Spatial distribution and quantitation of free luminal [Ca] within the $InsP_3$-sensitive internal store of individual BHK-21 cells: ion dependence of $InsP_3$-induced Ca release and reloading

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ABSTRACT **Free [Ca] within organelles of permeabilized BHK-21 cells was measured using ratio imaging of compartmentalized mag-fura-2. In BHK-21 cells, this dye monitors free [Ca] in principally one type of ATP-dependent Ca-sequestering organelle in which intrastore Ca was released uniformly and entirely by 100 nM thapsigargin or removal of ATP or Ca from the bath, and was reduced by 85% upon treatment with a supramaximal dose of $InsP_3$ (6 μM). Examination of the spatial distribution of $InsP_3$-sensitive Ca stores showed that $InsP_3$ released Ca throughout all regions of the cell, although we often noted a perinuclear region (which we speculate may correspond to the Golgi apparatus) with reduced responsiveness to $InsP_3$. $InsP_3$-induced changes of intraluminal Mg could not be detected. Cyclic ADP-ribose, ryanodine, caffeine, mitochondrial inhibitors, and GTP, agents known to influence intraorganellar Ca sequestration in other cell types, were all without effect on the mag-fura-2 ratio. In situ calibration of the mag-fura-2 ratio with Ca ionophores revealed that the average free intraorganellar [Ca] was initially 188 ± 21 μM in the presence of 170 nM free Ca and 3 mM ATP, and was reduced to 25 ± 5 μM upon stimulation with 6 μM $InsP_3$. The ionic dependence of the release and reloading process was also investigated. The presence of either K, Na, or Cl could consistently support both $InsP_3$-induced release and the refilling of stores with Ca, but physiological concentrations of HCO_3 were effective in sustaining the response in only 24% of cells examined.—Hofer, A. M., Schlue, W.-R., Curci, S., Machen, T. E. Spatial distribution and quantitation of free luminal [Ca] within the $InsP_3$-sensitive internal store of individual BHK-21 cells: ion dependence of $InsP_3$-induced Ca release and reloading. *FASEB J.* 9, 788–798 (1995)**

Key Words: ratio imaging • thapsigargin • Ca ionophores

Virtually all eukaryotic cells possess subcellular stores of compartmentalized Ca that are available for mobilization into the cytoplasm during agonist-stimulated Ca signaling events. Ca stores that can be released by inositol 1,4,5-trisphosphate ($InsP_3$)[5] are nearly ubiquitous, but other types of releasable Ca pools are known to coexist in the same cell type, for example, stores that are sensitive to ryanodine or caffeine, cyclic ADP ribose, or GTP (see, for example, refs 1, 2 for review). The particular complement of Ca-sequestering pools present depends on the cell type.

We have developed a fluorescence technique that allows direct measurement of free [Ca] within the $InsP_3$-releasable pool using the Ca indicator mag-fura-2 (3, 4). This probe becomes compartmentalized in intracellular organelles when loaded as the acetoxymethyl ester derivative, where, by virtue of its relatively high K_d for Ca (53 μM; refs 5, 6) it reports changes in free [Ca] in this space. Our previous studies were performed on gastric epithelilal cells, which have a relatively complex collection of compartments capable of ATP-dependent Ca accumulation as measured by this probe. In addition to the $InsP_3$-sensitive store, gastric cells were shown to have an unusually robust mitochondrial Ca uptake activity, as well as a Ca-sequestering pool that was determined to be distinct from either the $InsP_3$-sensitive pool or the mitochondrial store. A disadvantage of using these cells is that the presence of multiple pools complicates the quantitation of free [Ca] in any one given pool, as described in detail in ref 4.

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[5]Abbreviations: $InsP_3$, inositol 1,4,5–trisphosphate; SL–O, streptolysin–O; NMG, *N*–methyl glucamine.

0892-6638/95/0009-0788/$01.50.

A previous paper concluded from measurements on compartmentalized fura-2 that Ca was released uniformly from the internal stores by $InsP_3$, but quantitation of free [Ca] changes was difficult due to the low K_d of fura-2 (7). Moreover, there was no consideration of the issue of the dye becoming compartmentalized in multiple pools, with ensuing complications in interpreting the data. Thus, precise determination of the intraluminal free [Ca] of the internal store has not been possible before, but this value is of potential interest to many investigators, e.g., for understanding the kinetics of the $InsP_3$-induced release and subsequent refilling of stores. Furthermore, many luminal enzymes and resident proteins of organelles bind Ca, and in some cases a regulatory role for Ca binding has been suggested. For instance, in the endoplasmic reticulum BiP, protein disulfide isomerase, reticulocalbin, and reticuloplasmins are luminal Ca binding proteins, the location of which may coincide with the $InsP_3$-sensitive store (8–10). It would be interesting to know whether the binding affinities for Ca lie within a range that might suggest regulation by [Ca] changes occurring during the $InsP_3$-induced release.

We report here on a fibroblastic cell type, the BHK-21 cell line, that appears to have essentially one functional type of store capable of accumulating Ca in an ATP-dependent manner as measured by the mag-fura-2 technique. The fact that mag-fura-2 monitors [Ca] in mainly one pool in this cell type greatly simplifies the interpretation of data and permits the estimation of the amount of Ca released during $InsP_3$ stimulation. Furthermore, the flattened morphology of these cells makes it possible to examine the spatial distribution of $InsP_3$-responsive stores using imaging techniques and conventional optics.

We also investigated the ionic dependence of $InsP_3$-induced Ca release and reloading in BHK-21 cells. Because Ca pumping by the Ca-ATPase is an electrogenic process, it is expected that refilling the internal store will require the movement of a counterion. Similarly, a counterion would also be expected to accompany Ca during the massive release of Ca from the internal store during agonist stimulation. In fact, it has been established in a number of cell types that a permeant ion must be present in order to elicit a response, but the particular ionic requirements for this process and the pharmacological characteristics of these parallel pathways appear to vary depending on cell type. For example, it has been reported that Cl is required for the refilling of ATP-dependent Ca pools in pancreatic and gastric cells (3, 11). The coupling of Ca fluxes to univalent cations, with particular emphasis on the role of K in the release process, has also been examined in liver, brain, and platelets (12–15). To our knowledge, the participation of HCO_3 ions has not been investigated. Here we report that Cl, Na, and K all participate in the release and reloading process after $InsP_3$ stimulation in BHK-21 cells. Physiological concentrations of HCO_3 only occasionally were able to support the response.

MATERIALS AND METHODS

Cell culture, dye loading, and permeabilization

BHK-21 cells were grown in Eagle's MEM with Earle's BSS containing 10% fetal calf serum and 10% Difco tryptose phosphate broth, and were maintained in a humidified incubator at 37°C in the presence of 5% CO2/95% air. Cells were plated onto glass coverslips and used the following day for ratio imaging experiments. For dye loading, cells were incubated with 5 mM mag-fura-2-AM in tissue culture medium at 37°C for 20 min, after which they were placed for 1 min into cold permeabilization buffer (4°C) containing reduced streptolysin-O (SL-O). In some experiments cells were permeabilized at 37°C with 1 mM digitonin as described previously (3, 4). Both permeabilization protocols produced equivalent results. Coverslips were mounted immediately into a metal flow-through perfusion chamber that has been described previously in more detail (16) and perfused continuously with "intracellular buffer" at 37°C. SL-O binds to the plasma membrane at 4°C, but is unable to form the perforating pore complex in the plasma membrane until the temperature is raised to 37°C (17). This procedure permits selective permeabilization of the plasma membrane, allowing cytosolic dye to leak out but leaving compartmentalized mag-fura-2 behind in intact organelles.

