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Free [Ca^{2+}] dynamics measured in agonist-sensitive stores of single living intact cells: a new look at the refilling process

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Free [Ca^{2+}] in agonist-sensitive internal stores of single intact cells was measured *in situ* in order to examine the role of [Ca^{2+}] in modulating the store refilling process. BHK-21 fibroblasts were loaded with the low-affinity fluorescent calcium indicator mag-fura-2-AM such that >80% of the dye was trapped in organelles, where it reported [Ca^{2+}] changes solely in an agonist- and thapsigargin-sensitive internal store. The rates of store reloading following stimulation by 100 nM bradykinin were essentially unchanged when cytosolic [Ca^{2+}] was clamped to resting values with BAPTA-AM. In control cells, recharging of stores totally depended on the presence of external Ca^{2+}, but pre-loading the cells with BAPTA-AM permitted efficient refilling in Ca^{2+}-free, EGTA-containing external medium. Our results show: (i) Ca^{2+} stores normally are recharged by Ca^{2+} which must first transit the cytoplasm; (ii) an elevation in cytoplasmic [Ca^{2+}] is not required to replenish Ca^{2+} stores; (iii) the activation of the plasma membrane Ca^{2+} pump during the Ca^{2+} spike ordinarily results in complete extrusion of released Ca^{2+}; and (iv) the buffering capacity of the cytoplasm is an essential component of the store refilling process. An interesting finding was that acute treatment of cells with BAPTA-AM activated capacitative Ca^{2+} entry at the plasma membrane, due to its efficient hydrolysis in the stores, and the ensuing decrease in the endoplasmic reticulum [Ca^{2+}].

Keywords: BAPTA-AM/calcium homeostasis/mag-fura-2/oscillations/receptor desensitization

Introduction

In intact cells, a multiplicity of intracellular Ca^{2+} transport and buffer systems serve to shape intracellular Ca^{2+} signals and maintain the low resting [Ca^{2+}] typically found in the cytoplasm (Berridge, 1993; Pozzan *et al.*, 1994; Clapham, 1995). At rest, the principal players are Ca^{2+} pumps and leaks residing in both the plasma membrane and the endoplasmic reticulum (ER). During agonist activation, however, there are dramatic increases in the Ca^{2+} permeability of both the ER, for example through the opening of inositol 1,4,5-trisphosphate ($InsP_3$) receptors, and secondarily at the plasma membrane via store-operated or capacitative Ca^{2+} entry (Putney, 1990; Berridge, 1995). Endogenous Ca^{2+} buffers (including 'physiological' buffering by other organelles such as mitochondria; Mohr and Fewtrell, 1990; Friel and Tsien, 1994; Drummond and Fay, 1996; Babcock *et al.*, 1997; Hoth *et al.*, 1997) further modulate these Ca^{2+} signals. The fact that many of the elements of the Ca^{2+} signaling apparatus are themselves regulated by Ca^{2+} ions gives rise to additional complexity. While the integrated actions of many of these various elements on cytosolic Ca^{2+} dynamics are assessed readily by direct measurement of free Ca^{2+} in that compartment (e.g. using fluorescent indicators; Grynkiewicz *et al.*, 1985; Tsien and Pozzan, 1989), some experimental questions (e.g. the influence of cytosolic Ca^{2+} on the release and reloading of intracellular stores) can be addressed directly only by monitoring free [Ca^{2+}] changes in the ER lumen.

Two main strategies have been adopted to attack the problem of measuring ER [Ca^{2+}]: (i) targeting of Ca^{2+}-sensitive proteins, mainly aequorins (Kendall *et al.*, 1994; Montero *et al.*, 1995, 1997), and very recently green fluorescent protein (GFP)-based indicators (Miyawaki *et al.*, 1997; Persechini *et al.*, 1997); and (ii) the use of trapped fluorescent low-affinity Ca^{2+} indicators, such as mag-fura-2. A major drawback of the latter approach is that since the probe accumulates in both the cytoplasm and organelles, permeabilization of the plasma membrane is generally necessary in order to eliminate the cytosolic indicator. The permeabilization process obviously destroys the opportunity to measure native interactions of ER transporters with those of the plasma membrane and with other organelles. We report here a modification of the mag-fura-2 technique which permits sensitive, non-invasive measurements of intraluminal ER Ca^{2+} dynamics in intact BHK-21 cells, a fibroblastic cell line. Stimulation with a Ca^{2+} mobilizing agonist [bradykinin (BK) or ATP] resulted in a large, rapid drop in the mag-fura-2 ratio, consistent with the indicator being trapped in, and reporting Ca^{2+} changes primarily from, agonist-sensitive stores. This afforded us the opportunity to follow changes in free [Ca^{2+}] in the ER of single intact cells following physiological stimulations with Ca^{2+}-releasing agonists.

