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Technique for *in situ* measurement of calcium in intracellular inositol 1,4,5-trisphosphate-sensitive stores using the fluorescent indicator mag-fura-2

(gastric glands/thapsigargin/heparin)

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ABSTRACT **Stimulation of cells with calcium-mobilizing agonists frequently results in inositol 1,4,5-trisphosphate (InsP_3)-mediated discharge of Ca from an internal store. We report here a technique for directly monitoring Ca within this and other stores in gastric epithelial cells. This technique takes advantage of the propensity of the acetoxymethyl ester derivative of the fluorescent dye mag-fura-2 (which is sensitive to Ca concentrations above 5 μM) to accumulate in subcellular compartments where it can report changes in the free Ca concentration. Intact dye-loaded cells responded to cholinergic stimulation with a decrease in the 350 nm/385 nm excitation ratio, as measured in individual cells with a digital imaging microscope, consistent with reduced Ca concentration in one or more cellular compartments. When cells were permeabilized with digitonin and incubated in an "intracellular buffer," the cytoplasmic dye was released, leaving the mag-fura-2 in the internal store. InsP_3 caused the ratio from the trapped indicator to decrease (i.e., Ca was released) in a dose-dependent manner, and this effect was blocked by the InsP_3 receptor antagonist heparin. Ca sequestration into the internal store was ATP-dependent, and reuptake into the InsP_3-sensitive pool was blocked by thapsigargin, a specific inhibitor of the Ca-ATPase of the internal store. We used this technique to investigate the role of Cl on the release and reloading of the InsP_3-sensitive internal store and found that Ca uptake was reduced in Cl-free solutions, suggesting an important function for Cl in the refilling of this pool.**

The use of Ca-sensitive fluorescent indicators such as fura-2 to measure cytosolic Ca has revealed much about intracellular Ca signaling and the release and reloading of inositol 1,4,5-trisphosphate (InsP_3)-sensitive Ca pools (1, 2). While enormous advances have been made toward understanding these complex processes through measurement of the cytosolic Ca concentration alone, a useful complement to this approach would be the ability to monitor the free Ca concentration directly within these pools in single cells. In some cases, direct measurement of intracompartmental Ca is the only practical way to resolve certain types of questions regarding the Ca metabolism of internal pools, particularly in cases where competition from other Ca-transporting mechanisms (i.e., at the plasma membrane) may obscure the response of interest. Although isotopic techniques can be used to monitor Ca transport in purified microsomal vesicles derived from subcellular compartments (3, 4), as well as in permeabilized cells (4, 5), these methods have many drawbacks: isotopic methods cannot be used to measure the free Ca concentration; they do not allow the comparison of different, single cells in a heterogeneous population; and they do not have the time resolution that is possible using fluorescence techniques.

To measure organellar Ca with fluorescent indicators, a Ca-sensitive probe must somehow be introduced into these compartments, and the K_d of the indicator must be in the appropriate range for monitoring the relatively high Ca concentrations which are likely to exist in these spaces. mag-fura-2, a fluorescent indicator with spectral properties similar to fura-2, has been used in the past, primarily for measuring cytoplasmic Mg (6, 7). However, this dye is also quite sensitive to free Ca concentrations above 5 μM (K_d for Ca = 53 μM; refs. 8 and 9), and we have taken advantage of this property to measure the Ca concentration of the internal store of cells in isolated rabbit gastric glands. When loaded as the acetoxymethyl (AM) ester, this dye distributes into both the cytoplasm and organelles, including the InsP_3-sensitive pool. Upon permeabilization with digitonin, the cytoplasmic dye is released, leaving entrapped mag-fura-2 in subcellular compartments where it can report changes in ionized Ca. We have used the above method to examine the role of Cl in the release and reloading of Ca in the internal stores (IS) and were able to draw conclusions using this technique that might have otherwise been difficult to discern by measuring only cytoplasmic Ca.