Ratio imaging experiments

After permeabilization with SL-O or digitonin, the cells were then ready for ratio imaging measurements, using a system described previously in more detail (16). The chamber containing the coverslip was fitted onto the heated stage of a Zeiss IM 35 microscope and cells were observed with a 40X objective. Because cells were perfused continuously with intracellular buffer (the turnover time of the perfusion chamber was approximately 2 s) both cytosolic mag-fura-2 and and dye that might leak from subcellular compartments were eliminated. Cells were excited alternately at 350 nm and 385 nm, and video images of the resulting fluorescence emission from each excitation wavelength (collected at 510 nm) were recorded by a silicon-intensified target camera (model 66, Dage-MTI, Michigan City, Ind.). Raw images were forwarded to a Gould FD5000 image processor (Cleveland, Ohio) that produced a background-corrected pseudocolor image of the 350/385 ratio throughout the cell. Images were generally acquired every 10 s. The 350/385 ratio is proportional to the free [Ca] or [Mg] in subcellular compartments resistant to permeabilization. The records shown depict the averaged response of 1–10 individual cells in a single experiment; "*n*" refers to the number of independent experimental runs. Errors are ± SEM.

Solutions and materials

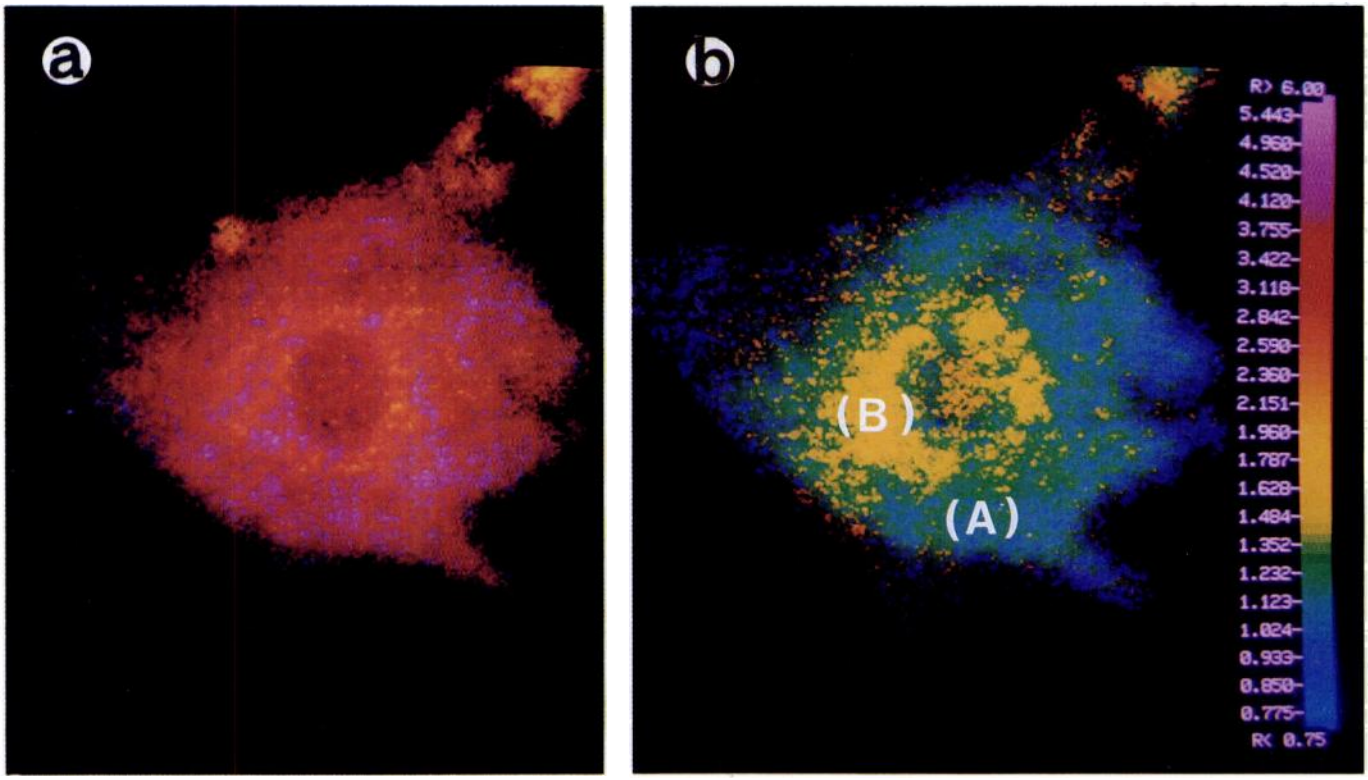
Permeabilization buffer contained (in mM): 125 KCl, 25 NaCl, 10 HEPES, pH 7.40, and 1.5 units/ml reduced streptolysin-O (Murex Diagnostics Limited, Dartford, England). Intracellular buffer contained (in mM): 125 KCl, 25 NaCl, 10 HEPES, 3 Na_2ATP (unless otherwise stated), 0.1 $MgCl_2$, pH 7.20, with free [Ca] clamped to 170 nM using CaEGTA buffers that were prepared by methods described previously (18). Calibration solutions (as in Fig. 4) had the same composition as the intracellular buffer, except that ATP and EGTA were omitted, and the solutions were supplemented with the indicated amount of $CaCl_2$ and the Ca/Mg ionophore 4-Br-A23187. Zero Ca calibration buffer contained 0.8 mM EGTA. The free [Ca] already present in our nominally Ca-free solution was estimated to be approximately 5 μM. HCO_{3e}-containing solutions (as in Fig. 5*B*) were prepared with 25 mM added $NaHCO_3$ ($NaCl$ replacement) and were bubbled with 5% CO_2/95% air. For experiments using Na and/or K-free solutions (as in Figs. 5*C*, *D*), *N*-methyl glucamine (NMG) chloride or choline chloride were used as replacements, and the tris-salt of ATP was used. For Cl-free solutions (as used in Fig. 5*A*), the corresponding gluconate salt was used. The sucrose-based intracellular solutions used in experiments of the type shown in Fig. 6*A* contained (in mM): 300 sucrose, 10 HEPES, 3 Tris ATP, 0.1 $MgCl_2$, pH 7.20, and 170 nM free Ca. Iso-osmolar replacement of sucrose with the indicated amounts of K gluconate, Na gluconate, or NMG Cl were made for experi-

ments depicted in Figs. 6A, 6B. HCO_3-containing sucrose buffer used in Fig. 6C was prepared by adding 20 mM NMG free base to the sucrose buffer and bubbling with either 95% O_2/5% CO_2 or 95% air/5% CO_2 gas mixture until a stable pH of 7.20–7.30 was achieved. We noted better results with the CO_2/air mixture than with the CO_2/O_2 mixture, and speculate that this may arise from accelerated photobleaching of the fluorophore in the presence of O_2. For some experiments using HCO_3-containing solutions, BAPTA was used instead of EGTA (which is highly pH-sensitive) to buffer free [Ca] to 170 nM. Cyclic ADP-ribose was a generous gift from H. C. Lee, University of Minnesota. Thapsigargin was from L.C. Services (Woburn, Mass.). All other reagents were obtained from Sigma (St. Louis, Mo.) unless otherwise stated.

