Antigen and thapsigargin promote influx of Ca^{2+} in rat basophilic RBL-2H3 cells by ostensibly similar mechanisms that allow filling of inositol 1,4,5-trisphosphate-sensitive and mitochondrial Ca^{2+} stores

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In single, Fura 2-loaded RBL-2H3 cells, antigen and thapsigargin depleted the same intracellular pool of Ca^{2+} in the absence of external Ca^{2+}; provision of external Ca^{2+} induced immediate increases in levels of free Ca^{2+} ($[Ca^{2+}]_i$). These increases were dependent on the presence of external Ca^{2+} and, presumably, on influx of Ca^{2+} across the cell membrane. Both stimulants enhanced intracellular accumulation of $^{45}Ca^{2+}$ through ostensibly similar mechanisms because accumulation was blocked to similar extents by various multivalent cations or by depolarization with K^+. Because thapsigargin blocked reuptake of Ca^{2+} into inositol 1,4,5-trisphosphate sensitive stores, uptake occurred independently of the refilling of these stores. Uptake was dependent instead on sequestration of $^{45}Ca^{2+}$ in a pool of high capacity that was insensitive to thapsigargin, caffeine, GTP and inositol 1,4,5-trisphosphate but sensitive to ionomycin and mitochondrial inhibitors. The existence of an inositol 1,4,5-trisphosphate-insensitive pool was also apparent in permeabilized cells; at 0.1 μM $[Ca^{2+}]_i$, uptake of $^{45}Ca^{2+}$ was largely confined (> 80%) to the inositol 1,4,5-trisphosphate-sensitive pool, but at 2 μM $[Ca^{2+}]_i$ uptake was largely (> 60%) into the inositol 1,4,5-trisphosphate-insensitive pool. Provision of mitochondrial inhibitors along with thapsigargin to block uptake into both pools, did not impair the thapsigargin-induced increase in $[Ca^{2+}]_i$ or influx of Ca^{2+}, as indicated by changes in Fura 2 fluorescence, but did block the intracellular accumulation of $^{45}Ca^{2+}$. The studies illustrate the utility of simultaneous measurements of $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake for a full accounting of Ca^{2+} homoeostasis as exemplified by the ability to distinguish between influx and mitochondrial uptake of Ca^{2+}.

INTRODUCTION

The secretion of granules from antigen-stimulated mast cells, basophils and related cultured cell lines is dependent on release of intracellular Ca^{2+}, influx of external Ca^{2+} and an elevation in concentration of free Ca^{2+} in cytosol or medium $[Ca^{2+}]_i$ [1]. These events have been studied in most detail in RBL-2H3 cells, a tumour analogue of rat mucosal mast cells [2–4], in which antigen stimulation causes a transient increase in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} [5,6] and a more sustained increase in $[Ca^{2+}]_i$ [5,7,8] in the presence of external Ca^{2+}. These increases occur abruptly after a delay of 50–100 s [7] which has been attributed to the time required for the aggregation of IgE receptors and the subsequent activation of phospholipase Cγ by cytosolic tyrosine kinase(s) [9].

The transient increase in $[Ca^{2+}]_i$ can be attributed to release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate for the following reasons. First, the increase in $[Ca^{2+}]_i$ coincides with an early appearance of this inositol phosphate [10,11]. Secondly, Ca^{2+} is released from permeabilized RBL-2H3 cells by submicromolar amounts of inositol 1,4,5-trisphosphate in a co-operative fashion [12]. Thirdly, the increase is not observed when the intracellular stores are first depleted by carbachol in transfected RBL-2H3 cells that express muscarinic m1 receptors [6]; carbachol causes simultaneous increases in $[Ca^{2+}]_i$ and inositol 1,4,5-trisphosphate within seconds of addition of drug [13].

The sustained elevation in $[Ca^{2+}]_i$ in RBL-2H3 cells, as determined by use of Ca^{2+}-sensitive fluorescent probes, is totally dependent on influx of external Ca^{2+}. Blockade of Ca^{2+}-influx by use of EGTA or La^{3+} causes a rapid decline in $[Ca^{2+}]_i$ to basal levels and subsequent neutralization of these reagents rapidly restores $[Ca^{2+}]_i$ to its former elevated levels [8]. The increase in $[Ca^{2+}]_i$ is associated with an increase in total intracellular Ca^{2+} due, possibly, to uptake of cytosolic Ca^{2+} into mitochondrial stores when $[Ca^{2+}]_i$ is elevated above basal levels [14].

Influx of Ca^{2+} is also evident from an accelerated uptake of $^{45}Ca^{2+}$ by RBL-2H3 cells for several minutes after the addition of antigen [15–17]. Other cations impede Ca^{2+} uptake, either by blocking entry of Ca^{2+} at the cell surface, e.g. La^{3+} and Zn^{2+}, or by competing for Ca^{2+} entry into the cell, e.g. Sr^{2+}, Ba^{2+} and Mn^{2+} ([17] and references cited therein). The cation-influx pathway is apparently not voltage-activated [15,18] nor blocked by organic blockers of voltage-activated Ca^{2+} channels [19]. Indeed, entry of Ca^{2+} is suppressed when cells are depolarized with high concentrations of external K^+ [15,18] and a repolarizing current may be required to maintain influx [18,20–24].

Influx of Ca^{2+} into RBL-2H3 cells is reminiscent of that observed in other types of electrically non-excitable cells [13], including rat peritoneal mast cells [17], in which influx is closely associated with the emptying of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores [25,26], probably through the generation of a diffusible messenger molecule [27,28] from intracellular organelles. Influx is thought to occur through a low-conductance current, designated I_{crac} for 'calcium-release-activated calcium current', which has been characterized in mast cells [29] and RBL-2H3 cells [30]. This current appears to be highly selective

Abbreviations used: $[Ca^{2+}]_i$ or $[M^{n+}]$, concentration of free calcium or metal ion in cytosol or medium; $[Ca^{2+}]_m$, calculated concentration of calcium in the mitochondria; DNP–BSA, antigen consisting of 24 molecules of dinitrophenol conjugated with one molecule of BSA, DNP, *O*-dinitrophenol.

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for Ca^{2+} ions [29] and its activation is dependent on GTP, possibly acting via a low-molecular-mass G-protein [30,31]. To date, Ca^{2+} conductance, including I_{crac}, has been examined in mast cells by use of reagents such as GTP and the chemical secretagogue, compound 48/80, [29,30,32,33] but there is no reason to suspect that I_{crac} would not be activated in antigen-stimulated mast cells.

As part of a continuing study of mechanisms of Ca^{2+} homoeostasis in RBL-2H3 cells, we have compared antigen-induced influx with that induced by thapsigargin, which depletes the intracellular, inositol 1,4,5-trisphosphate-sensitive stores by inhibiting re-uptake of Ca^{2+} into these stores [31,34]. We found that thapsigargin not only stimulates uptake of Ca^{2+} through a non-selective pathway ostensibly identical to that noted above for antigen-stimulated RBL-2H3 cells, but that it also promotes sustained accumulation of $^{45}Ca^{2+}$ in mitochondrial-like stores. While this accumulation differs from that recently described in HeLa cells in which transient high elevations of Ca^{2+} are generated in mitochondrial domains ($[Ca^{2+}]_m$) that are believed to lie in close proximity to inositol 1,4,5-trisphosphate-sensitive channels [35], both studies implicate the mitochondrion as a regulator of Ca^{2+} homoeostasis in stimulated cells.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: calcium ionophore, A23187, and *N*-ethylcarboxamidoadenosine from Sigma, St. Louis, MI, U.S.A.; ionomycin from Calbiochem Corp., La Jolla, CA, U.S.A.; phorbol 12-myristate 13-acetate and thapsigargin from LC Services Corporation, Woburn, MA, U.S.A.; GTP and inositol 1,4,5-trisphosphate from Boehringer–Mannheim Biochemicals, Indianapolis, IN, U.S.A.; reduced streptolysin O from Burroughs Wellcome, Research Triangle Park, NC, U.S.A.; the fluorescent Ca^{2+} probes, quin 2 and Fura 2 from Molecular Probes, Eugene, OR, U.S.A.; radiolabelled compounds from Dupont–NEN, Boston, MA, U.S.A. The antigen, dinitrophenol (DNP)–BSA, and DNP-specific monoclonal IgE were kindly supplied by Dr. Henry Metzger (NIAMS, National Institutes of Health, Bethesda, MD, U.S.A.).

Stock solutions of drugs, A23187 and phorbol 12-myristate 13-acetate were prepared in dimethylsulphoxide. They were diluted directly into the buffer to give the required final concentration of drug and a concentration of dimethylsulphoxide of $< 0.1\%$ (v/v). Appropriate controls were prepared with vehicle. All other reagents were dissolved in the buffer alone.