MATERIALS AND METHODS

Preparation of Glands and Dye Loading. Isolated rabbit gastric glands are long tubular structures consisting of several secretory cell types, including acid-secreting parietal cells and zymogen-secreting chief cells. Glands were prepared by collagenase digestion as described (10). Briefly, following retrograde perfusion of the descending aorta, the mucosa was scraped from the underlying muscularis, minced, and digested with type I collagenase (Sigma). After filtration through a nylon mesh, glands were rinsed several times and incubated with 2 μM mag-fura-2-AM or 2 μM fura-2-AM (Molecular Probes) for 30 min at room temperature. This was followed by several rinses in dye-free medium. An aliquot of glands was permitted to settle onto a glass coverslip, which was then mounted in a metal flow-through perfusion chamber, which has been described elsewhere in more detail (11). In this way, solutions could be maintained at 37°C and exchanged rapidly (1- to 2-sec turnover time), and artifacts due to leaked dye were eliminated.

Ratio Imaging Measurements. Simultaneous fluorescence measurements from 8 to 12 individual cells (both parietal and nonparietal cells) within the gland were made using a ratio imaging system (11). Both fura-2- and mag-fura-2-loaded cells were excited alternately at 350 nm and 385 nm on the stage

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Abbreviations: AM, acetoxymethyl; BAPTA, bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; InsP_3, inositol 1,4,5-trisphosphate; IS, internal store(s).

of a Zeiss IM35 microscope. The ratio of emitted light (>510 nm) from these two wavelengths (350 nm/385 nm) provides a measure of ionized Ca (or Mg) (refs. 8 and 12). Video images at each wavelength were acquired with a silicon-intensified target camera (model 66; Dage-MTI, Michigan City, IN) and processed by a model FD5000 image processor (Gould, Cleveland) to yield a background-corrected pseudocolor image reflecting the 350/385 ratio. Images were typically acquired every 5–10 sec. Fluorescence measurements reported here were always >20 times larger than background (autofluorescence), as determined on unloaded glands. In experiments employing the Ca chelator bis(2-aminophenoxy) ethane-*N*,*N*,*N'*,*N'*-tetracetic acid (BAPTA; Molecular Probes), cells were loaded with the AM ester by incubating with the compound at 10 μM for 20 min at room temperature. Throughout this manuscript *n* refers to the number of similar experimental runs. During each run, data were typically collected from at least two glands in the field. All experiments were repeated with glands from at least two different animals on separate days. The traces shown represent the average response of 8–12 individual cells in a single gland; similar responses were obtained for both parietal and nonparietal cells.

Solutions and Materials. NaCl Ringer's solution contained 145 mM NaCl, 2.5 mM K_2HPO_4, 10 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose at pH 7.4. "Intracellular buffer" contained 125 mM KCl, 25 mM NaCl, 10 mM Hepes, 3 mM Na_2ATP, and 0.1 mM $MgCl_2$, with the free Ca concentration clamped to 170 nM by using CaEGTA buffers (13) at pH 7.30. Cl-free Ringer's solution was the same as NaCl Ringer's described above, except that Cl was replaced with gluconate. NaCl and KCl were replaced with the corresponding gluconate salts for the Cl-free intracellular buffer, which still contained 0.2 mM Cl (as $MgCl_2$). Cells were permeabilized by adding 10 μM digitonin, a concentration that is known to selectively perforate the plasma membrane in gastric cells (14). Unless otherwise noted, all reagents were obtained from Sigma. Thapsigargin was from LC Services (Woburn, MA).

RESULTS AND DISCUSSION

We originally observed that the responses of intact mag-fura-2-loaded cells to cholinergic stimulation were consistent with this dye being able to report changes within the InsP_3-sensitive IS. When glands were loaded with the AM ester of mag-fura-2, the dye appeared to be uniformly distributed throughout the cell (Fig. 1*A*). Upon stimulation with the cholinergic agonist carbachol (100 μM, a maximal dose that causes the rapid and sustained release of all the Ca in the IS; refs. 15 and 16), there was a transient peak followed by a small, sustained decrease in the mag-fura-2 ratio (Fig. 2*A*). Although these changes measured in intact cells could have been due to alterations of the cytoplasmic Mg concentration, the initial rapid increases in the ratio seen in cells loaded with either fura-2 [which is insensitive to Mg and monitors primarily the cytoplasmic Ca concentration (ref. 12); Fig. 2*C*] or mag-fura-2 (Fig. 2*A*) were quite similar. As shown in Fig. 2 *B* and *D*, these initial peaks were absent when glands were coloaded with BAPTA-AM (K_d for Ca = 107 nM), which effectively buffers rapid transients in the cytoplasmic Ca concentration (17). It was also observed that the carbachol-induced decrease in the mag-fura-2 ratio was larger in the BAPTA-loaded cells (Fig. 2*B*) compared to control (Fig. 2*A*). Similar observations have been reported recently in mag-fura-2- and mag-fura-2- plus BAPTA-loaded rat pancreatic and salivary gland acini in response to cholinergic agonists (18).