RESULTS

Spatial distribution of $InsP_3$-responsive Ca stores

One advantage of measuring [Ca] changes in internal stores directly using the mag-fura-2 technique (as opposed to measuring reciprocal changes in the cytoplasm) is that there is the potential to investigate the spatial localization of $InsP_3$-sensitive pools using imaging techniques. The flattened morphology of BHK cells makes them particularly amenable to this type of experimentation. **Figure 1A** depicts a pseudocolor image of a typical BHK-21 cell that was loaded with mag-fura-2-AM and then permeabilized with SL-O in intracellular buffer. Cytosolic dye was eliminated as a consequence of plasma membrane permeabilization, leaving behind compartmentalized mag-fura-2. In subsequent sections of this paper we demonstrate that the mag-fura-2 ratio is a measure of intraluminal free [Ca] with minimal interference from Mg under our experimental conditions. In the presence of 3 mM ATP and 170 nM free Ca, internal stores were loaded with Ca as indicated by the relatively high ratio. The darkened central area of the image corresponded to the nucleus, which was devoid of organelles and thus did not accumulate mag-fura-2 in the permeabilized cell. It was noted that there were subtle variations in the ratio throughout the resting cell, reflecting an inho-



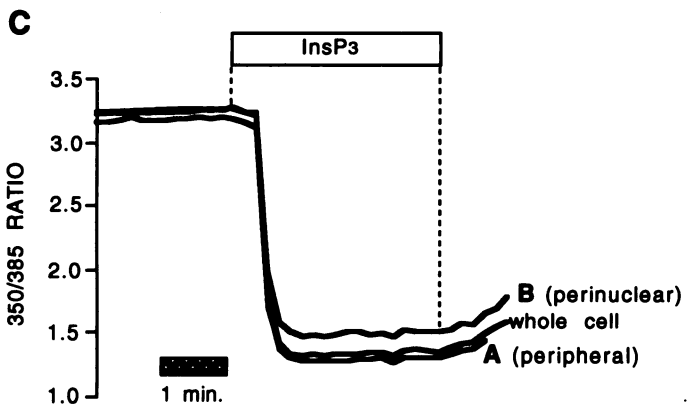


Figure 1. *a*) Pseudocolor ratio image of streptolysin O–permeabilized BHK–21 cell bathed in intracellular buffer containing ATP and 170 nM free Ca. Cytosolic dye has been washed out leaving mag–fura–2 entrapped in organelles. The relatively high ratio (corresponding to the magenta color) indicates that the free intraluminal [Ca] is elevated in all areas of the cell. *b*) Same cell 130 s after stimulation with 6 μM $InsP_3$. The mag–fura–2 ratio decreases throughout the cell as indicated by the blue or yellow color, although not to the same extent in all regions. We often (in about 30% of cells) noticed a perinuclear region with attenuated responsivenes to $InsP_3$ (yellow color) that was especially prominent in this particular cell. *c*) The mag– fura–2 ratio was collected from selected areas of the cell (marked "(A)" and "(B)" in the micrograph *B* corresponding to peripheral (A) and perinuclear (B) zones and plotted with respect to time. Zone (B) exhibits a smaller decrement in the ratio upon $InsP_3$ treatment than zone (A). Shown for comparison is the averaged response throughout the entire cell ("whole cell").

mogeneous distribution of Ca in subcellular compartments. These variations disappeared when cells were calibrated with ionophores (as in Fig. 4), indicating that the difference in ratio was due to an actual difference in free [Ca], and was not a consequence of aberrant behavior of mag-fura-2 in differing environments. As seen in Fig. 1*B*, treatment with 6 μM $InsP_3$ (a supramaximal dose) resulted in a rapid decrease in the ratio in all portions of the cell, indicating that $InsP_3$-responsive stores are distributed throughout the cell, and are not localized to discrete regions in this cell type.

We often observed a perinuclear region that was slightly less responsive to $InsP_3$ than the periphery of the cell. Such a region was conspicuous in approximately 30% of cells and was also apparent in the cell depicted in Fig. 1*B*. Figure 1*C* compares the ratio change upon $InsP_3$ stimulation in selected regions near the nucleus, at the periphery, and an average of the entire cell. The magnitude of the $InsP_3$-induced decrease in these perinuclear "hotspots" was 75 ± 6% of that observed in the remainder of the cell (n = 7). This zone of reduced responsiveness typically constituted a small proportion of the total area and, as such, did not greatly influence the overall ratio change measured in the whole cell. Our estimate of the free [Ca] within the $InsP_3$-sensitive pool is not affected to a large extent by these unresponsive zones, as discussed further below.

Treatment with 100 nM thapsigargin caused an even greater reduction in the ratio than stimulation with 6 μM $InsP_3$; this decrease in the ratio was observed in all portions of the cell, both at the periphery and in the perinuclear region (not shown).

Characterization of pools in BHK-21 cells: evidence for one type of ATP-dependent Ca-sequestering pool sensitive to thapsigargin

We used a pharmacological approach to investigate whether there were additional Ca stores present in BHK-21 besides