Cell culture and experimental conditions

The cells were maintained in culture and plated in 24-well or 6-well plates in growth medium by use of previously described techniques [36]. Cultures in the multiwell plates contained DNP-specific IgE and, when required, [^{3}H]5-hydroxytryptamine to label intracellular granules and [^{14}C]arachidonic acid to label phospholipids [36,37]. Experiments were performed in medium that contained 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 5.6 mM glucose, 25 mM Pipes, 40 mM NaOH (to pH 7.2) and 1 mM Ca^{2+} (Ca^{2+}-containing medium) or one that contained 0.1 mM EGTA instead of Ca^{2+} (Ca^{2+}-free medium) [8]. When the concentration of KCl was varied, NaCl was added to give a final concentration of monovalent cations of 165 mM. For experiments with permeabilized cells, however, a potassium glutamate, Pipes-buffered medium was used. This medium contained 140 mM potassium glutamate, 5 mM ATP, 7 mM magnesium acetate, 5 mM glucose, 20 mM potassium Pipes, 1 mM EGTA and the calculated concentration of Ca^{2+} to give the desired concentration of free Ca^{2+} [38].

Measurement of release of radiolabelled 5-hydroxytryptamine and arachidonic acid

Radiolabelled cultures (24-well plates) were washed and incubated in the required medium for 10 min before addition of stimulant. The cultures were incubated for an additional 15 min. Samples of medium and cell lysates were assayed for radioactivity to determine the percentage of radiolabel that was released into the medium [36]. Values were corrected for the spontaneous release that occurred in the absence of stimulant (less than 3%).

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

$[Ca^{2+}]_i$ was measured in suspensions of Quin-2-loaded cells by use of previously described calibration procedures [34]. For qualitative observations of changes in $[Ca^{2+}]_i$ in single cells, the cells were grown on coverslips overnight with DNP-specific IgE as required. They were then loaded with Fura 2 acetoxymethyl ester (1 μM for 45 min). The coverslip was placed in a Dvorak–Stottler chamber (heated at 37 °C). A plastic spacer was placed in the chamber to reduce the free volume to 50 μl. Reagents were dissolved in the Ca^{2+}-containing or Ca^{2+}-free medium. Fura 2 fluorescence was measured at 510 nM (excitation at 340 and 380 nM) and fluorescence was expressed as a ratio of the fluorescence at the two excitation wavelengths [5,6]. Fluorescence was determined in a Deltascan fluorimeter with photon detectors (Photon Technology International, NJ, U.S.A.). Consistent with previous observations [5], whole-cell imaging indicated no apparent accumulation of Fura 2 (or its ester) in secretory granules or other intracellular organelles in RBL-2H3 cells (M. A. Beaven, unpublished work). Therefore changes in fluorescence ratio were assumed to be indicative of alterations in free $[Ca^{2+}]_i$. However attempts to calibrate intracellular $[Ca^{2+}]_i$ against the ratio of fluorescence by use of ionomycin [39] were suspect because thapsigargin at concentrations of 100 nM or greater consistently yielded fluorescent ratios in excess of those achieved with ionomycin. All experiments shown were representative of traces obtained from at least three cells on the same day. The observations were verified by additional experiments on separate days as noted in the figure legends.

Uptake of $^{45}Ca^{2+}$

Cultures in 24-well plates were incubated overnight at 37 °C in growth medium with DNP-specific IgE. The cultures were washed and growth medium replaced with 0.2 ml of the Ca^{2+}-containing buffer. After a 10 min incubation (37 °C) the buffer was replaced with buffer that contained $^{45}Ca^{2+}$ (1 μCi/0.2 ml) and the indicated concentration of stimulant and, where stated, metal ion. At the indicated times, the reaction was stopped by washing the cultures with ice-cold Ca^{2+}-free buffer that contained 100 μM La^{3+}. Cells were then lysed with 0.5 ml of deionized water for assay of intracellular $^{45}Ca^{2+}$. As in previous studies [17], the amount of Ca^{2+} taken up per culture (approx. 300000 cells/culture) was calculated on the assumption that the specific activity of Ca^{2+} taken up was the same as that in the medium (5 nCi/nmol of Ca^{2+}). An assumed cell volume, 1.1 μl/10^6 cells ([8], and M. A. Beaven, unpublished work), was used to estimate the intracellular concentrations of accumulated Ca^{2+} in the presence of stimulant. In experiments with inhibitors, values were expressed as a percentage of maximal uptake in the absence of inhibitor. Curve fitting (second-order polynominal) was done by a computer program [17].

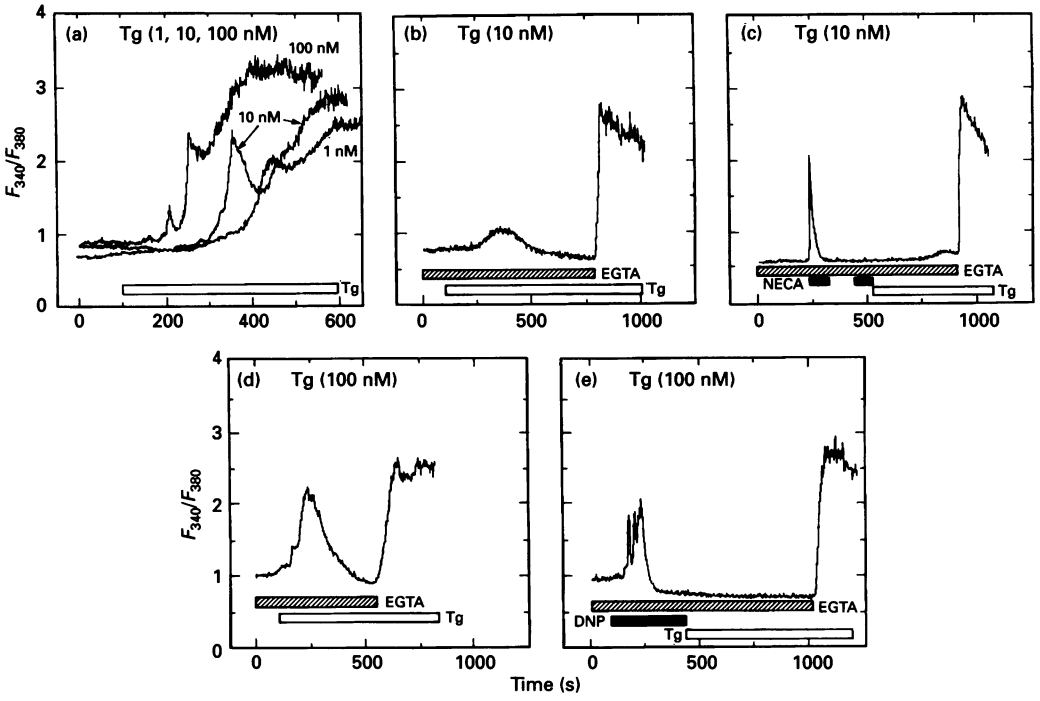

Figure 1 Depletion of Ca^{2+} from the same internal stores with thapsigargin, *N*-ethylcarboxamidoadenosine and the antigen, DNP–BSA

(**a**) The indicated concentration of thapsigargin (Tg) was added to the perfusion medium and changes in $[Ca^{2+}]_i$ were determined in individual Fura 2-loaded cells. The three traces shown are representative of responses of three cells. Additional experiments revealed that the maximal response to 100 nM thapsigargin varied from 3 to 4.5 (fluorescence ratio) but the differences in the time course of response for each concentration of thapsigargin was similar to those shown here. For reasons discussed in the Materials and methods section estimation of $[Ca^{2+}]_i$ was not attempted for studies with single cells. (**b**–**e**). In addition to thapsigargin (10 or 100 nM as indicated), 10 μM *N*-ethylcarboxamidoadenosine (NECA), 10 ng/ml DNP–BSA (DNP) and 0.1 mM EGTA (instead of 1 mM Ca^{2+}) were included in the perfusion fluid during the periods indicated by the different bars. All panels: changes in $[Ca^{2+}]_i$ were monitored from changes in ratio of fluorescence at 340 nM and 380 nM (excitation wavelengths) as measured at an emission wavelength of 510 nM. Additional experiments showed that after release of Ca^{2+} from intracellular stores in the presence of EGTA and thapsigargin [i.e. as in (**b**)], *N*-ethylcarboxamidoadenosine and antigen failed to elicit increases in $[Ca^{2+}]_i$. The traces shown here were representative of responses in at least six cells from experiments performed on separate occasions.

Efflux of Ca^{2+} from intact and permeabilized cells

Procedures for the measurement of efflux were based on those devised by Fewtrell and associates [14,18]. Suspensions of cells (0.5×10^6 cells/ml) were labelled by incubation with $^{45}Ca^{2+}$ (2 μCi/ml) for 90 min in complete growth medium. Alternatively, the cells were incubated with the same concentration of $^{45}Ca^{2+}$ in the presence of thapsigargin (100 nM) for 15 min. The cells were washed with Ca^{2+}-containing buffer and resuspended in the same buffer (0.5×10^6 cells/ml) for each experiment. The $^{45}Ca^{2+}$-loaded cells were incubated (37 °C) for 10 min before the addition of stimulant. Samples (100 μl) were removed at the indicated times and centrifuged through 400 μl of a mixture of dibutylphthalate and bis(2-ethylhexyl)phthalate (6:4, v/v) for determination of intracellular $^{45}Ca^{2+}$ exactly as described [18].