Because mag-fura-2 is quite sensitive to Ca concentrations >5 μM and the AM ester method of loading is known to sometimes result in compartmentalization into organelles (19), we suspected that the dye was reporting the Ca concentration within both the cytoplasm and the IS. These observations in intact cells prompted us to investigate these phenomena further in gastric glands that had been permeabilized with digitonin.

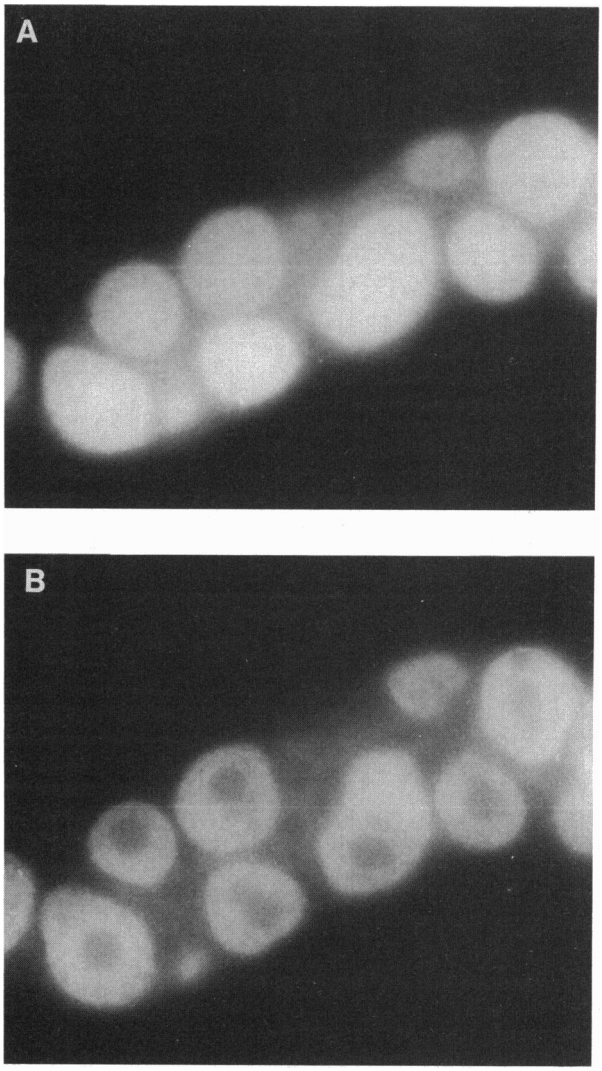

FIG. 1. Fluorescence micrographs (corrected for autofluorescence) of mag-fura-2-loaded rabbit gastric gland in NaCl Ringer's solution (*A*) and in intracellular buffer after permeabilization with 10 μM digitonin (*B*). Glands exist as long tubular structures containing several epithelial cell types. The very bright, large cells are parietal cells. Approximately 30% of the mag-fura-2 fluorescence remains following permeabilization. Similar punctate distribution of fluorescence corresponding to sequestered dye was also evident after permeabilization in cells incubated in fura-2 (not shown).

When mag-fura-2-loaded cells were incubated in intracellular buffer, digitonin caused a large loss of fluorescence (Fig. 1*B*). Sixty percent of the mag-fura-2 signal was eliminated upon digitonin treatment (not shown), as assessed by measurement of the 350-nm excitation, which is the isoexcitation point for this dye. Addition of the fluorescence quencher Mn (0.5 mM) only reduced the signal by an additional 12%, suggesting that most of the cytosolic contribution was eliminated by digitonin permeabilization alone. The remaining fluorescence (at least 28%) likely resides in various membrane-bounded compartments within the cell. This residual dye signal (corresponding to entrapped probe) was then measured using fluorescence ratio imaging while clamping the Ca and Mg concentrations in the perfusing solution. As