In the present study, we have examined the relationship between Ca^{2+} entry and cytoplasmic Ca^{2+} on the recharging of agonist-sensitive Ca^{2+} stores. Our results indicate that stores refill efficiently in the absence of elevations in cytoplasmic Ca^{2+}, but that there is normally an absolute requirement for external Ca^{2+} for this process. Increasing the intracellular buffer capacity of the cell, however, allowed recycling of released Ca^{2+} and refilling of internal stores, even in Ca^{2+}-free external solutions containing

EGTA. These data provide an original insight into the physiological coordination of plasma membrane Ca^{2+} extrusion mechanisms, ER Ca^{2+} reuptake mechanisms and Ca^{2+} entry pathways during Ca^{2+} signaling events.

Results

One of the drawbacks of loading Ca^{2+} indicators into cells as their AM-ester derivative is that the accumulation of dyes in intracellular organelles often confounds the interpretation of cytosolic measurements (Roe *et al.*, 1990). This feature can be turned around to yield a serendipitous bonus, however, when Ca^{2+} handling by organelles is to be addressed. Many groups have taken advantage of this phenomenon (see Hofer and Schulz, 1996, and references therein), although the overwhelming signal from cytoplasmic dye generally requires drastic procedures such as dilution of the cytosolic dye via the patch–clamp pipette (Tse *et al.*, 1994; Chatton and Stucki, 1995; Hofer *et al.*, 1998), or plasma membrane permeabilization (Hofer and Machen, 1993; Hirose and Iino, 1994; Hajnóczky and Thomas, 1997) in order to get usable information. This represents a significant obstacle to understanding organelle function *in situ*. We thus undertook a systematic search of cell types and dye-loading protocols that would maximize the trapping within intracellular $InsP_3$-sensitive stores, thus allowing sensitive measurement of store [Ca^{2+}] in single, intact, living cells. Among the many cell types examined, the BHK-21 fibroblast was that which approached our goal most closely. Golovina and Blaustein (1997) recently reported a similar approach using organelle-trapped mag-fura-2 in intact smooth muscle cells.

When BHK-21 cells were incubated for extended periods (45–60 min) with mag-fura-2-AM at 37°C (or at higher temperatures, 39–41°C, for briefer periods), in the absence of any additional treatment, cells displayed a distribution of fluorophore consistent with preferential dye accumulation in organelles. We noticed that following 20 min of loading at 37°C, cells had a uniform fluorescence, but between 20 and 45 min there was a progressive loss of cytoplasmic dye, as evidenced by the reduced fluorescence in the nuclear (reflecting the cytoplasmic) region with respect to the periphery of the cell. Similar observations were made when cells were loaded in this manner with indo-1-AM and fura-2-AM (not shown). Dye accumulation in the cytoplasm, on the other hand, was encouraged by reducing the loading time (20 min) and temperature (25°C). As seen in Figure 1A, the fluorescence of mag-fura-2 after 45–60 min of loading at 37°C was clearly localized in the peripheral regions of the cell and in a non-uniform manner, often with the appearance of a delicate reticulum. The nuclear regions were, on the other hand, nearly devoid of fluorescence, with the nuclear membrane plainly delineated.

As shown in Figure 1B, cells loaded with mag-fura-2 according to the protocol described above had high resting mag-fura-2 ratios, indicating that the signal was dominated by compartment(s) with elevated resting [Ca^{2+}]. A rapid drop in the ratio followed the treatment with the Ca^{2+}-mobilizing agonist BK (100 nM), as expected from a probe residing primarily in the $InsP_3$-sensitive store. In order to assess what fraction of the probe remained in organellar versus cytosolic compartments, we then