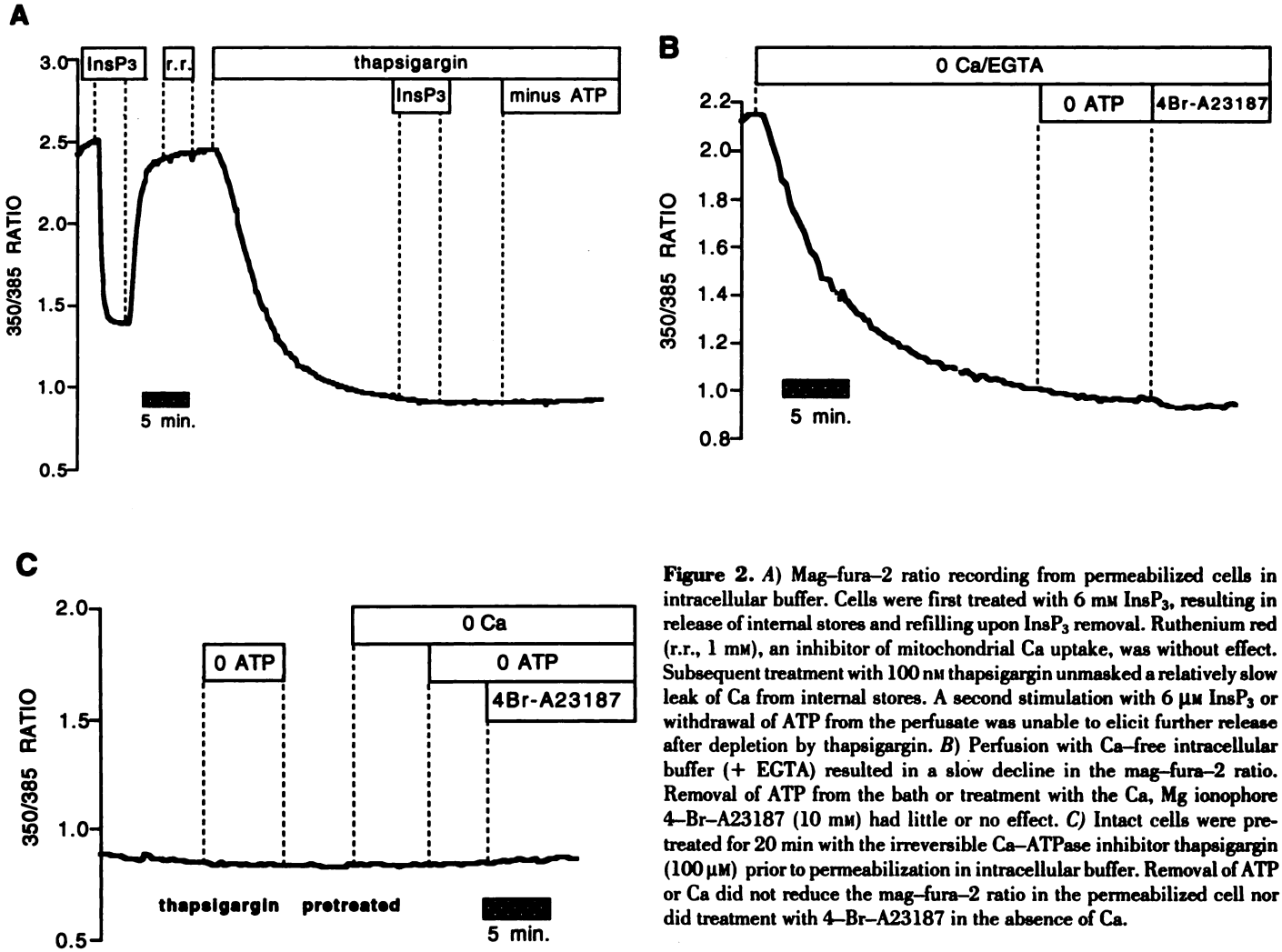


Figure 2. *A*) Mag–fura–2 ratio recording from permeabilized cells in intracellular buffer. Cells were first treated with 6 mM $InsP_3$, resulting in release of internal stores and refilling upon $InsP_3$ removal. Ruthenium red (r.r., 1 mM), an inhibitor of mitochondrial Ca uptake, was without effect. Subsequent treatment with 100 nM thapsigargin unmasked a relatively slow leak of Ca from internal stores. A second stimulation with 6 μM $InsP_3$ or withdrawal of ATP from the perfusate was unable to elicit further release after depletion by thapsigargin. *B*) Perfusion with Ca–free intracellular buffer (+ EGTA) resulted in a slow decline in the mag–fura–2 ratio. Removal of ATP from the bath or treatment with the Ca, Mg ionophore 4–Br–A23187 (10 mM) had little or no effect. *C)* Intact cells were pretreated for 20 min with the irreversible Ca–ATPase inhibitor thapsigargin (100 μM) prior to permeabilization in intracellular buffer. Removal of ATP or Ca did not reduce the mag–fura–2 ratio in the permeabilized cell nor did treatment with 4–Br–A23187 in the absence of Ca.

the $InsP_3$-sensitive pool. It was also essential to validate that our technique provided a measure of intraluminal free [Ca] for this cell type. Because mag-fura-2 is also sensitive to changes in free [Mg] (K_d for Mg = 1.5 mM, ref 6), it was necessary to determine the contribution of intrastore [Mg], if any, to the measured ratio.

Figure 2A shows the response of a permeabilized BHK cell to 6 μM $InsP_3$. Maximal release was achieved within 64 ± 5 s (data from 15 records), and the effect of $InsP_3$ was blocked by 100mg/ml heparin (n = 2; not shown), a competitive antagonist of $InsP_3$ binding to its receptor (19). Rapid refilling occurred upon removal of $InsP_3$. Subsequent treatment with 100 nM thapsigargin, a specific blocker of the Ca-ATPase in the internal store membrane (20), resulted in a relatively slow decline in the mag-fura-2 ratio (reflecting a passive leak from the store) that typically required 19 ± 2 min (n = 6) for completion. Cyclopiazonic acid, a reversible blocker of the ATPase (21), had a similar action (not shown; n = 4). As seen in the trace, $InsP_3$ was ineffective in eliciting any response after 20 min in the presence of thapsigargin (n = 5), indicating that the $InsP_3$-sensitive pool had been completely emptied. Removal of ATP from the bathing solution (which usually produced a relatively slow decline in the ratio in control cells) also had no further effect on the mag-fura-2 ratio in thapsigargin treated cells (n = 7).

We interpreted the ratio changes seen in Fig. 2A as reflecting movements of sequestered Ca into and out of the internal store. These experiments indicated the presence of one functional type of ATP-dependent Ca pool in BHK-21 cells that was sensitive to thapsigargin and cyclopiazonic acid. However, it was important to understand whether there were additional releasable Ca stores in these cells and to what extent intraluminal [Mg] might be influencing our measurements.

In the intact cell under physiological conditions, cytosolic free [Mg] has been reported to be in the range of 0.5 to 1.0 mM (22). The intracellular buffer used in our experiments included 0.1mM $MgCl_2$, which was necessary because the thapsigargin-sensitive Ca-ATPase uses Mg-ATP

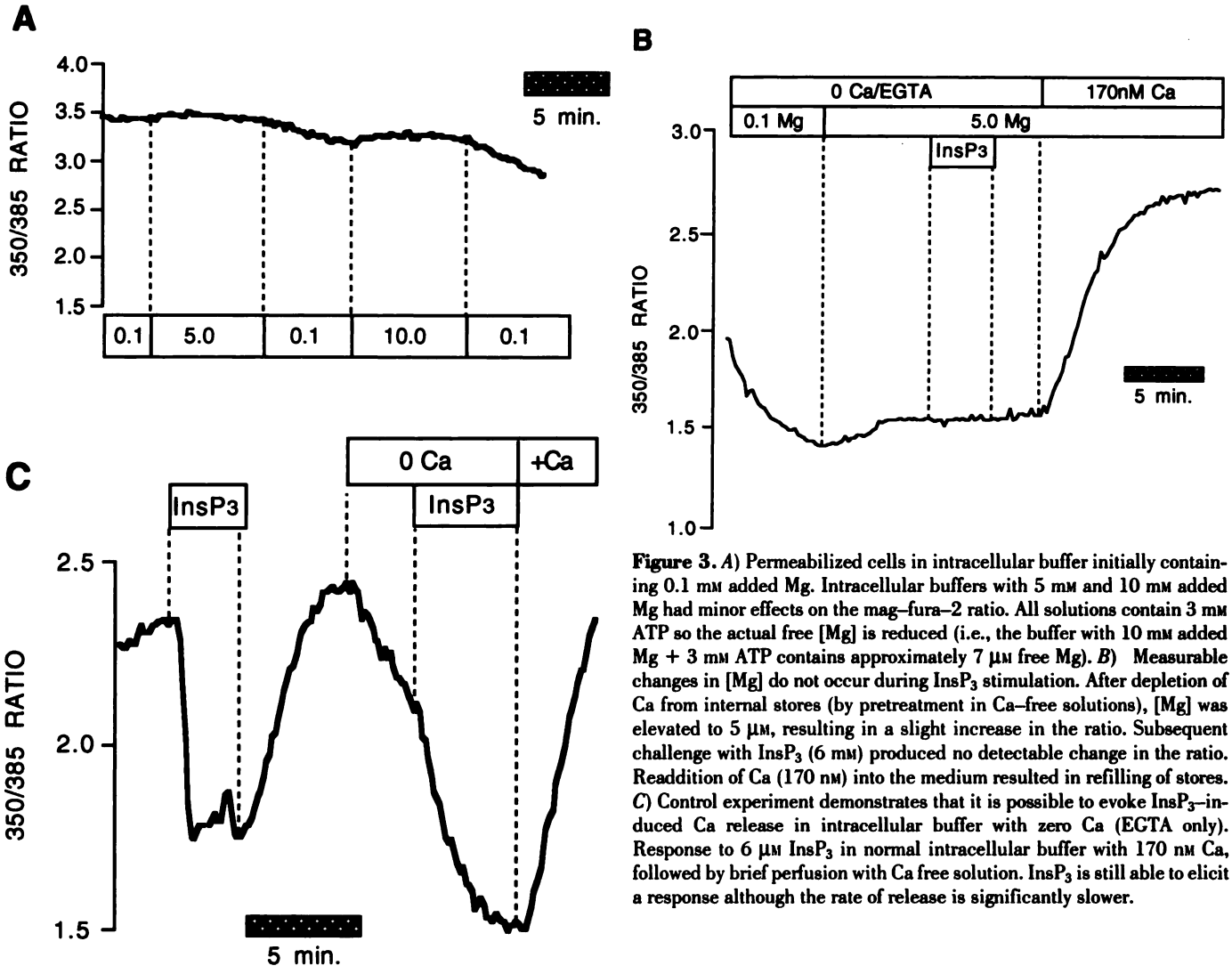


Figure 3. *A*) Permeabilized cells in intracellular buffer initially containing 0.1 mM added Mg. Intracellular buffers with 5 mM and 10 mM added Mg had minor effects on the mag–fura–2 ratio. All solutions contain 3 mM ATP so the actual free [Mg] is reduced (i.e., the buffer with 10 mM added Mg + 3 mM ATP contains approximately 7 μM free Mg). *B*) Measurable changes in [Mg] do not occur during $InsP_3$ stimulation. After depletion of Ca from internal stores (by pretreatment in Ca–free solutions), [Mg] was elevated to 5 μM, resulting in a slight increase in the ratio. Subsequent challenge with $InsP_3$ (6 mM) produced no detectable change in the ratio. Readdition of Ca (170 nM) into the medium resulted in refilling of stores. *C*) Control experiment demonstrates that it is possible to evoke $InsP_3$–induced Ca release in intracellular buffer with zero Ca (EGTA only). Response to 6 μM $InsP_3$ in normal intracellular buffer with 170 nM Ca, followed by brief perfusion with Ca free solution. $InsP_3$ is still able to elicit a response although the rate of release is significantly slower.