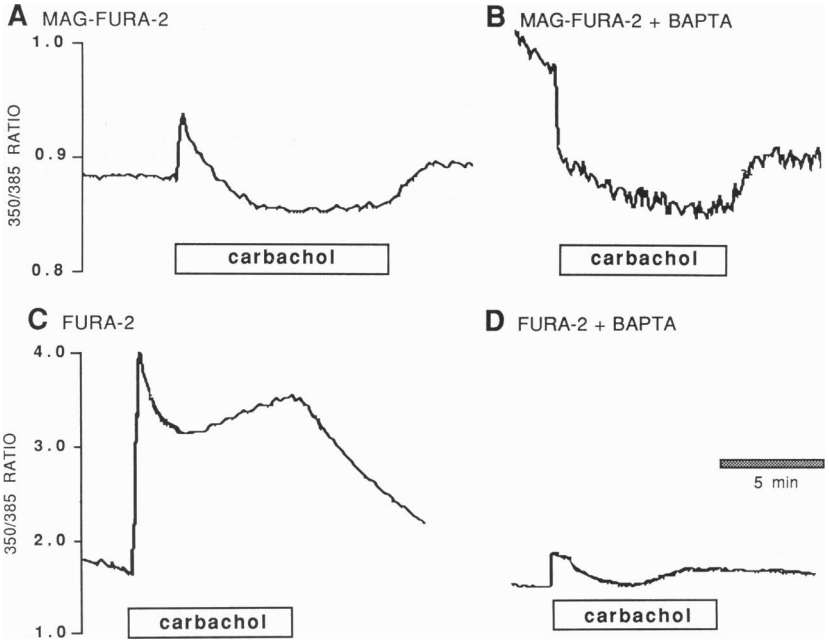

FIG. 2. Responses of *intact* cells from the isolated rabbit gastric gland to carbachol (100 μM) in NaCl Ringer's solution. (*A*) mag-fura-2-loaded cells displayed a transient spike in the 350/385 ratio after carbachol addition. This was followed by a distinctive excursion below the initial baseline, with recovery back to the initial value when the agonist was removed (n = 47). (*B*) When BAPTA and mag-fura-2 were coloaded, the initial spike was absent, and the decrease below the starting value was larger and persisted until agonist was removed (n = 36). (*C*) Ca response of fura-2-loaded cells within a single gland after carbachol treatment. These cells exhibited the typical peak/plateau behavior characteristic of secretory cells after cholinergic stimulation (n = 13). (*D*) BAPTA effectively buffers rapid, carbachol-induced transients in the cytoplasmic Ca concentration, as seen in these fura-2-loaded cells (n = 5).

shown in Fig. 3*A*, there was an increase in the ratio upon permeabilization, consistent with the idea that this probe was now monitoring only compartments in the cell where the calcium concentration was high. Maintenance of this elevated ratio was absolutely dependent on the presence of both ATP *and* Ca in the intracellular buffer (data not shown), suggesting active sequestration of Ca into compartment(s) where dye had also accumulated. Using the method of Grynkiewicz *et al.* (12) to calibrate ratios from compartmentalized mag-fura-2 (and assuming a K_d of 53 μM), we estimate that a ratio of 1.75 (which was a typical starting value in the presence of ATP and 170 nM Ca) corresponds to a free Ca concentration in the IS of $\approx$127 μM. This value for the Ca concentration in the IS may be somewhat overestimated due to the possible presence of Mg in the internal store, since mag-fura-2 responds similarly to both ions.

When these permeabilized cells were stimulated with 5 μM InsP_3, the ratio decreased rapidly, corresponding to a change in Ca concentration of $\approx$49 μM. These estimated changes in the IS are consistent with observations that the cytosolic Ca concentration can be elevated to levels >1 μM during agonist stimulation (15), assuming that the IS occupies around 2% of the total cell volume* and that Ca buffering in the IS and the cytosol is comparable. When InsP_3 was removed from the perfusion, the ratio increased, indicating that there had been a reversible release and then reuptake of Ca from a digitonin-resistant store. Maximal release was elicited by 1–5 μM InsP_3, with apparent half-maximal stimulation at 260 nM (data not shown). In addition, 30 μM sphingosine and 100 μM GTP, which are known releasers of Ca from IS (5, 22), both mobilized Ca from the IS (data not shown).