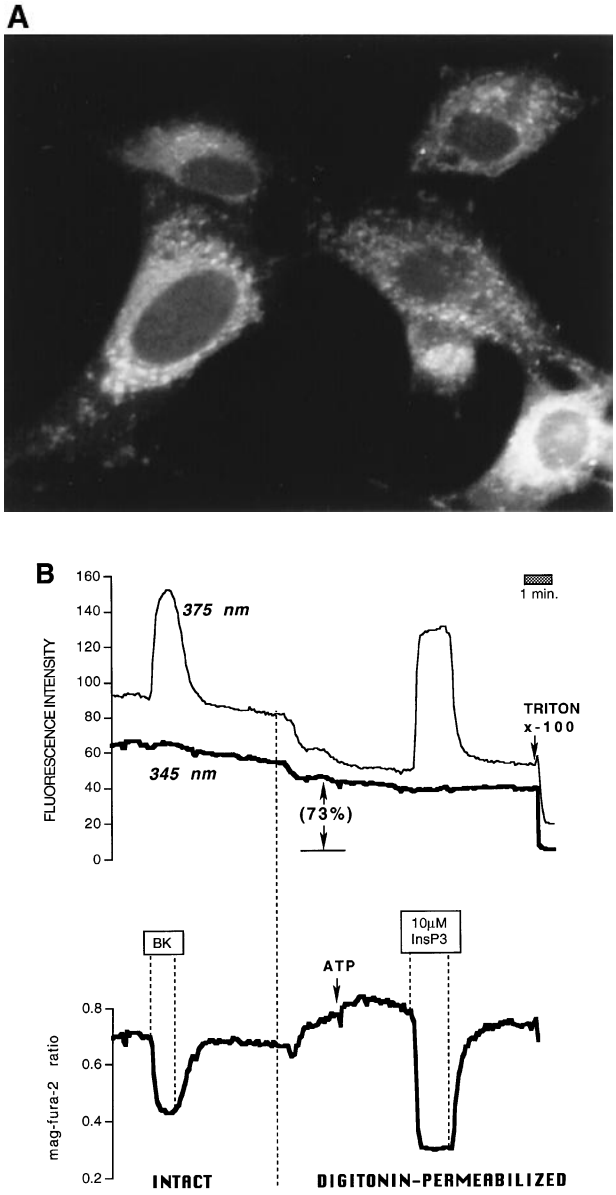

Fig. 1. (**A**) Mag-fura-2 fluorescence (excitation 351 nm) from intact BHK-21 fibroblasts loaded with dye at 37°C according to procedures described in the text. (**B**) Individual fluorescence intensities at 345 nm (Ca^{2+}-insensitive) and 375 nm (Ca^{2+}-sensitive) excitation wavelengths (top panel) and the calculated 345/375 ratio (lower panel) from mag-fura-2-AM-loaded fibroblasts. Intact cells were bathed in a standard NaCl Ringer's solution, and then stimulated with 100 nM BK, resulting in a drop in the mag-fura-2 ratio. Cells were then permeabilized with digitonin in intracellular buffer (see Materials and methods), initially in the absence of ATP to avoid stimulation of purinergic receptors. The drop in the fluorescence intensity at 345 nm is a measure of the amount of cytosolic dye released. Note that in this cell 73% of the total Triton X-100-releasable fluorescence was retained following permeabilization, which was less than the average amount of compartmentalized dye (83%) for cells used in this study. After supplementing the medium with ATP (arrow) to maintain Ca^{2+} uptake into stores, accumulated Ca^{2+} was released with 10 μM $InsP_3$.

permeabilized the same cells with digitonin in an intracellular-like buffer (Hofer *et al.* 1995), and monitored the release of cytosolic indicator at the Ca^{2+}-insensitive excitation wavelength of 345 nm. In the cell under study in Figure 1B, 73% of the probe was retained following permeabilization. On average, 83.7 ± 3.2% of the