The determination of uptake and release of $^{45}Ca^{2+}$ in permeabilized cells was based upon the procedures of Gill and coworkers [40]. Cells were incubated overnight in 24-well plates exactly as described for the measurement of $^{45}Ca^{2+}$ uptake and then washed with the KCl buffer (Ca^{2+} buffered to 0.1 μM) before permeabilizing the adherant cells with streptolysin O (0.2 unit/ml) for 5 min [38]. The permeabilized cells were washed twice and the buffer was replaced with one that contained $^{45}Ca^{2+}$ (2 μCi/0.2 ml), EGTA (60 μM), polyethylene glycol and oligomycin as described by others [40] except that Ca^{2+} was buffered at 0.1 or 2.0 μM. Uptake of Ca^{2+} was started by addition of ATP (1.5 mM) 2 min later. Stimulants were added 10 min after addition of ATP. Cell-bound $^{45}Ca^{2+}$ was assayed as described above for the uptake studies. The stated times indicate minutes after the addition of stimulant.

RESULTS

Antigen and thapsigargin rely on the same sources of Ca^{2+} to increase $[Ca^{2+}]_i$

RBL-2H3 cells were unusually sensitive to thapsigargin. Measurement of $[Ca^{2+}]_i$ in suspensions of Quin 2-loaded cells indicated that as little as 3 nM thapsigargin elicited increases in $[Ca^{2+}]_i$ (from 0.1 μM to > 1.5 μM) in excess of those obtained with optimal concentrations of antigen (an increase from 0.1 μM to 1.2 μM; range of values for 13 experiments with 20 ng/ml DNP–BSA). Still higher concentrations of thapsigargin (10 nM or greater) induced increases in excess of 3 μM $[Ca^{2+}]_i$ (results not shown). Measurements in individual Fura 2-loaded cells indicated that the increases in $[Ca^{2+}]_i$ were of slow onset and reached a maximum by 300 s at high (> 100 nM) concentrations and by 600 s at low (< 10 nM) concentrations of thapsigargin (examples are shown in Figure 1a). Thereafter $[Ca^{2+}]_i$ slowly declined but it remained significantly elevated for at least 45 min (results not shown). As in other types of cells (e.g. [28]), a transient rise in $[Ca^{2+}]_i$ occurred over the course of 300–600 s in the absence of external Ca^{2+} and this was presumed to be due to outflow of Ca^{2+} from intracellular stores while re-uptake into these stores was blocked. The extent of this rise was dependent

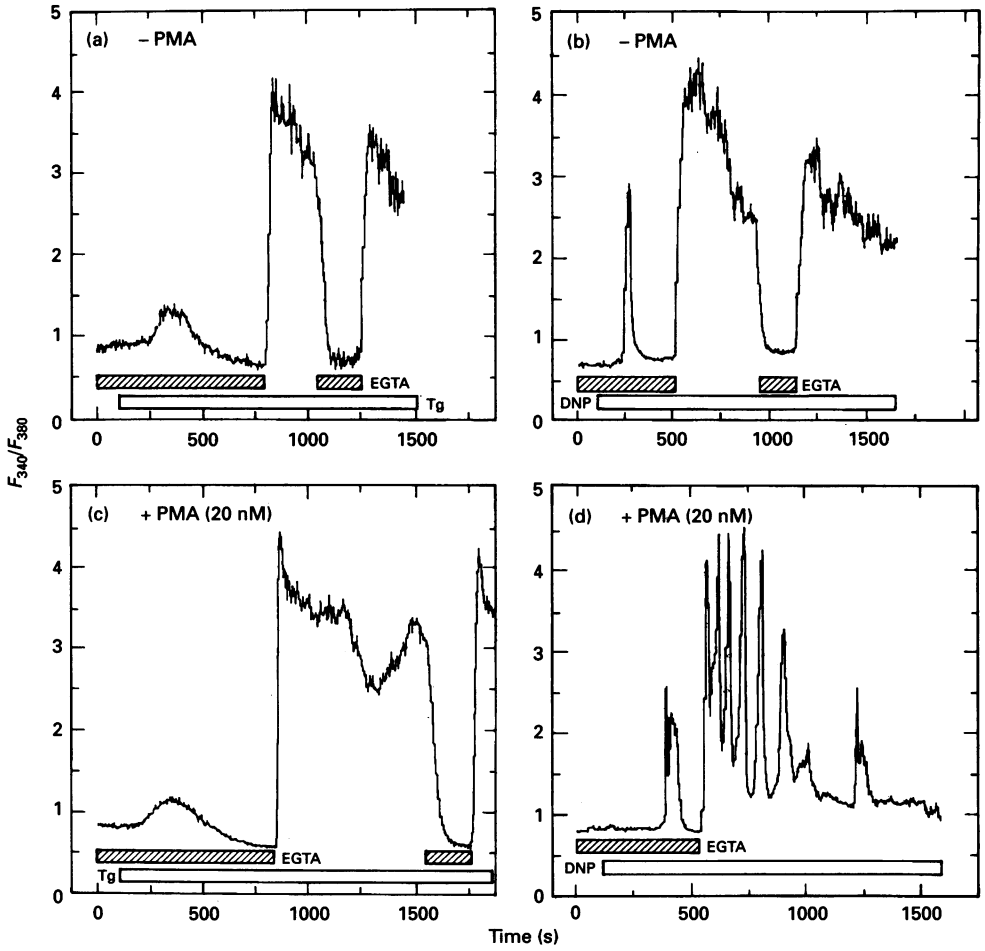

Figure 2 Regulation of $[Ca^{2+}]_i$ through influx and efflux of Ca^{2+} across the cell membrane is not impaired by thapsigargin or phorbol 12-myristate 13-acetate

$[Ca^{2+}]_i$ was assessed from changes in ratio of fluorescence at 340 nM and 380 nM (excitation wavelengths; emission at 510 nM) in individual Fura 2-loaded cells. The perfusion medium contained 0.1 mM EGTA (instead of Ca^{2+}), 10 mM thapsigargin (Tg) or 10 ng/ml DNP–BSA (DNP) during the periods shown by the bars and, for (**c**) and (**d**), the perfusion fluids contained 20 nM phorbol 12-myristate 13-acetate (PMA) for the entire perfusion period. The traces shown here were representative of responses in at least six cells from experiments performed on separate occasions: Differences were noted between experiments in the maximal response (F_{340}/F_{380} varied from 3.5 to 4.5) and in the number of oscillations in (**d**) (these ceased by as early as 300 s to as late as 750 s after provision of Ca^{2+}).

on the concentration of drug [results not shown but compare, for example, Figures 1(b) and 1(d)]. When $[Ca^{2+}]_i$ had returned to basal values, provision of Ca^{2+} then resulted in a rapid rise in $[Ca^{2+}]_i$ (Figure 1b) to indicate that for prolonged elevation in $[Ca^{2+}]_i$ influx of external Ca^{2+} was necessary.

The transient elevation in $[Ca^{2+}]_i$ that was observed in the absence of external Ca^{2+} was not apparent when intracellular stores were first depleted by prior exposure of cells to stimulants such as antigen or 5-*N*-ethylcarboxamidoadenosine (Figures 1c and 1e). Both of these agents were known to cause rapid depletion of intracellular stores through generation of inositol 1,4,5-trisphosphate [5,6,41]. Conversely, when cells were exposed to thapsigargin first, neither antigen nor 5-*N*-ethylcarboxamidoadenosine elicited further increases in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} (results not shown). Therefore, all three stimulants appeared to deplete a common, presumably inositol 1,4,5-trisphosphate-sensitive, pool of intracellular Ca^{2+}. Subsequent provision of Ca^{2+} resulted in rapid and prolonged increase in $[Ca^{2+}]_i$ even when provision of Ca^{2+} was delayed 10 min or longer (Figures 1c and 1e). Therefore, the mechanism for enhanced influx remained intact long after the initial depletion of the internal stores of Ca^{2+}.

Other characteristics of Ca^{2+}-influx in thapsigargin- and antigen-stimulated cells

Another indication of the durability of the influx mechanism after depletion of the intracellular stores was that removal of external Ca^{2+} caused a rapid decline in $[Ca^{2+}]_i$ to basal values and reprovision of external Ca^{2+} led to rapid restoration of $[Ca^{2+}]_i$ to its former elevated levels in either thapsigargin- (Figure 2a) or antigen- (Figure 2b) stimulated cells. Such changes in $[Ca^{2+}]_i$ were induced also by temporarily elevating extracellular K^+ concentrations to 75 mM in the presence of Ca^{2+}. The effects of high extracellular K^+ concentrations were indistinguishable from those of Ca^{2+}-deprivation in Figure 2. Thus influx of Ca^{2+}, whether induced by antigen or thapsigargin, was inhibited by

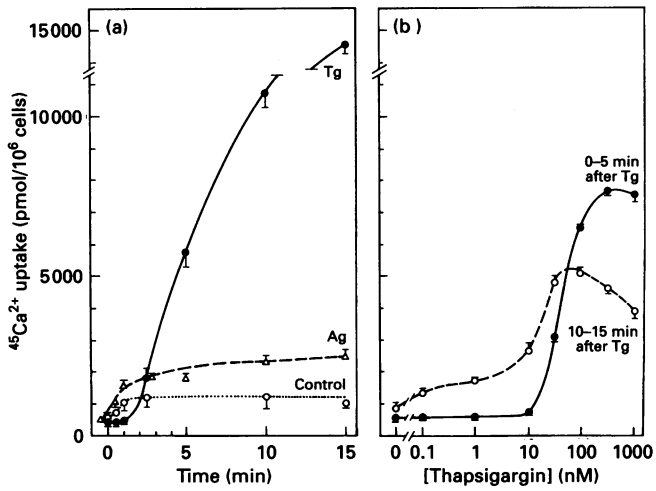

Figure 3 Time course of accumulation of $^{45}Ca^{2+}$ in RBL-2H3 cells that were unstimulated or simulated with antigen or thapsigargin (a); effect of dose of thapsigargin on accumulation of $^{45}Ca^{2+}$ (b)