When similar experiments were performed in permeabilized fura-2-loaded cells, InsP_3 had no effect, even though this probe was also compartmentalized. Possibly the relatively high Ca concentration found in the IS saturated this dye, which has a much lower K_d for Ca. Thus, it appears (at least for this cell type) that changing Ca levels within the IS do not interfere with fura-2 measurements of cytoplasmic Ca, although such interference from compartmentalized fura-2 has been described in hepatocytes (23).

Fig. 3*A* also shows that removal of ATP from the intracellular buffer caused a larger decrease in the ratio than did maximal stimulation with InsP_3, and under these ATP-free conditions InsP_3 had no further effect on release of Ca from the IS. Thus, this technique monitors Ca in both InsP_3-sensitive and -insensitive (but ATP-dependent) pools. The InsP_3 receptor antagonist heparin (4) blocked or attenuated Ca release when added prior to InsP_3 and reversed the effects of InsP_3 when applied during stimulation (Fig. 3*B*). Thapsigargin, which specifically and irreversibly inhibits the Ca-ATPase of the InsP_3-sensitive IS of many cell types, including rabbit gastric cells (24–26), induced a relatively slow decline in the ratio in permeabilized cells (Fig. 3*C*), and subsequent treatment with InsP_3 released residual Ca from the IS. However, addition of thapsigargin to cells following maximal InsP_3 stimulation had no further effect on the mag-fura-2 ratio (Fig. 3*D*). After thapsigargin exposure, the recovery normally seen after InsP_3 removal was absent (compare Fig. 3 *D* and *A*).

The results obtained with InsP_3, heparin, and thapsigargin in mag-fura-2-loaded, permeabilized cells indicate the following: (*i*) mag-fura-2 monitors the Ca concentration in an InsP_3-sensitive IS. (*ii*) InsP_3 and thapsigargin affect Ca transport in the same IS.† (*iii*) Under control conditions, there is a relatively small, but finite and constant, leak of Ca from the IS, which is balanced by continual reaccumulation of Ca into this space. Since similar results were obtained using AtT-20 cells, a neuroendocrine cell line, and also HT-29/B6 cells, a colon carcinoma cell line (data not shown), the method described here may be generally applicable to a wide variety of cell types.

We had previously noted that the calcium response of gastric cells to carbachol stimulation (as assessed by fura-2) was greatly attenuated in the absence of extracellular Cl (P. A. Negulescu and T.E.M., unpublished observations). In the present work, brief treatment with Cl-free solutions in intact cells allowed nearly normal carbachol-induced increases in the fura-2 ratio, but longer-term treatment (15 min) with Cl-free solution blocked the carbachol response (Fig. 4*A*). The carbachol response could be partially restored by

*This is the estimated fractional volume of the "calciosome," an organelle that has been proposed as the Ca-storing compartment involved in InsP_3-induced Ca release (21).

†Although the InsP_3- and thapsigargin-sensitive Ca pools appear to be coextensive in gastric cells, in some other cells types the InsP_3-sensitive pool appears to be a subset of a larger thapsigargin-sensitive store (25); thus, this property may be cell-type dependent.