(23, 24). Because ATP is an effective chelator of Mg, the free [Mg] in our solutions was estimated to be only 1.7 μM in the presence of 3 mM ATP. Removal of 0.1 mM Mg from the bath elicited a slow decrease in the mag-fura-2 ratio with a time course that was similar to that observed when ATP was omitted from the bath (not shown). We interpreted this change as a slow leak of Ca resulting from the loss of pump activity by the Mg-dependent Ca-ATPase in the internal store ($n = 3$; not shown). As seen in Fig. 2*B*, removal of Ca from the intracellular buffer (but with ATP and 0.1 mM Mg still present) also elicited a slow decline in the ratio that required 21 ± 3 min for completion. After this time, withdrawal of ATP or treatment with the Ca, Mg ionophore 4-Br-A23187 (10 mM) did not result in any further decrease in the ratio ($n = 4$).

When cells were pretreated with thapsigargin (which is irreversible) and then perfused with a Ca free solution in the absence or presence of 4-Br-A23187 (10 mM), as depicted in Fig. 2*C*, there was no addtional change in the ratio ($n = 4$). Because 4-Br-A23187 carries both Ca and Mg, this result also demonstrates that under our experimental conditions there is no detectable residual pool of Ca or Mg in these cells in the absence of ATP (or after thapsigargin treatment). These results are consistent with the existence of one major type of Ca-sequestering compartment that requires ATP for filling and that can be depleted entirely by thapsigargin.

We were interested to know whether the mag-fura-2 technique could also be used to study Mg transport in the $InsP_3$-sensitive store. However, the following experiments suggest that the permeability of internal store membranes to Mg is low compared to that for Ca, and that there is no measurable Mg flux through the $InsP_3$ receptor. First we examined the effect of altering [Mg] in the intracellular solution under resting conditions where internal stores of Ca were full, i.e., in the presence of 170 nM free Ca and 3 mM ATP. As seen in **Fig. 3***A*, switching between intracellular buffers containing 0.1, 5.0, or 10.0 mM added Mg had little effect on the mag-fura-2 ratio of resting cells.

To test whether fluxes of Mg were occurring during $InsP_3$-stimulation, we first removed Ca from the bathing solution (still in the presence of 0.1 mM added Mg and ATP), which resulted in a large decrease in the mag-fura-2 ratio (Fig. 3*B*). Addition of 5 mM Mg had minimal effects on the ratio, again suggesting that the permeability of the store to Mg was relatively small. Subsequent treatment with $InsP_3$ in the absence of Ca (but in the presence of 3 mM ATP and 5 mM Mg) had no effect on the ratio. However, because the $InsP_3$ receptor is known to be modulated by cytosolic [Ca] such that Ca release is diminished in the absence of Ca (25, 26), we were concerned that the protocol used here might not have revealed Mg movements due to inactivation of the $InsP_3$ receptor. As seen in Fig. 3*C*, internal stores of BHK-21 cells were capable of responding to $InsP_3$ in the absence of cytosolic Ca, although the response (determined in the same cells) was on average 4.2 ± 0.7 ($n = 6$) times slower than was observed when the intracellular buffer contained 170 nM free Ca.

Based on the experiments depicted in Figs. 2*B*, *C* and Figs. 3*A–C*, it appears unlikely that intraluminal [Mg] changes interfere with the assessment of $InsP_3$-induced Ca release under our experimental conditions. However, because many of our experiments were conducted in the absence of Ca, we cannot exclude entirely the possibility that there is a mechanism for Mg uptake that requires Ca for its operation.

Testing for the presence of other types of Ca stores

In previous studies of gastric epithelial cells using the mag-fura-2 technique we noted a relatively large component of ATP-dependent Ca-sequestration, which based on the actions of mitochondrial inhibitors was ascribed to mitochondrial uptake (4). In contrast, these inhibitors had no effect in permeabilized BHK-21 cells. As seen in Fig. 2*A*, 1 μM ruthenium red (a potent blocker of the mitochondrial Ca uniporter; ref 27) elicited no change in the mag-fura-2 ratio ($n = 11$). Other agents known to release Ca from mitochondrial stores (27) were also tested: 1 μM FCCP ($n = 4$), 10 μM valinomycin ($n = 4$), 10 μM oligomycin + 5 mM azide ($n = 6$) were all without effect (not shown). Thus, there is no detectable contribution of intramitochondrial Ca to the mag-fura-2 ratio in BHK-21 cells. This result was also anticipated based on the experiments shown in Fig. 2*C*, where it was demonstrated that ionophores were unable to release any additional Ca from thapsigargin-insensitive stores. It is unclear what accounts for the difference between gastric cells and BHK-21 cells with respect to mitochondrial Ca uptake. It is possible that BHK-21 cells simply possess very few mitochondria compared to gastric cells, or perhaps these mitochondria do not sequester detectable amounts of Ca or dye.

To define the nature of Ca stores within these cells more fully, we also tested other agents that have been reported to influence Ca release in other cell types. Many cell types possess Ca pools sensitive to caffeine and ryanodine (2, 28, 29), which are thought to participate in the phenomenon of calcium-induced-calcium release. However, neither caffeine (10 mM; $n = 5$) nor ryanodine (1 μM; $n = 3$) had any effect in BHK-21 cells. Cyclic ADP-ribose, which has been shown to be a potent Ca releaser in a variety of cell types (30), was similarly unable to elicit any response in BHK cells when used at a dose of 2 μM ($n = 3$). In some permeabilized cell preparations, GTP releases Ca and/or modulates the size of the $InsP_3$-releasable store (31, 32); however, these effects of GTP (used at a concentration of 100 μM) were not observed in BHK cells ($n = 4$).

It appears from these data that BHK-21 cells possess a simple complement of Ca sequestering compartments when compared to other cell types such as gastric epithelial cells, pancreatic acinar cells, or PC12 cells (4, 28, 29, 33, 34). Our findings demonstrate that mag-fura-2 monitors free [Ca] in a subcellular compartment in which Ca could be released entirely by thapsigargin and reduced by approximately 85% with 6 mM $InsP_3$.

Calibration of intrastore free [Ca]

Having established the presence of one ATP-dependent Ca-sequestering compartment sensitive to thapsigargin and having excluded interference by Mg, we next performed in situ calibrations of store [Ca] using the ionophore 4-Br-A23187 (10 μM) and calibration solutions containing different amounts of Ca. Shown in **Fig. 4** is the release of Ca from internal stores in response to 6 mM $InsP_3$, and corresponding refilling of stores after $InsP_3$ washout. Cells were then treated with ionophore and calibration solutions containing 300, 200, 100, 50, or 0 μM added Ca. The inset summarizes data from 15 such records, and relates the mag-fura-2 ratio to the added [Ca] for this particular series of experiments. Some inconsistency in the starting ratio and size of the $InsP_3$-induced transient was observed from day to day, so that this calibration curve cannot be applied to all of the records presented in this report. Calibrations were performed in the presence and absence of 0.1 mM Mg, with no observable difference. Because mag-fura-2 is monitoring free [Ca] within the store, we converted the total [Ca] in our buffer to free [Ca] assuming an activity coefficient of 0.88. We observed some variability in the size of the response from cell to cell, but on average, treatment with 6 μM $InsP_3$ caused the resting intraluminal free [Ca] to decrease from 188 ± 21 μM to 25 ± 5 μM. In this estimate we assumed that we monitored [Ca] changes from a single contiguous pool of Ca. Another possible scenario is that Ca is liberated entirely from one subset of Ca-sequestering compartments, whereas other physically separated domains are unresponsive. However, because the $InsP_3$-induced release constituted such a large proportion of the total sequestered Ca (approximately 85%), the error in our estimate will be minimized if nonresponding pools are present.