(**a**) $^{45}Ca^{2+}$ (1 μCi) and vehicle (control), 10 ng/ml DNP–BSA (Ag) or 100 nM thapsigargin (Tg) were added (at 0 min) to washed cultures of RBL-2H3 cells (0.2×10^6 cells/0.2 ml per well) in Pipes-buffered medium that contained 1 mM Ca^{2+} (or 200 nmol of Ca^{2+}/well). Uptake of $^{45}Ca^{2+}$ was measured, at the times indicated, as described in the Materials and methods section. (**b**) The indicated concentration of thapsigargin was added at 0 min and 1 μM $^{45}Ca^{2+}$ was added either at the same time or 10 min after addition of thapsigargin when $[Ca^{2+}]_i$ was expected to have reached a maximum for all doses of thapsigargin (Figure 1a). Uptake was determined 5 min after the addition of $^{45}Ca^{2+}$. Values are mean ± S.E.M. of three cultures from one of three similar experiments. In this and the following experiments, calculation of the amount of intracellular $^{45}Ca^{2+}$ was based on the assumed dilution of radiolabel with Ca^{2+} in the medium (see the Materials and methods section).

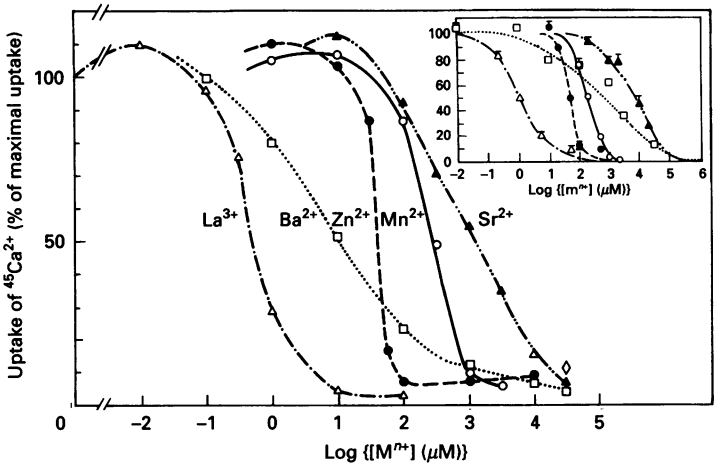

Figure 4 Inhibition of accumulation of $^{45}Ca^{2+}$ by various metal ions in thapsigargin-stimulated cells

Uptake of $^{45}Ca^{2+}$ was determined in cell cultures 5 min after addition of 100 nM thapsigargin and $^{45}Ca^{2+}$ in the absence or presence of the indicated concentrations of the various metal ions. Values (mean ± S.E.M. of three cultures) were representative of two series of experiments and were expressed as a percentage of uptake in the absence of metal ion (6700–8400 pmol of $^{45}Ca^{2+}/10^6$ cells; range of values over all experiments). For comparison, the inset shows results from a previous study [17] of the effects of the metal ions on accumulation of $^{45}Ca^{2+}$ in antigen-stimulated cells.

concentrations of K^+ that were known to depolarize RBL-2H3 cells [18].

The only noticeable difference in the Ca^{2+}-influx mechanisms was that the prolonged increase in $[Ca^{2+}]_i$ in antigen-stimulated cells was probably dependent on production of inositol phosphates whereas thapsigargin-induced influx was not. Thapsigargin induced no detectable hydrolysis of inositol phospholipids at 10 nM and only minimal (< 2%) hydrolysis of these lipids at 100 nM (results not shown). Phorbol 12-myristate 13-acetate, which was known to suppress hydrolysis of the inositol phospholipids in antigen-stimulated RBL-2H3 cells [42] by suppressing tyrosine phosphorylation of phospholipase Cγ1 [43], had no discernable effect on the release of intracellular Ca^{2+} or on the rates of influx or efflux in thapsigargin-treated cells. This was evident from the rapid decline and restoration of $[Ca^{2+}]_i$ on omission or reprovision of Ca^{2+} in the absence or presence of phorbol ester (compare Figure 2a with Figure 2c). The phorbol ester, however, markedly altered the calcium response to antigen (compare Figures 2b and 2d). Antigen still elicited a transient spike in $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, but instead of the normal prolonged elevation of $[Ca^{2+}]_i$, provision of external Ca^{2+} resulted in oscillations in $[Ca^{2+}]_i$ which declined in extent and frequency (i.e. as in Figure 2d) until cells became quiescent within a period of 5 to 12 min. The response was almost totally abrogated when the phorbol ester was added several minutes before addition of antigen which then elicited two to five transient spikes of $[Ca^{2+}]_i$ but no sustained increase in $[Ca^{2+}]_i$ (results not shown). An incidental conclusion from these results was that release of Ca^{2+} from intracellular stores and the consequent influx of external Ca^{2+} were not highly regulated by phorbol ester-sensitive isoenzymes of protein kinase C.

Enhanced accumulation of $^{45}Ca^{2+}$ in cells treated with thapsigargin

The influx of Ca^{2+} was examined by direct measurement of the accumulation of $^{45}Ca^{2+}$. In unstimulated cells a significant but modest accumulation of $^{45}Ca^{2+}$ occurred within a few minutes of addition of the labelled nucleide (Figure 3a). Although antigen induced a further modest increase (3–5-fold) in accumulation of $^{45}Ca^{2+}$, most of this additional accumulation occurred within 2–4 min of addition of antigen. In contrast, high concentrations (100 nM) of thapsigargin caused, after a delay of 1–2 min, a substantial accumulation of $^{45}Ca^{2+}$ which continued for at least 15 min to achieve estimated intracellular concentrations of Ca^{2+} (see the Materials and methods section) in the millimolar range (8–13 mM). Under non-steady-state conditions, such as those employed in these experiments, the estimates must be regarded as qualitative. Nevertheless, the estimated values exceeded previous estimates of intracellular Ca^{2+} in unstimulated (0.5 mM to 1 mM) or antigen-stimulated (approx. 3.5 mM) RBL-2H3 cells (values calculated from data in [14,18]). The data, therefore, allow the conclusion that most of the accumulated Ca^{2+} was sequestered in intracellular stores or depots because free $[Ca^{2+}]_i$ would be expected to remain in the micromolar range.

The delay in uptake of $^{45}Ca^{2+}$ (Figure 3a), like the delay in increase of $[Ca^{2+}]_i$ (Figure 1a), was most evident with low concentrations of thapsigargin. For example, when $^{45}Ca^{2+}$ and thapsigargin were added simultaneously and measurements then made 5 min later, accumulation of the $^{45}Ca^{2+}$ was evident only when cells were exposed to high concentrations of thapsigargin (> 10 nM). If, however, $^{45}Ca^{2+}$ was added 10 min after the addition of thapsigargin when the elevation in $[Ca^{2+}]_i$ was at a maximum for all concentrations of thapsigargin (e.g. Figure 1a), accumulation was evident with as little as 0.1 nM thapsigargin (Figure 3b). The accumulation of $^{45}Ca^{2+}$, therefore, appeared to

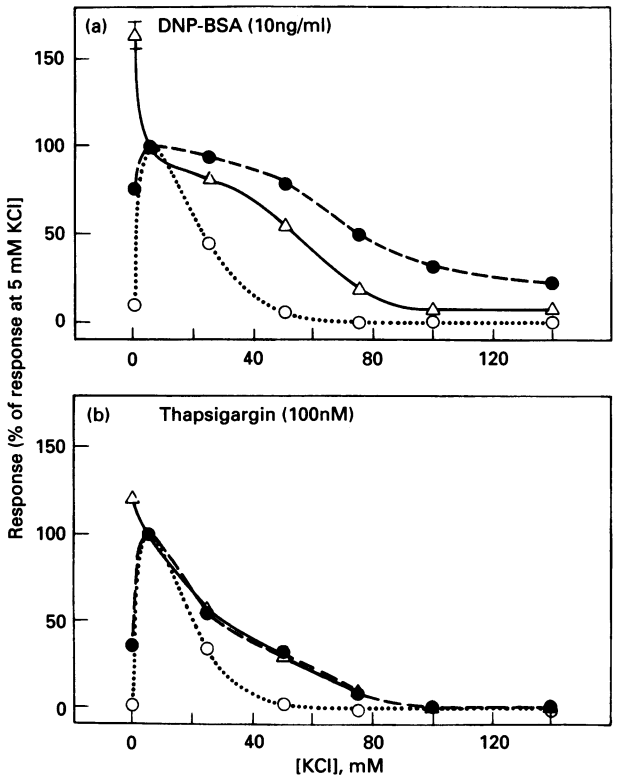

Figure 5 Effect of external K^+ uptake of $^{45}Ca^{2+}$ and other responses of cells stimulated with antigen (a) or thapsigargin (b)

These experiments were performed in medium that contained the indicated concentration of KCl and sufficient NaCl to give a final concentration of monovalent cations of 165 mM as described in the Materials and methods section. Uptake of $^{45}Ca^{2+}$ (○) was determined 5 min after addition of 10 ng/ml DNP–BSA or 100 nM thapsigargin and $^{45}Ca^{2+}$ as described in the legend of Figure 2. Secretion, as measured through release of [^{3}H]5-hydroxytryptamine ([^{3}H]5HT) into the medium (●) and the activation of phospholipase A_2, as measured through release of [^{14}C]arachidonic acid (△), were determined in matched cultures. Values are mean ± S.E.M. of three cultures.

