in intracellular calcium concentration in fura-2–loaded human platelets. J. Biol. Chem. *259*, 15350–15363.

Sagi-Eisenberg, R., Lieman, H., and Pecht, I. (1985). Protein kinase C regulation of the receptor-coupled calcium signal in histamine-secreting rat basophilic leukaemia cells. Nature *313*, 59–60.

Seagrave, J.C., and Oliver, J.M. (1990). Antigen-dependent transition of IgE to a detergent-insoluble form is associated with reduced IgE receptor-dependent secretion from RBL-2H3 mast cells. J. Cell Physiol. *144*, 128–136.

Stump, R.F., Oliver, J.M., Cragoe, E.J., Jr., and Deanin, G.G. (1987). The control of mediator release from RBL-2H3 cells: roles for Ca^{2+}, Na^+, and protein kinase C. J. Immunol. *139*, 881–886.

Takemura, H., Hughes, A.R., Thastrup, O., and Putney, J.W., Jr. (1989). Activation of calcium entry by the tumor promotor thapsigargin in parotid acinar cells. J. Biol. Chem. *264*, 12266–12271.

Tepikin, A.V., Voronina, S.G., Gallacher, D.V., and Petersen, O.H. (1992a). Acetylcholine-evoked increase in the cytoplasmic Ca^{2+} concentration and Ca^{2+} extrusion measured simultaneously in single mouse pancreatic acinar cells. J. Biol. Chem. *267*, 3569–3572.

Tepikin, A.V., Voronina, S.G., Gallacher, D.V., and Petersen, O.H. (1992b). Pulsatile Ca^{2+} extrusion from single pancreatic acinar cells during receptor-activated cystolic Ca^{2+} spiking. J. Biol. Chem. *267*, 14073–14076.

Wilson, B.S., Deanin, G.G., Standefer, J.C., Vanderjagt, D., and Oliver, J.M. (1989). Depletion of guanine nucleotides with mycophenolic acid supresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. J. Immunol. *143*, 259–265.

Wong, A., Cook, M.N., Foley, J.J., Sarau, H.M., Marshall, P., and Hwang, S.M. (1991). Influx of extracellular calcium is required for the membrane translocation of 5-lipoxygenase and leukotriene synthesis. Biochemistry *30*, 9346–9354.

Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K.S., and Casteels, R. (1994). A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. J. Biol. Chem. *269*, 1410–1416.