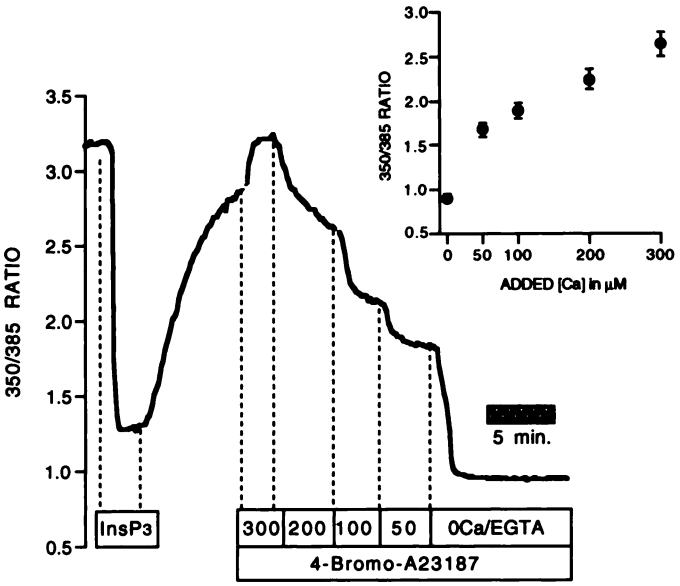


Figure 4. Calibration of $InsP_3$–induced Ca release from internal stores. Cells were first treated with 6 mM $InsP_3$ (a supramaximal dose) to establish the magnitude of the response, followed by perfusion with calibration solutions containing 300, 200, 100, or 50 μM added Ca (no EGTA) and 10 μM 4–Br–A23187. The zero Ca calibration solution contained 0.8 mM EGTA. The inset summarizes data from 15 records relating the mag–fura–2 ratio to the [Ca]. Errors are ± SEM.

Ionic dependence of Ca release and reloading

Both the efflux of Ca through the $InsP_3$ receptor during stimulation and the refilling of internal stores are electrogenic processes, and thus a counterion must accompany the movement of Ca in order to neutralize charge. The following experiments were designed to test the ionic requirements for the release and reloading of Ca in BHK cells by comparing the response to $InsP_3$ in solutions of varying ionic composition. **Figure 5*A*** shows a control stimulation with $InsP_3$ in normal intracellular buffer containing Cl (total [Cl] = 150 mM), followed by $InsP_3$-induced release and reloading of internal stores in the absence of Cl (gluconate replacement; trace typical of four similar records). Cl-free solutions had no significant effect on either the efflux of Ca from stores or the refilling phase after $InsP_3$ washout, indicating that there was no absolute requirement for Cl during these events. We also compared the $InsP_3$ response in HCO_3-containing solutions (bubbled with 5% CO_2) with that in nominally HCO_3-free conditions and also observed no significant difference (Fig. 5*B*, n = 5). Similarly, the $InsP_3$ response was unaltered when experiments were conducted in Cl-free solutions (gluconate replacement) that contained HCO_3 (20 mM) as the major permeant anion (n = 4; not shown).

Because omission of Cl had no obvious effect on the $InsP_3$ response it was inferred that cations might play a role in the release and reloading process, so we next performed experiments in Na-free solutions (Fig. 5*C*). There was no discernable difference in the $InsP_3$-induced discharge in the presence of 31 mM Na or in the absence of Na ions (n = 4). However, as seen in Fig. 5*D*, there was a marked attenuation of the response when cells were perfused with an intracellular solution deficient in K, where the replacement cation was either NMG (n = 7) or choline (n = 4). On average the magnitude of the $InsP_3$-induced ratio change in K-free solutions was 46±4% of that observed under control conditions in K-containing medium. In intracellular solution in which Na and K were both removed (NMG or choline replacement), $InsP_3$ elicited a decrease in ratio that was similar to that obtained under K-free conditions (45±7% of control; n = 4, not shown). No difference in the response was observed when Cs was used as the replacement for K (n = 6; not shown).

From the data presented above we concluded that the release and reloading process did not depend entirely on any one ion, although because there was a considerable attenuation in the absence of K this ion was likely playing the most important role. To assess more directly whether Cl, HCO_3, K, or Na were serving as counterions, we used sucrose-based solutions in which only one species of permeant ion was added at a time. In a nominally ion-free intracellular solution containing only 300 mM sucrose, 3 mM Tris-ATP, 170 nM free Ca (using Ca-EGTA buffers),

and 0.1 mM $MgCl_2$, there was no response to $InsP_3$ (Fig. 6*A*; $n = 14$). However, when 60 mM K was subsequently introduced (as K-gluconate), the $InsP_3$ response was restored ($n = 22$). As illustrated in Fig. 6*B*, addition of 45 mM Na-gluconate was also sufficient to initiate a partial release and reloading of sequestered Ca ($n = 7$). Shown for comparison is the result of $InsP_3$ stimulation in the presence of 100 mM K-gluconate in the same cell. Addition of 60 mM NMG.Cl to the sucrose solution also restored Ca mobilization and the subsequent refilling of the pool ($n = 4$), although the response was less robust than that observed in the presence of Na or K, also shown in Fig. 6*B*. In contrast, sucrose solutions containing approximately 15 mM HCO_3 (bubbled with 5% CO_2) supported the $InsP_3$ response in only 20 of 84 cells. Representative records from from responding and nonresponding cells in HCO_3/sucrose buffers are shown in Fig. 6*C*. Note that the onset of the $InsP_3$-induced release occurred after a considerable lag in those cells that released Ca.

From these data we conclude that compensatory movements of K, Na, and Cl (and possibly HCO_3) can occur during the release and reloading of Ca during $InsP_3$ stimulation. Although we did not investigate in detail the relative abilities of these ions to support this process, it appeared that Na and K substituted more readily than Cl and much better than HCO_3 for both efflux and reuptake of Ca.

We also investigated the effects of a number of K channel blockers on $InsP_3$-induced release in normal intracellular solution. Quinidine (100 μM, $n = 3$), apamin (0.5 mM, $n = 2$), 4-aminopyridine (2 mM, $n = 2$), and tetraethylammonium (TEA, 10 mM, $n = 2$) had no effect on the $InsP_3$ response compared to control responses in the same cells (not shown).

DISCUSSION

In the present study we used a fluorescence technique recently developed in our laboratory to monitor free [Ca]