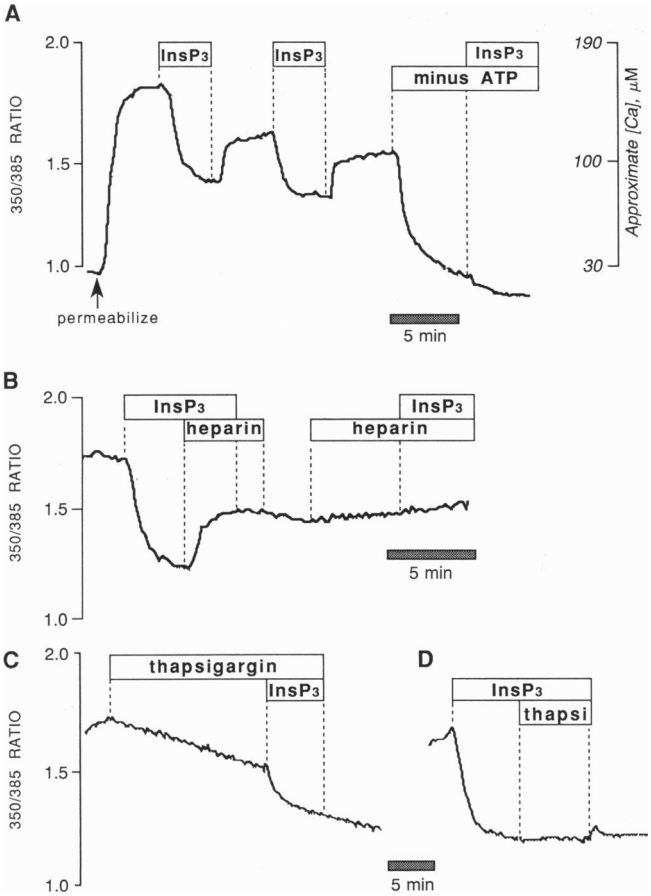

FIG. 3. mag-fura-2 monitors Ca in the InsP_3-sensitive IS of digitonin-permeabilized cells. (*A*) Effects of InsP_3 in permeabilized, mag-fura-2-loaded rabbit gastric glands. Cells were initially perfused with NaCl Ringer's solution followed by a brief change to intracellular buffer without digitonin or ATP. The solution was then switched to intracellular buffer containing 3 mM ATP and 10 μM digitonin. This protocol permitted introduction of impermeant compounds (i.e., ATP and InsP_3) and also improved the resolution of the signal emanating from the IS because probe had been eliminated from the cytosolic compartment. The sustained increase in the 350/385 ratio was dependent on the presence of both ATP and Ca in the bath (not shown). Thus, ATP promotes sequestration of Ca into digitonin-resistant pool(s), which was released repeatedly by 5 μM InsP_3, with reuptake of Ca between stimulations. Removal of ATP from the solution also led to the apparent release of Ca (possibly by reversal of the Ca-ATPase; ref. 20), and subsequent treatment with 5 μM InsP_3 elicited no further release (n = 10). (*B*) The effect of InsP_3 is reversed by heparin. Cells were permeabilized and then stimulated with 5 μM InsP_3. Addition of heparin (100 μg/ml) during InsP_3 treatment reversed the effect of InsP_3 (n = 5). Pretreatment with heparin blocked or attenuated the InsP_3 response (n = 7). (*C*) Treatment of permeabilized cells with 1 μM thapsigargin reveals a slow leak of Ca from the IS with additional Ca release elicited by 5 μM InsP_3 (n = 6). (*D*) Reloading of Ca into the IS is blocked by thapsigargin. Gastric cells were permeabilized, and 5 μM InsP_3 was applied, followed by perfusion with 1 μM thapsigargin. No recovery upon InsP_3 removal was observed following thapsigargin treatment (n = 29). thapsi, thapsigargin.

readdition of Cl to the bath (data not shown). These experiments suggested that Cl might be required for refilling of the IS.

The lack of a plateau phase in the fura-2 response (corresponding to Ca entry from the extracellular space; ref. 15) seen under Cl-free conditions is suggestive of impaired Ca entry across the plasma membrane and could possibly account for inhibition of refilling of the IS. However, since it has been established previously for this cell type that removal