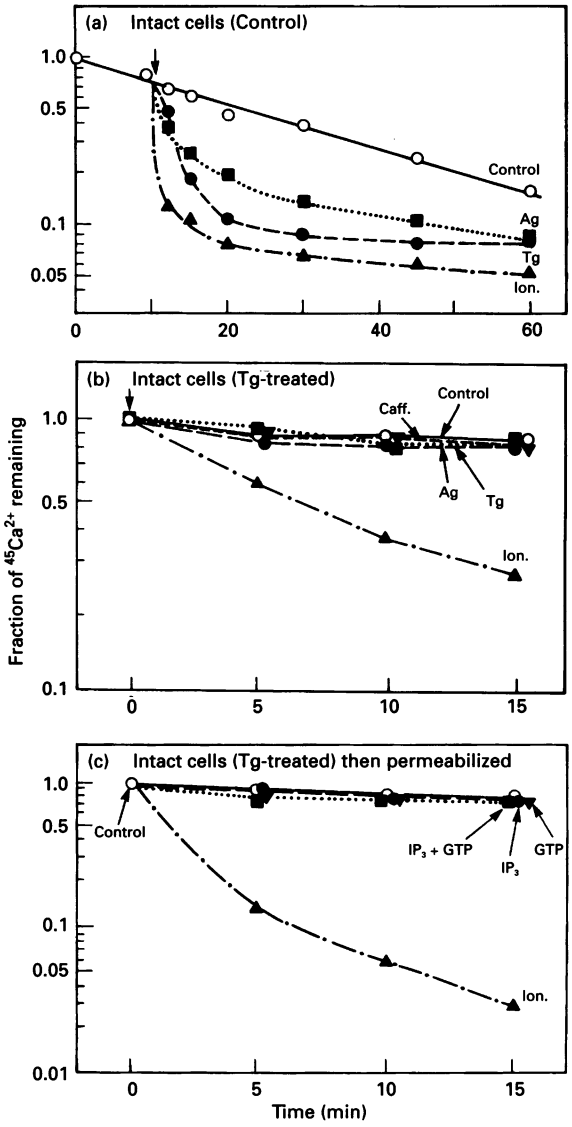

Figure 6 Releasibility of accumulated $^{45}Ca^{2+}$ from cells previously labelled in the absence or presence of thapsigargin (Tg)

Cells in suspension (0.5×10^6 cells/ml of Pipes-buffered medium with 1 mM Ca^{2+}) were exposed to 2 μCi of $^{45}Ca^{2+}$ for 90 min without thapsigargin (**a**) or for 15 min in the presence of 100 nM thapsigargin (**b** and **c**). Cells were washed with the Pipes-buffered medium (1 mM Ca^{2+}) before incubation in the same buffer [(**a**) and (**b**)]. Alternatively, the cells were washed with a potassium glutamate buffer that contained 0.1 μM $[Ca^{2+}]_i$ and were then permeabilized with streptolysin O in the same buffer (**c**). Stimulants were added at 10 min (**a**) or 0 min [(**b**) and (**c**)], namely, 20 ng/ml DNP–BSA (Ag.), 100 nM thapsigargin (Tg), 200 nM ionomycin (Ion.), 10 mM caffeine (Caff.), 10 μM inositol 1,4,5-trisphosphate (IP_3) and 50 μM GTP. The retained intracellular $^{45}Ca^{2+}$ was determined by centrifugation of cells through oil as described in the Materials and methods section. Values are mean ± S.E.M. of three sets of cell suspensions and indicated the fraction of intracellular $^{45}Ca^{2+}$ at the start of the experiment [i.e. at 0 min, 460 pmol/10^6 cells for (**a**) 6750 pmol/10^6 cells for (**b**) and 6355 pmol/10^6 cells for (**c**)] that was retained in cells at the indicated times. Similar results were obtained in two additional experiments. Keys to symbols: (**a**) and (**b**); ○, controls (no stimulant); ■, antigen; ●, thapsigargin; ▲, ionomycin; ▼, caffeine. (**c**) ○, Controls (no stimulant); ●, inositol 1,4,5-trisphosphate; ▼, GTP; ■, inositol 1,4,5-trisphosphate plus GTP.

coincide with, or even precede, the elevation in $[Ca^{2+}]_i$ as would be expected if influx was a consequence of depletion of intracellular stores of Ca^{2+} [31].

Similarities in the uptake mechanism in cells stimulated with thapsigarin and antigen

The thapsigargin-induced uptake of $^{45}Ca^{2+}$ was inhibited by La^{3+}, Ba^{2+}, Zn^{2+}, Mn^{2+} and Sr^{2+} (Figure 4). Except for Ba^{2+} (see below), these cations appeared to be equally effective inhibitors whether cells were stimulated with thapsigargin or antigen (Figure 4, inset). Uptake was inhibited to the same extent in cells stimulated with antigen or thapsigargin by depolarizing cells with high concentrations of external K^+. An incidental, but expected, finding was that the functional responses of these cells, such as release of arachidonic acid and secretion of 5-hydroxytryptamine, were also impaired by high K^+ (Figure 5), as had been observed previously in antigen-stimulated cells [17,18].

Paradoxically, Ba^{2+} was a much more effective inhibitor of thapsigargin-induced uptake of Ca^{2+} (50% inhibition at approx. 10 μM) than of antigen-induced uptake of Ca^{2+} (50% inhibition at approx. 400 μM). The reason for this difference is unclear but it may be related to the ability of Ba^{2+} to inhibit, at micromolar concentrations, a K^+-selective outward rectifying current [21], and as a consequence repolarization of the plasma membrane [22], in RBL-2H3 cells. The repolarization is thought to counteract the depolarizing effects of Ca^{2+} influx and thereby facilitate this influx [18,20]. Thapsigargin-stimulated cells might be par-

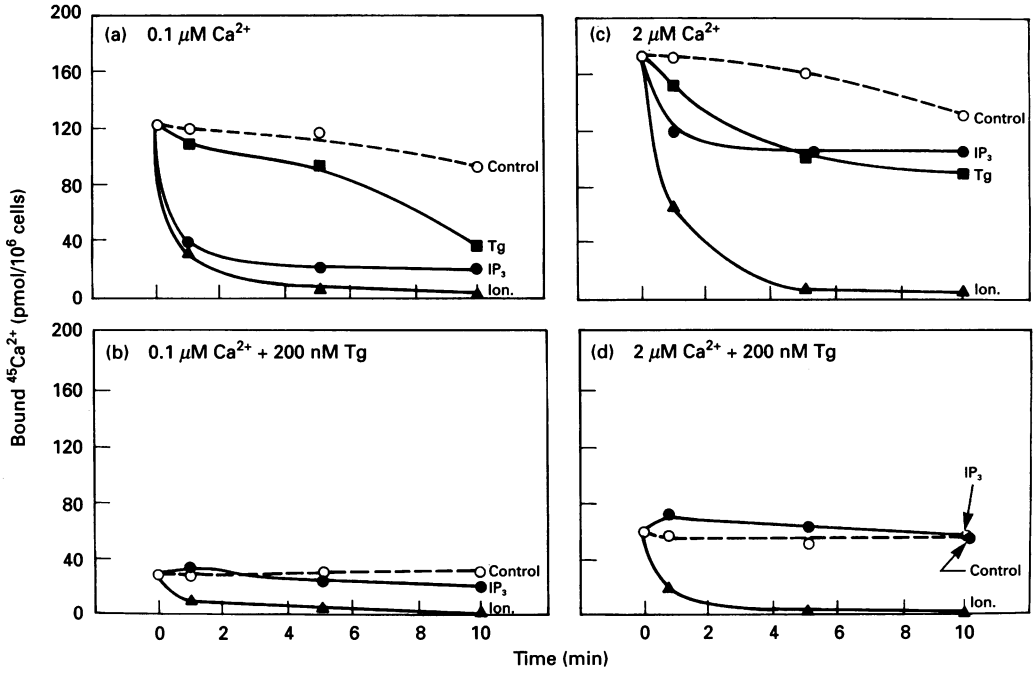

Figure 7 Releasibility of $^{45}Ca^{2+}$ that was sequestered by permeabilized cells in the absence or presence of thapsigargin

Cultured RBL-2H3 cells (0.2×10^6 cells/0.2 ml per well) were permeabilized with streptolysin *O* and medium replaced with one that contained 2 μCi of $^{45}Ca^{2+}$, 50 μM Ca^{2+} (in total or 10000 pmol of Ca^{2+}/well) and EGTA to buffer free Ca^{2+} at 0.1 μM [(**a**) and (**b**)] or 2.0 μM [(**c**) and (**d**)], without or with 200 nM thapsigargin (Tg). ATP was added to allow sequestration of $^{45}Ca^{2+}$ by the permeabilized cells and stimulants were then added 10 min later to monitor release of $^{45}Ca^{2+}$ as described in the Materials and methods section. The times indicated are minutes after the addition of stimulants. These included 10 μM inositol 1,4,5-trisphosphate (IP_3), 200 nM thapsigargin (Tg) and 200 nM ionomycin (Ion.). Values are means of two identical experiments which were representative of preliminary experiments of this design.

ticularly dependent on repolarizing systems because the net uptake of Ca^{2+} is substantially greater than that in antigen-stimulated cells (Figure 3a).