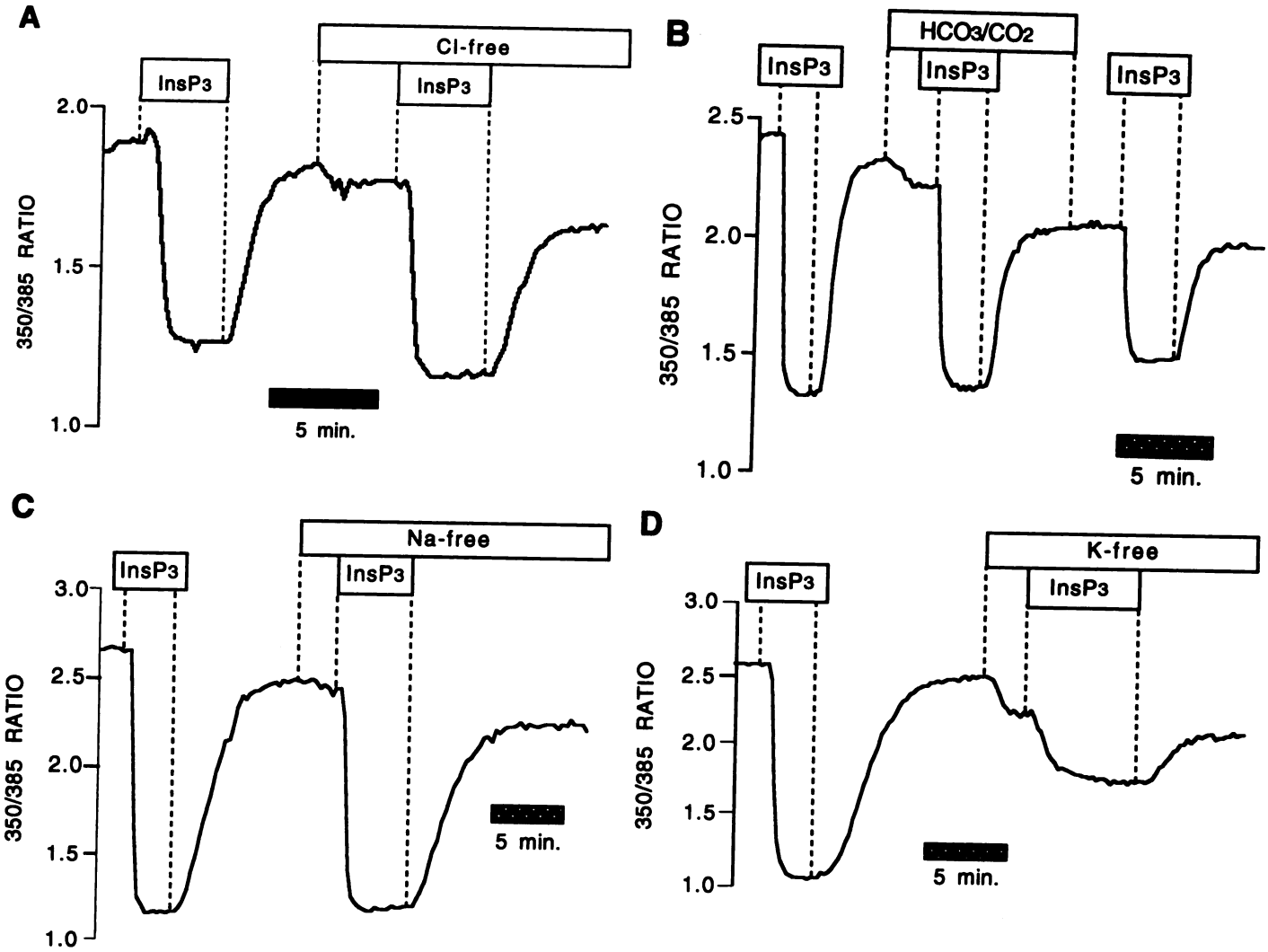


Figure 5. Effects of removal of individual ions from intracellular solution on the $InsP_3$ response. *A*) Control response to $InsP_3$ in normal intracellular solution is followed by stimulation in Cl–free buffer (gluconate replacement). *B*) compares response in nominally HCO_3–free solution (bubbled with air) with that in HCO_3/5% CO_2, where [HCO_3] is approximately 15 mM. Responses in absence of Na (*C*) or K (*D*) where NMG was used as a replacement cation.

within organelles of single BHK-21 cells permeabilized with SL-O or digitonin. We found that the Ca indicator mag-fura-2 reported [Ca] changes in principally one type of Ca sequestering compartment that could be released encently developed in our laboratory to monitor free [Ca] within organelles of single BHK-21 cells permeabilized with SL-O or digitonin. We found that the Ca indicator mag-fura-2 reported [Ca] changes in principally one type of Ca sequestering compartment that could be released entirely by the Ca-ATPase inhibitor thapsigargin. Calibration of the mag-fura-2 ratio with ionophores indicated that the resting free intraluminal [Ca] was initially 188 ± 21 μM, and was rapidly reduced to 25 ± 5 μM with $InsP_3$ stimulation. Fluxes of intraluminal Mg upon stimulation were not detected with this method. Agents affecting mitochondrial Ca accumulation (valinomycin, FCCP, oligomycin + azide, and ruthenium red) were all without effect on the mag-fura-2 ratio, as were compounds that modulate the ryanodine receptor, caffeine, and ryanodine. Similarly, no actions of cyclic ADP-ribose (a potent releaser of Ca in some cell types) or GTP (which may promote interstore communication, and affect $InsP_3$-induced release) were observed in BHK-21 cells. Thus, this cell type appeared to have a relatively simple Ca store, and may therefore be useful for studying properties of the $InsP_3$ receptor and other phenomena related to $InsP_3$-induced release under more physiological conditions in single semiintact cells using the mag-fura-2 technique. It would be interesting to quantitate the effects of luminal [Ca] on the sensitivity of the $InsP_3$ receptor (35) and further investigate the nature of quantal or incremental release (36, 37) from these stores.

By directly visualizing changes in Ca sequestering compartments with mag-fura-2, we were also able to monitor the spatial distribution of Ca release sites within permeabilized BHK-21 cells. Other investigators have previously exploited the compartmentalization of fura-2-AM (which has a relatively lowK_d) for this purpose (7, 38), although they did not consider the problem of multiple Ca pools in interpreting their data. As we have shown previously (4), this issue needs to be addressed for each cell type individually when using compartmentalized fura-2 or mag-fura-2. Consistent with the results of others (39, 40), we found that $InsP_3$ elicited changes in store [Ca] throughout the entire cell, although our experiments showed that not all regions responded to the same extent. In particular, we sometimes observed a perinuclear region that appeared to be less responsive to $InsP_3$ than peripheral areas of the cell. The morphology and location of this region, which was conspicuous in about 30% of cells, was highly suggestive of the Golgi apparatus, which has been localized in this cell type by a number of independent methods (see, for example, ref 41). This organelle is known to sequester Ca, but it is not generally believed that the Golgi apparatus constitutes a release site for Ca mediated by $InsP_3$ (42, 43). However, a