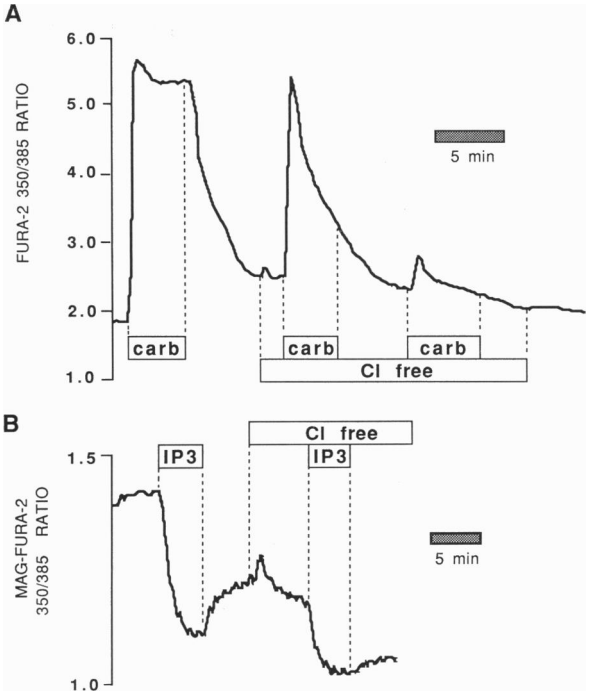

FIG. 4. Refilling of the IS is inhibited in the absence of Cl. (*A*) Fura-2-loaded cells were first given a brief control stimulation with 100 μM carbachol, and cells responded with the typical peak in cytoplasmic Ca (due to release from the IS) followed by a sustained plateau phase (corresponding to entry of Ca from the extracellular space via the plasma membrane; refs. 15 and 16). Subsequent stimulation after a brief incubation with Cl-free solution produced a comparable peak in Ca, although the plateau was attenuated. However, attempts to restimulate in the continued absence of Cl were unsuccessful (n = 3). (*B*) mag-fura-2-loaded glands were permeabilized as in Fig. 3 and treated initially with 5 μM InsP_3 to establish a control response, with recovery upon InsP_3 removal. Switching to Cl-free intracellular buffer resulted in a transient increase in the mag-fura-2 ratio, which was not due to perturbations in the volume of the IS (because adding 75 mM sucrose to increase buffer osmolarity, testing for shrinkage effects, had no influence on the ratio), but may be due to a nonspecific effect of the Cl-free solution on the dye (see text for details). When cells were then restimulated with InsP_3, Ca release was similar to the control, but reuptake into the IS was highly attenuated (n = 9). IP3, InsP_3; carb, carbachol.

of extracellular Cl results in rapid (2–3 min) depletion of cytosolic Cl (27), we considered the possibility that there is an additional, direct effect of Cl-free treatment on the refilling of the InsP_3-sensitive pool. Using only fura-2 to assess cytoplasmic Ca, it would have been difficult to ascertain whether this effect of Cl-free treatment on refilling of the IS was due to reduced Ca entry at the plasma membrane, inhibition of Ca pumping into the IS, or a combination of both. Thus, mag-fura-2 was used to test whether Cl-free conditions were directly affecting Ca transport of the IS (Fig. 4*B*).

When permeabilized cells were treated with Cl-free intracellular buffer, there was a transient increase followed by a slow decrease in the mag-fura-2 ratio, indicating that the long-term effect of the Cl-free treatment was to cause a loss of Ca from the IS. The initial increase may be due to a nonspecific effect of the Cl-free solution on the mag-fura-2 fluorescence, since similar increases were observed *in vitro* when switching from Cl-containing to Cl-free buffer (A. Hofer and T. Machen, unpublished observations). However, subsequent treatment with InsP_3 in the Cl-free intracellular buffer still elicited a decrease in the ratio, consistent with the release of Ca from the IS, while the reaccumulation of Ca by the IS normally seen upon InsP_3 removal was blocked.

It has been shown previously that Ca accumulation and ATPase activity of isolated endoplasmic reticulum membranes are reduced by Cl-free solutions (3). Here we have extended these findings to show an important role for Cl in the reloading of Ca into the $InsP_3$-sensitive IS in permeabilized cells. A possible interpretation of these results is that reuptake of Ca by the Ca-ATPase is prevented because Ca cannot be pumped into the IS in the absence of a permeant counterion. This phenomenon may help to explain recent observations of abnormal Ca signaling in cystic fibrosis, a disease associated with reduced membrane Cl conductance at both the plasma membrane and in internal organelles (28).

mag-fura-2 offers many advantages for assessing the Ca transport activity of the IS: an actual measurement of the free Ca concentration is obtained, the time resolution is good, and measurements can be made on single, intact (though permeabilized) cells. This approach should have wide application. Because mag-fura-2 accumulates in many subcellular compartments, including mitochondria (unpublished observations), the judicious use of pharmacological tools should allow the investigation of the Ca transport properties of these different organelles individually, especially in flat cells where the different subcellular compartments may be localized with the imaging technique. Moreover, since disruption of intracompartmental Ca gradients has profound effects on both protein secretion and cell growth (29, 30), this technique is also of potential use in investigating the role(s) of organellar Ca concentrations in these processes. It may also be possible to exploit such uptake of dyes into the IS for investigating the subtle physiological features of Ca release and reloading in intact, nonpermeabilized cells and to extend this approach to measure pH and Na and Cl concentrations in intracellular organelles in single cells using other fluorescent indicators (2).

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