Evidence that $^{45}Ca^{2+}$ accumulates in two distinct stores of Ca^{2+} in thapsigargin-stimulated cells

The sustained uptake of $^{45}Ca^{2+}$, even when uptake of Ca^{2+} into the inositol 1,4,5-trisphosphate-sensitive store was blocked by thapsigargin, indicated that $^{45}Ca^{2+}$ had accumulated in another type of store. This possibility was tested by allowing $^{45}Ca^{2+}$ to fully equilibrate (i.e. 90 min incubation) with intracellular stores in the absence and presence of thapsigargin. The releasibility of the sequestered $^{45}Ca^{2+}$ was then determined after replacing medium with one that contained unlabelled Ca^{2+} (Figure 6). In cells equilibrated in the absence of thapsigargin, $^{45}Ca^{2+}$ exchanged slowly with external Ca^{2+} [half-life ($t_{\frac{1}{2}}$) for the decline in $^{45}Ca^{2+}$ was approx. 20 min]. This exchange was markedly accelerated when the cells were stimulated with antigen (see also [14,18,20]), thapsigargin or ionomycin, although release in response to thapsigargin was slightly delayed when compared with the other stimulants (Figure 6a). When cells were equilibrated with $^{45}Ca^{2+}$ in the presence of 100 nM thapsigargin, however, virtually all of the accumulated $^{45}Ca^{2+}$ was not released by antigen, thapsigargin or caffeine (Figure 6b) or, after permeabilizing the cells, by inositol 1,4,5-trisphosphate, GTP and the combination of inositol 1,4,5-trisphosphate and GTP (Figure 6c). Only ionomycin released Ca^{2+} from this apparently resistant pool of Ca^{2+}. Furthermore, this pool did not rapidly exchange with either external Ca^{2+} when cells were left intact (Figure 6b) or 0.1 μM free Ca^{2+} when cells were permeabilized (Figure 6c) as indicated by the slow rate of spontaneous loss of sequestered $^{45}Ca^{2+}$.

There appeared, therefore, to be two distinct pools of Ca^{2+}; one that was sensitive to inositol 1,4,5-trisphosphate and thapsigargin (hereafter referred to as the mediator-sensitive pool) and one that was insensitive to these agents (hereafter referred to as the mediator-resistant pool). The latter pool was also relatively impermeable to external Ca^{2+} except in thapsigargin-treated cells, where presumably the intracellular concentrations of free Ca^{2+} were maintained at high levels for prolonged periods of time. It should be noted, however, that the agonist-induced release that was observed in untreated cells (i.e. Figure 6a) might not be discernible in thapsigargin-treated cells because these cells contained substantially greater amounts of sequestered $^{45}Ca^{2+}$ than did untreated cells (see legend to Figure 6).

The properties of the two pools were examined directly in permeabilized cells. Here uptake of $^{45}Ca^{2+}$ was initiated by addition of ATP in the presence of 0.1 μM or 2 μM $[Ca^{2+}]_i$ to simulate $[Ca^{2+}]_i$ in respectively, unstimulated and thapsigargin-stimulated cells. At 0.1 μM $[Ca^{2+}]$, uptake was largely restricted to the mediator-sensitive pool and could be depleted by inositol 1,4,5-trisphosphate, ionomycin and at a lower rate by thapsigargin (Figure 7a). Little uptake was observed in the presence of thapsigargin to indicate that thapsigargin was still active, even when added to permeabilized cells. The little $^{45}Ca^{2+}$ that was taken up was not released by inositol 1,4,5-trisphosphate (Figure 7b). At 2 μM $[Ca^{2+}]$, $^{45}Ca^{2+}$ was taken up into both pools because less than 50% of the sequestered $^{45}Ca^{2+}$ was released by inositol 1,4,5-trisphosphate or thapsigargin (Figure 7c); in the presence of thapsigargin uptake was largely confined to the mediator-

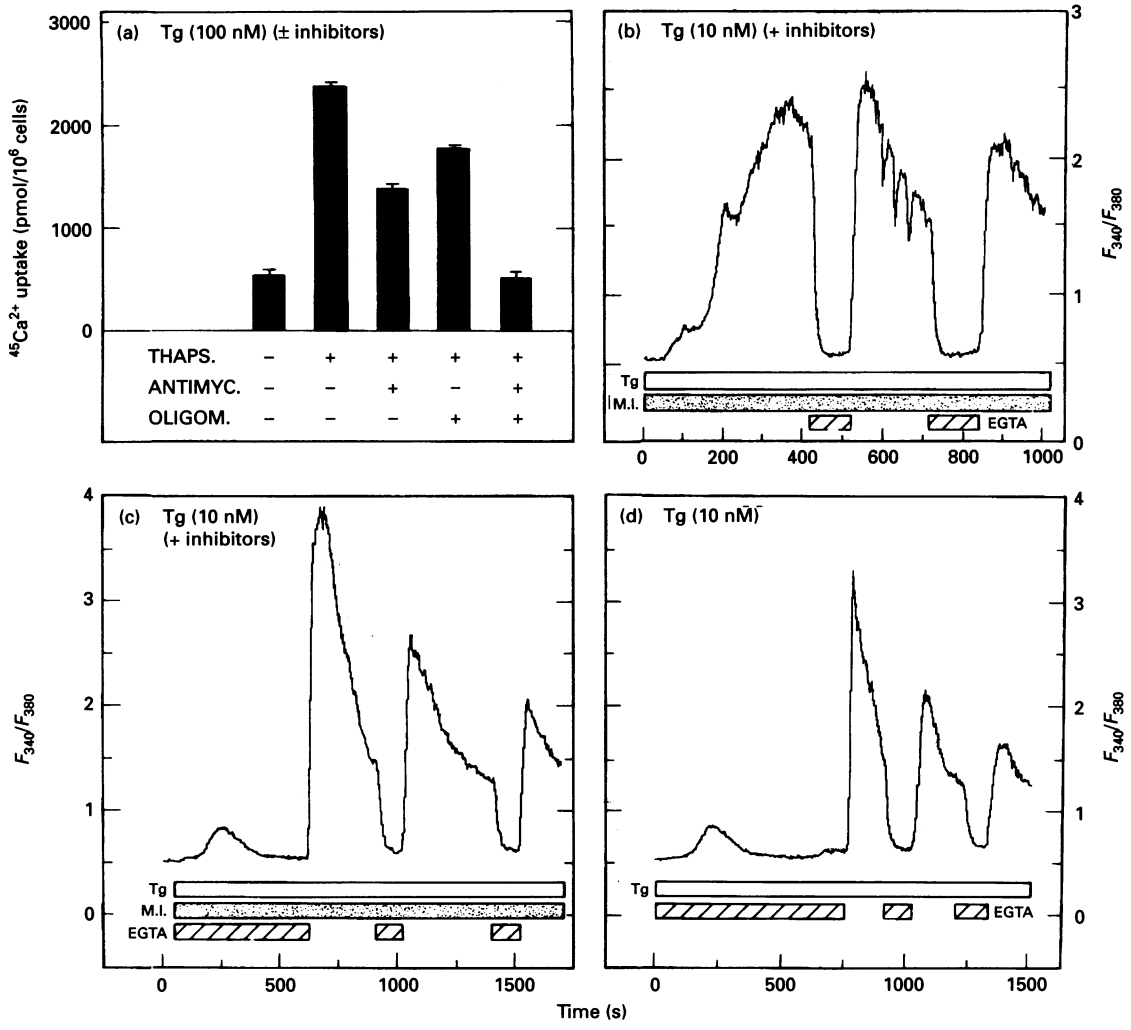

Figure 8 Effect of mitochondrial inhibitors (antimycin A and oligomycin) on uptake of $^{45}Ca^{2+}$ and changes in $[Ca^{2+}]_i$ with thapsigargin

(**a**) Uptake of $^{45}Ca^{2+}$ (for 5 min) was determined in cell cultures as described in the legend of Figure 3 in the absence or presence of 100 nM thapsigargin (Thaps.), 100 nM antimycin A (Antimyc.) or 1 μM oligomycin (Oligom.). Values are mean ± S.E.M. of three cultures and are representative of two additional experiments. In (**b**), (**c**) and (**d**), changes in $[Ca^{2+}]_i$ were assessed in individual Fura 2-loaded cells. The perfusion medium contained 10 nM thapsigargin (Tg), the two mitochondrial inhibitors (M.I.) at the concentrations noted above or 0.1 mM EGTA instead of 1 mM Ca^{2+} during the periods shown by the bars. The traces were representative of responses in at least six cells from experiments performed on separate occasions. Additional experiments (results not shown) in which 10 ng/ml DNP–BSA was perfused, in the presence of mitochondrial inhibitors and EGTA, before addition of thapsigargin [i.e. as in (**c**)], showed the same responses in $[Ca^{2+}]_i$ as those shown in Figure 1e. Therefore, the mitochondrial inhibitors did not appear to impair signals mediated by antigen.

resistant pool (Figure 7d). Therefore, uptake into the resistant pool was most apparent at high $[Ca^{2+}]_i$ (compare Figure 7b with Figure 7d). Finally, as noted previously, both pools were depleted by ionomycin [Figures 7(a–d)].