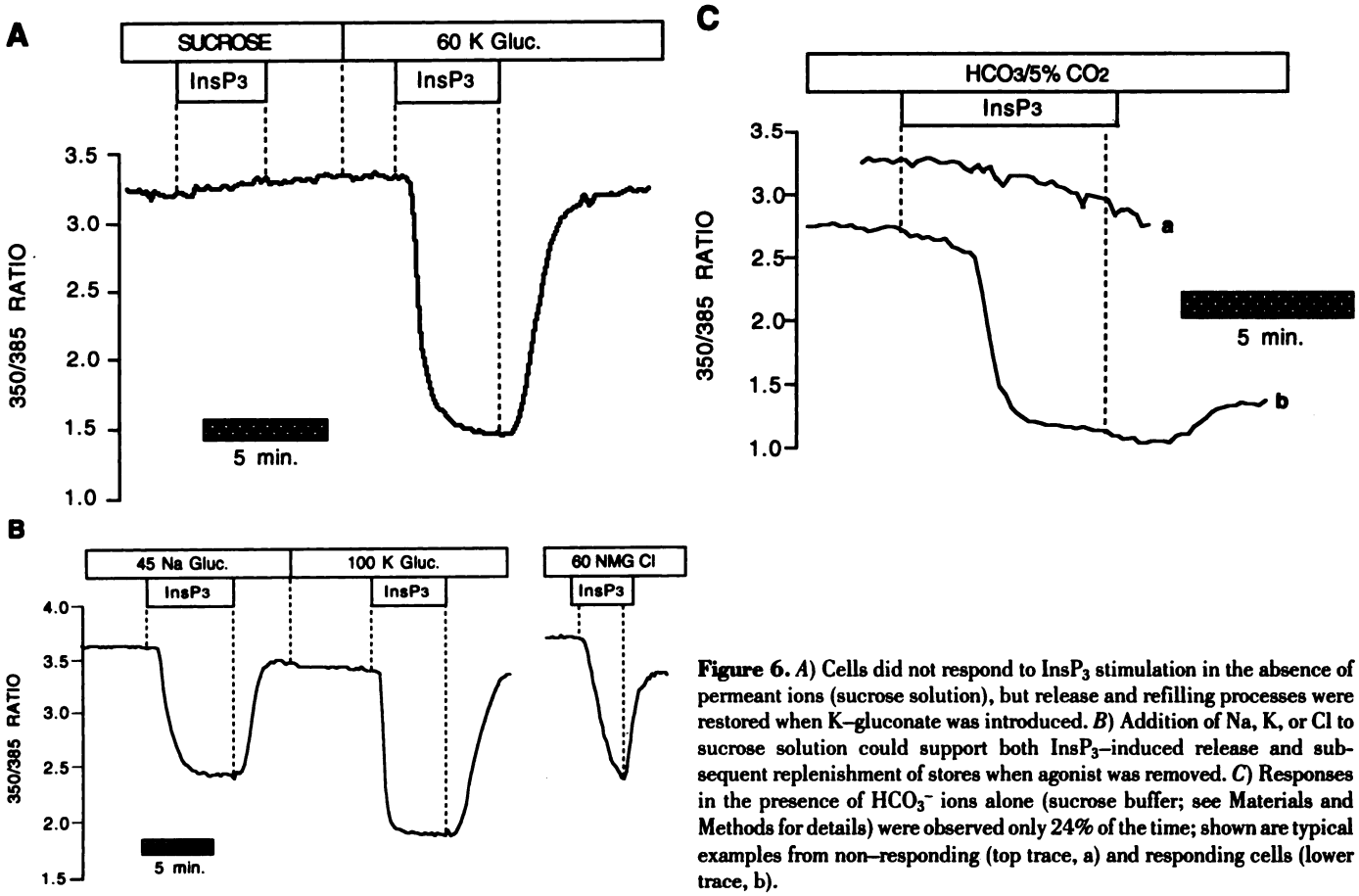


Figure 6. *A*) Cells did not respond to $InsP_3$ stimulation in the absence of permeant ions (sucrose solution), but release and refilling processes were restored when K–gluconate was introduced. *B*) Addition of Na, K, or Cl to sucrose solution could support both $InsP_3$–induced release and subsequent replenishment of stores when agonist was removed. *C*) Responses in the presence of HCO_3^- ions alone (sucrose buffer; see Materials and Methods for details) were observed only 24% of the time; shown are typical examples from non–responding (top trace, a) and responding cells (lower trace, b).

recent study using ion imaging techniques in RBL cells did come to the conclusion that the Golgi apparatus was involved in InsP3-induced Ca release (see ref 44). An alternative explanation, however, is that $InsP_3$-sensitive stores (possibly the endoplasmic reticulum?) are superimposed on unresponsive regions that may correspond to the Golgi apparatus. It is interesting that thapsigargin released Ca from all regions of the cell, including the unresponsive perinuclear zone. Residual Ca that remained in stores after $InsP_3$ stimulation at the periphery of the cell was also released by this agent.

We were concerned that the presence of Ca-sequestering organelles that were insensitive to $InsP_3$ might influence our quantitative estimate of the change in intraluminal free [Ca] in $InsP_3$-sensitive stores. However, the analysis of images as presented in Fig. 1*C* indicates that perinuclear hotspots of Ca remaining after stimulation have little effect on the quantitation; the difference between responses in selected regions near the nucleus and at the periphery is not great, and this region generally comprises a small part of the total cell area. Thus, we believe our estimate of the [Ca] change in the store is reliable, although ideally intraluminal [Ca] should be quantified in the intact cell under physiological conditions.

Another aspect of the present work relates to the coupling of $InsP_3$-induced Ca movements with other ions. Integral membrane proteins involved in ion transport, including channels, exchangers, and pumps, that have traditionally been studied at the level of the plasma membrane (for example by patch clamp, or by measurement of cytosolic ions) may or may not be active as they transit through the secretory pathway to be delivered to the plasma membrane. It has been reported, for example, that anion exchangers destined for the cell surface can function in a pre-Golgi compartment (45). Alternatively, very specific ion transport mechanisms may exist in these organelles that are distinct from those found in the plasma membrane. Relatively little is known about the transport of Na, K, Cl, and HCO_3 in subcellular compartments such as the endoplasmic reticulum and the role of these ions in organellar function. Although the precise anatomical correlate of the $InsP_3$-sensitive Ca store is open to debate, there is much evidence to indicate that the endoplasmic reticulum is the organelle serving this function (46, 47). Looking at the ionic dependence for release and reloading of Ca is important not only from the perspective of Ca signaling, but it may also provide an indirect way to examine the ion transport properties of the ER membrane in situ. The results presented here can be considered a first step in defining these pathways in BHK-21 cells.

We found that the presence of a permeant counterion was necessary for the movement of Ca into and out of the store. Na, K, Cl, and occasionally HCO_3 ions were able to provide a shunt for both the release and refilling of the $InsP_3$-sensitive pool. Data in Figs. 3*A*, 3*B* indicate that the Mg permeability of the Ca store is quite low, even in $InsP_3$-treated conditions, so Mg fluxes will probably not contribute significantly to maintenance of electroneutrality. The fact that monovalent cations could support refilling while anions could participate in the release of Ca indicates that the coupling may be more complex than a simple conductive exchange of these ions for Ca. That the $InsP_3$ response in the presence of Cl alone (and to an even lesser extent, HCO_3 ions) was not as robust as that seen with cations may indicate that anions must first permeate the compartment before they can travel with Ca out of the store during the opening of the $InsP_3$ receptor. Similarly, for cations to be effective as counterions during store repletion, they must be available at the luminal face of the membrane, but in this case perhaps cytosolic cations can access the store by first exchanging with Ca during the release phase.

In the presence of Na, K, and Cl conductances in parallel to the $InsP_3$ receptor in BHK-21 cells, it would not be expected in BHK-21 cells that pharmacological blockade of any one type of ion channel or absence of one particular ion would then abolish Ca signaling. We tested several known blockers of K channels, including Cs, apamin, quinidine, 4-aminopyridine, and TEA (Ba could not be used because it interferes with mag-fura-2 fluorescence), but did not discern any effects on the $InsP_3$-induced release and reloading. However, our findings are in contrast to previous studies in other cell types in which specific K channel blockers could markedly inhibit Ca release. For example, in platelet membrane vesicles apamin abolished $InsP_3$-induced Ca release (48), and in rat liver and brain microsomes TEA has been shown to influence release and reuptake (13, 49). These results emphasize *1*) that Na, K, and Cl channel activity in the internal store may play a physiological role in controlling Ca release during stimulant treatment and *2*) that these channels may be heterogeneous from cell to cell.

Our data show that free [Ca] within the store decreased by approximately 160 μM upon $InsP_3$ treatment. Given that two univalent ions must move for every Ca ion that crosses the store membrane, and that the buffering of Ca within this compartment could be considerable (say 100-fold), it is possible that there are significant and measurable (i.e., 30 mM) fluctuations in the intraluminal concentration of counterions during the release and reloading process. Our data therefore allow us to speculate about the events involved in the release of Ca from the store. The store membrane has large endogenous permeabilities to K, Na, and Cl but lower permeabilities to Ca, Mg, and HCO_3. $InsP_3$-induced release of Ca from the store occurs because Ca permeability increases, and there is rapid exchange (to maintain electroneutrality) of K, Na, and Cl through the permeability pathways that are always present in the membrane of the Ca store. The coordination of these ion movements and the mechanisms whereby the volume of the store is simultaneously maintained during the stimulatory cycle are interesting areas deserving further investigation. FJ

This work was supported by a grant from the National Institutes of Health (DK19520). W.-R.S. was supported by the Miller Foundation (Visiting Miller Professor).

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Received for publication December 10, 1994.

Accepted for publication March 30, 1995.