Evidence that the mediator-resistant pool is associated with mitochondria

Previous studies by others showed that accumulation of $^{45}Ca^{2+}$ in antigen-stimulated RBL-2H3 cells was blocked by the combination of the respiratory chain inhibitor, antimycin A, and the ATP synthetase inhibitor, oligomycin, in the presence of glucose [14]. Such conditions did not reduce intracellular levels of ATP nor apparently impair influx of Ca^{2+} across the cell membrane, the increase in $[Ca^{2+}]_i$ or secretion in response to antigen. The reduction in accumulation of $^{45}Ca^{2+}$ was attributed to lack of sequestration of Ca^{2+} by mitochondria which, under these specific conditions, would be expected to become depolarized [14]. The same combination of inhibitors also suppressed the accumulation of $^{45}Ca^{2+}$ in thapsigargin-stimulated RBL-2H3 cells, whereas each inhibitor individually did not do so (Figure 8a).

Nonetheless, treatment with antimycin A and oligomycin did not block entry of Ca^{2+} into the cell, as indicated by the prolonged increases in $[Ca^{2+}]_i$ in response to thapsigargin (Figure 8b). Also, a rapid decline in $[Ca^{2+}]_i$ to basal levels and its rapid restoration to its former elevated levels, during and after temporary deprivation of cells of external Ca^{2+} (Figures 8b and 8c), suggested that elevated $[Ca^{2+}]_i$ under these specific conditions was sustained exclusively through a dynamic balance of influx and efflux of Ca^{2+} across the cell membrane as uptake into the mediator-sensitive and mediator-insensitive pools should have been blocked. Furthermore, the rates of influx and efflux were as rapid as those observed in the absence of inhibitors (results not shown but compare Figure 8c with Figure 8d).

DISCUSSION

Studies with Ca^{2+}-sensitive probes and $^{45}Ca^{2+}$ have indicated that a variety of agents cause depletion of Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive stores and stimulate influx of Ca^{2+} in RBL-2H3 cells and transfected RBL-2H3 cells that express muscarinic m1 receptors. These agents include antigen [17,44], 5′-(*N*-ethyl)carboxamidoadenosine ([17] and this paper), carbachol [13] and thapsigargin (this paper). Such studies point to a common influx mechanism for all these stimulants and one that is analogous to the Ca^{2+}-entry, sometimes referred to as retrograde entry [26] that is associated with depletion of inositol 1,4,5-trisphosphate-sensitive stores in other types of electrically insensitive cells. Influx is suppressed by high, depolarizing concentrations of K^+ and by La^{3+}, Zn^{2+}, Mn^{2+}, Ba^{2+} and Sr^{2+} ([13,17] and this paper). The influx pathway is permeable to Na^+, in the absence of Ca^{2+} [15], and Mn^{2+}, Ba^{2+} and Sr^{2+} compete with Ca^{2+} for entry into the cells [17]. Such findings appear to be at variance with the direct measurements of I_{crac} which is thought to be the mechanism of Ca^{2+} entry in RBL-2H3 and mast cells [29,30] and which is apparently highly selective for Ca^{2+} [29]. One possible explanation for this variance is that the multivalent cations effectively compete for Ca^{2+} entry but are not conducted at high rates through the Ca^{2+}-conducting channel. We note, however, that Mn^{2+} has been widely used as a surrogate ion for Ca^{2+} to monitor retrograde cation-influx activity in other types of electrically insensitive cells because of the ability of this cation to quench Fura 2 fluorescence 2 [5,45,46]. Also, entry of Mn^{2+}, Ba^{2+} and Sr^{2+} is significantly enhanced in antigen-stimulated RBL-2H3 cells ([17]; M. A. Beaven and O. H. Choi, unpublished work).

The studies with $^{45}Ca^{2+}$ did demonstrate, however, the existence of at least two pools of intracellular Ca^{2+}, a mediator-sensitive pool that readily equilibrated with external Ca^{2+} in unstimulated cells and a thapsigargin/mediator-insensitive pool that did not readily equilibrate with external Ca^{2+}, except when $[Ca^{2+}]_i$ was elevated. The first pool exhibited spontaneous turnover (Figure 6a) and was depletable by antigen, thapsigargin and, after permeabilization of the cells, inositol 1,4,5-trisphosphate (Figure 7a). The second pool had slow turnover, was impermeable to Ca^{2+} in the presence of mitochondrial inhibitors and was insensitive to all the above stimulants as well as GTP and caffeine (Figures 6b and 6c), indicating its probable location in mitochondrial rather than the GTP- [40,47] or caffeine/ryanodine- [48] sensitive stores. Only ionomycin acted on both pools. Uptake of $^{45}Ca^{2+}$ by permeabilized cells also indicated that sequestration of $^{45}Ca^{2+}$ in a mediator-sensitive pool at 0.1 μM $[Ca^{2+}]_i$ (Figure 7a) was blocked by thapsigarin (Figure 7b) and that uptake into the mediator-insensitive pool was a consequence of elevated $[Ca^{2+}]_i$ rather than a direct action of thapsigargin (compare Figure 7b with Figure 5d).

The spontaneous turnover of the mediator-sensitive pool was also apparent from the studies with Fura 2-loaded cells. It was evident that this pool was depleted of Ca^{2+} within minutes when uptake of Ca^{2+} was blocked by thapsigargin, as has been noted in other cells [34]. As might be expected from previous work, release from this pool was accelerated by agents that acted via inositol 1,4,5-trisphosphate, as demonstrated by the sharp transient increases in $[Ca^{2+}]_i$ in response to antigen, *N*-ethylcarboxamidoadenosine (Figure 2b), and carbachol [13].

The apparent accumulation of $^{45}Ca^{2+}$ in mitochondria in thapsigargin-treated cells might be attributable to the elevation of $[Ca^{2+}]_i$ above 1 or 2 μM for more than 10 min in such cells. In antigen-stimulated cells, $[Ca^{2+}]_i$ rarely exceeds these levels for more than a few minutes but even these cells accumulate some Ca^{2+} in mitochondria [14]. Of possible significance, the accumulation of $^{45}Ca^{2+}$ in cells occur during the first few minutes of stimulation with antigen (Figure 3a) or carbachol [13] when $[Ca^{2+}]_i$ is most likely to exceed 1 μM. Although $[Ca^{2+}]_i$ subsequently declines it remains elevated for at least 30 min but with little additional accumulation or loss of $^{45}Ca^{2+}$. Recalculation of data from studies of RBL-2H3 cells loaded to isotopic equilibrium with $^{45}Ca^{2+}$ [18] indicate that total Ca^{2+} might increase from 0.5 mM to as high as 2–3 mM after antigen stimulation and that much of this accumulated $^{45}Ca^{2+}$ remains in the cells 60 min after stimulation. Recent measurements by ion microscopy further indicate that much of this increase is confined to the cytoplasmic compartment of RBL-2H3 cells which, because of inadequate spatial resolution of the imaging system, contained organelles such as mitochondria, endoplasmic reticulum, granules and vesicles [49]. Small pools of Ca^{2+} (about 12.5 % of total cell Ca^{2+}) in the nucleus and Golgi also showed significant increases in total Ca^{2+}.

Mitochondria in resting cells contain little Ca^{2+} but do accumulate Ca^{2+} by a low-affinity, high-capacity system when $[Ca^{2+}]_i$ is markedly elevated and this system is thought to modulate mitochondrial metabolism [50–55]. The uptake *in vitro* is relatively slow when free Ca^{2+} is set at submicromolar concentrations. The concentration gradient for Ca^{2+} ($[Ca^{2+}]_m/[Ca^{2+}]_i$) across the mitochondria becomes progressively more positive when $[Ca^{2+}]_i$ is increased above 750 nM [53]. In addition to this uptake mechanism, there are indications of microdomains in mitochondria in close proximity to sites of Ca^{2+}-release from inositol 1,4,5-trisphosphate-sensitive stores that allow rapid reversible increases of $[Ca^{2+}]_m$ as measured with aequorin [35]. As with the accumulation of $^{45}Ca^{2+}$ in our studies, the increase in $[Ca^{2+}]_m$ was prevented by ablation of the mitochondrial membrane potential [35]. Unlike the accumulation of $^{45}Ca^{2+}$, however, the increases in $[Ca^{2+}]_m$ were reversible when ambient $[Ca^{2+}]_i$ declined to basal levels [35]. In the present studies, $^{45}Ca^{2+}$ that was taken up by mitochondria did not readily exchange with ambient Ca^{2+}. The $^{45}Ca^{2+}$, therefore, had become distributed or redistributed in highly sequestered stores. Whether high $[Ca^{2+}]_m$, as measured by aequorin, was similarly dissipated into sequestered stores or the cytosol was unclear. Irrespective of the uncertainty as to the final disposition of $^{45}Ca^{2+}$, the present data suggest that mitochondrial uptake is the major mechanism for accumulation of $^{45}Ca^{2+}$ in cells stimulated with thapsigargin and possibly other stimulants.

The results also indicate that influx and efflux mechanisms in RBL-2H3 permit rapid changes in $[Ca^{2+}]_i$ even when the inositol 1,4,5-trisphosphate-sensitive and mitochondrial pools are not functional. We propose the following simple model to account for the above findings. A non-selective cation influx mechanism, perhaps of low conductive capacity, exists in dynamic equilibrium with efflux mechanisms when inositol 1,4,5-trisphosphate-sensitive pools are fully depleted to achieve a steady-state level > 1 μM $[Ca^{2+}]_i$. Uptake into the inositol 1,4,5-trisphosphate pool reduces $[Ca^{2+}]_i$ to 0.1 μM. Influx still continues, albeit at reduced activity (see [17]), such that cells remain permeable to external $^{45}Ca^{2+}$. Because the mediator-sensitive pool continues to turnover even in unstimulated cells, $^{45}Ca^{2+}$ equilibrates with this pool. When $[Ca^{2+}]_i$ exceeds the set point for mitochondria (0.7–1.0 μM $[Ca^{2+}]_i$), transiently so in antigen-stimulated cells or for a sustained period in thapsigargin-treated cells, $^{45}Ca^{2+}$ then accumulates in mitochondria. In the presence of thapsigargin and mitochondrial inhibitors, there is little net uptake of $^{45}Ca^{2+}$, but there are rapid changes in $[Ca^{2+}]_i$ on omission or reprovision of external Ca^{2+}. Apparently these changes in $[Ca^{2+}]_i$ require miniscule fluxes in Ca^{2+} in relation to the amount of $^{45}Ca^{2+}$ that

can accumulate in intracellular stores. The studies illustrate how full assessment of Ca^{2+} homoeostasis may require measurement of both $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake.

REFERENCES

1 Beaven, M. A. and Cunha-Melo, J. R. (1988) Prog. Allergy **42**, 123–184
2 Siraganian, R. P., McGivney, A., Barsumian, E. L., Crews, F. T., Hirata, F. and Axelrod, J. (1982) Fed. Proc. Fed. Am. Soc. **41**, 30–34
3 Metzger, H., Alcaraz, G., Hohman, R., Kinet, J. P., Pribluda, V. and Quarto, R. (1986) Annu. Rev. Immunol. **4**, 419–470
4 Seldin, D. C., Adelman, S., Austen, K. F., Stevens, R. L., Hein, A., Caulfield, J. P. and Woodbury, R. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3871–3875
5 Millard, P. J., Ryan, T., Webb, W. W. and Fewtrell, C. (1989) J. Biol. Chem. **264**, 19730–19739
6 Jones, S. V. P., Choi, O. H. and Beaven, M. A. (1991) FEBS Lett. **289**, 47–50
7 Millard, P. J., Gros, D., Webb, W. W. and Fewtrell, C. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1854–1858
8 Beaven, M. A., Rogers, J., Moore, J. P., Hesketh, T. R., Smith, G. A. and Metcalfe, J. C. (1984) J. Biol. Chem. **259**, 7129–7136
9 Beaven, M. A. and Metzger, H. (1993) Immunol. Today **14**, 222–226
10 Cunha-Melo, J. R., Dean, N. M., Moyer, J. D., Maeyama, K. and Beaven, M. A. (1987) J. Biol. Chem. **262**, 11455–11463
11 Pribluda, V. S. and Metzger, H. (1987) J. Biol. Chem. **262**, 11449–11454
12 Meyer, T., Holowka, D. and Stryer, L. (1988) Science **240**, 653–656
13 Choi, O. H., Lee, J. H., Kassessinoff, T., Cunha-Melo, J. R., Jones, S. V. P. and Beaven, M. A. (1993) J. Immunol. **151**, 5586–5595
14 Mohr, F. C. and Fewtrell, C. (1990) Am. J. Physiol. **258**, C217–C226
15 Kanner, B. I. and Metzger, H. (1984) J. Biol. Chem. **259**, 10188–10193
16 Fewtrell, C. and Sherman, E. (1987) Biochemistry **26**, 6995–7003
17 Hide, M. and Beaven, M. A. (1991) J. Biol. Chem. **266**, 15221–15229
18 Mohr, F. C. and Fewtrell, C. (1987) J. Cell Biol. **104**, 783–792
19 Middleton, E., Jr., Drzewiecki, G. and Triggle, D. (1981) Biochem. Pharmacol. **30**, 2867–2869
20 Mohr, F. C. and Fewtrell, C. (1987) J. Biol. Chem. **262**, 10638–10643
21 McCloskey, M. A. and Quian, Y.-X. (1994) J. Biol. Chem. **269**, 14813–14819
22 Labrecque, G. F., Holowka, D. and Baird, B. (1989) J. Immunol. **142**, 236–243
23 Penner, R., Matthews, G. and Neher, E. (1988) Nature (London) **334**, 499–504
24 Romanin, C., Reinsprecht, M., Pecht, I. and Schindler, H. (1991) EMBO J. **10**, 3603–3608
25 Putney, J. W., Jr. (1990) Cell Calcium **11**, 611–624
26 Putney, J. W. (1993) Science **262**, 676–678
27 Randriamampita, C. and Tsien R. Y. (1993) Nature (London) **364**, 809–814
28 Parekh, A. P., Terlau, H. and Stumer, W. (1993) Nature (London) **364**, 814–818
29 Hoth, M. and Penner, R. (1992) Nature (London) **355**, 353–356
30 Fasolato, C., Hoth, M. and Penner, R. (1993) J. Biol. Chem. **268**, 20737–20740
31 Bird, G. S. J. and Putney, J. W. (1993) J. Biol. Chem. **268**, 21486–21488
32 Matthews, G., Neher, E. and Penner, R. (1989) J. Physiol. (London) **418**, 105–130
33 Kuno, M., Okada, T. and Shibata, T. (1989) Am. J. Physiol. **256**, C560–C568
34 Takemura, H., Hughes, A. R., Thastrup, O. and Putney, J. W., Jr. (1989) J. Biol. Chem. **264**, 12266–12271
35 Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Science **262**, 744–747
36 Yamada, K., Jelsema, C. L. and Beaven, M. A. (1992) J. Immunol. **149**, 1031–1037
37 Ali, H., Cunha-Melo, J. R., Saul, W. F. and Beaven, M. A. (1990) J. Biol. Chem. **265**, 745–753
38 Ali, H, Cunha-Melo, J. and Beaven, M. A. (1989) Biochim. Biophys. Acta **1010**, 88–99
39 Williams, D. A. and Fay, F. S. (1990) Cell Calcium **11**, 75–83
40 Bian, J. H., Ghosh, T. K., Wang, J. C. and Gill, D. L. (1991) J. Biol. Chem. **266**, 8801–8806
41 Ramkumar, V., Stile, G. L., Beaven, M. A. and Ali, H. (1993) J. Biol. Chem. **268**, 16887–16890
42 Beaven, M. A., Guthrie, D. F., Moore, J. P., Smith, G. A., Hesketh, T. R. and Metcalfe, J. C. (1987) J. Cell Biol. **105**, 1129–1136
43 Ozawa, K., Yamada, K., Kazanietz, M. G., Blumberg, P. and Beaven, M. A. (1993) J. Biol. Chem. **268**, 2280–2283
44 Cleveland, P. L., Millard, P. J., Showell, H. J. and Fewtrell, C. M. (1993) Cell Calcium **14**, 1–16
45 Meldolesi, J., Clementi E., Fasolato, C., Zachetti, D. and Pozzan, T. (1991) Trends Pharmacol. Sci. **12**, 289–292
46 Rink, T. J. (1990) FEBS Lett. **268**, 381–385
47 Gill, D. L., Ghosh, T. K. and Mullaney, J. M. (1989) Cell Calcium **10**, 363–374
48 McPherson, P. S. and Campbell, K. P. (1993) J. Biol. Chem. **268**, 13765–13768
49 Chandra, S., Fewtrell, C., Millard, P. J., Sandison, D. R., Webb, W. W. and Morrison, G. H. (1994) J. Biol. Chem. **269**, 15186–15194
50 Nicholls, D. and Akerman, K. (1982) Biochim. Biophys. Acta **683**, 57–88
51 Bygrave, F. L. (1978) Biol. Rev. Camb. Philos. Soc. **53**, 43–79
52 Rasmussen, H. and Barett, P. Q. (1984) Physiol. Rev. **64**, 938–984
53 Hansford, R. G. (1991) J. Bioener. Biomembr. **23**, 823–854
54 Brown, G. C. (1992) Biochem. J. **284**, 1–13
55 McCormack, J. G., Halestrap, A. P. and Denton, R. M. (1990) Physiol. Rev. **70**, 391–425

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