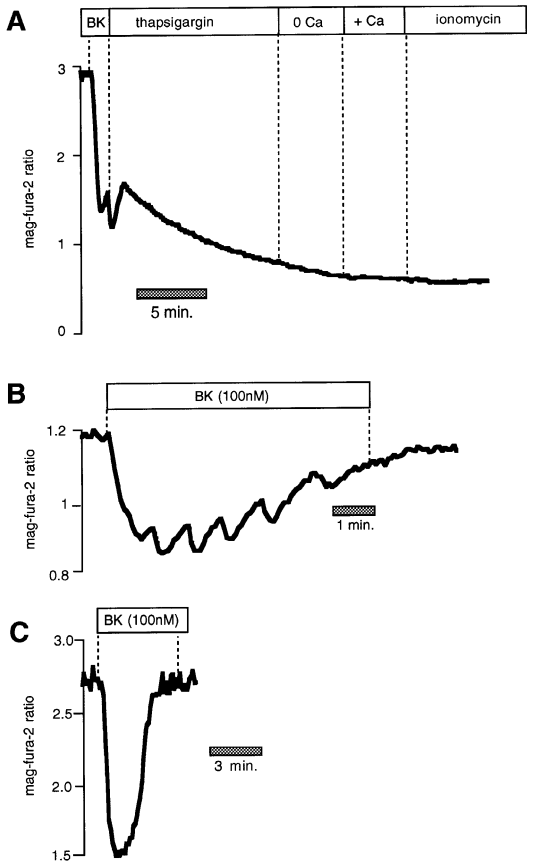

Fig. 2. (**A**) Mag-fura-2 measures [Ca^{2+}] changes only in thapsigargin- and agonist-releasable internal stores of intact BHK-21 cells. BK at 100 nM caused a rapid drop in the mag-fura-2 ratio, and evoked oscillations in store [Ca^{2+}] in this particular cell. Treatment with 100 nM thapsigargin caused additional release of Ca^{2+}, but removal and readdition of extracellular Ca^{2+} had no effect on the mag-fura-2 ratio. No further release of Ca^{2+} was detected upon addition of 10 μM ionomycin. (**B**) Agonist-induced oscillations of internal store [Ca^{2+}]. A subpopulation of BHK-21 cells exhibited oscillations in internal store [Ca^{2+}] upon treatment with 100 nM BK. (**C**) Receptor desensitization: stores can rapidly recover released Ca^{2+} in the continued presence of 100 nM BK. This is an example of a cell where the recovery was particularly rapid.

fluorophore was trapped in digitonin-resistant compartments (n = 16) and, in ~3 out of 16 cells, there was no detectable loss of dye from the cytoplasm. After supplementing the permeabilization buffer with ATP to maintain the uptake of Ca^{2+} into stores, the cells responded to $InsP_3$, which is membrane impermeant, demonstrating that cells were indeed permeabilized by digitonin (n = 4).

The data reported above clearly demonstrate that under the selected experimental conditions, trapped mag-fura-2 was reporting [Ca^{2+}] changes in an $InsP_3$-sensitive internal store. Although dye was not present to a significant extent in the cytoplasm, we could not, however, exclude that the probe was not also contained within structures other than the ER (e.g. mitochondria, which are notorious for compartmentalizing AM-esters). In order to test whether non-ER dye was significantly altering the kinetic behavior of the agonist-dependent ratio changes, the experiment presented in Figure 2A was carried out (n = 4). The intact cell was first challenged with BK. In this particular case, the agonist first elicited a rapid drop in the ratio, followed by a slow recovery upon which were superimposed two oscillations (see below). Addition of thapsigargin (100 nM), a selective inhibitor of the sarcoendoplasmic reticulum calcium ATPase (SERCA; Thastrup *et al.*, 1989), reversed the recovery phase following BK stimulation, and produced a relatively slow continuous drop in the ratio. During the falling phase of the thapsigargin-induced Ca^{2+} loss, the mag-fura-2 ratio remained insensitive to alterations in external [Ca^{2+}]. Thapsigargin is well known to activate capacitative Ca^{2+} entry in many cell types (including BHK-21 cells) by depleting internal Ca^{2+} stores (Putney, 1990). Therefore, under these conditions, the cytoplasmic as well as the intramitochondrial [Ca^{2+}] is predicted to increase greatly upon Ca^{2+} readdition. However, this maneuver had no consequence on the mag-fura-2 ratio. Addition of the Ca^{2+} ionophore ionomycin (10 μM) after the ratio had reached its minimum also had no effect, further confirming that, for all practical purposes, the dye reports [Ca^{2+}] movements only in the thapsigargin-sensitive internal store. The simplest explanation for this finding is that, because of the relatively low K_d of mag-fura-2 for Ca^{2+} (53 μM), changes in [Ca^{2+}] in the range of 1–10 μM (such as those occurring in the cytoplasm and in mitochondria) hardly affect the fluorescence of the dye which might be sequestered in those compartments.

There are two noteworthy observations regarding the profile of Ca^{2+} release and reloading in BHK-21 fibroblasts. The first is that following stimulation with 100 nM BK, the [Ca^{2+}] in internal stores of ~20% of cells was seen to oscillate with an average frequency of about one complete cycle per minute (Figure 2B; see also Figure 3A). Oscillations in intraluminal [Ca^{2+}] have been reported previously by a number of investigators (Tse *et al.*, 1994; Chatton *et al.*, 1995; Golovina and Blaustein, 1997; Hajnóczky and Thomas, 1997). Oscillations occurred upon a background of store refilling, but generally ceased before recovery was complete. These fluctuations were abolished by removal of external Ca^{2+} (illustrated in Figure 3A), indicating that Ca^{2+} entry and/or the replenishment of stores is important for sustaining oscillations.

The second point is that internal stores of BHK-21 cells were able to re-sequester Ca^{2+} effectively in the continued presence of agonists. The rate at which this reuptake occurred was highly variable from cell to cell. Shown in Figure 2C is a striking example of a cell in which stored Ca^{2+} was completely recovered in <2 min in spite of continuous exposure to 100 nM BK. This observation is explained most readily by the phenomenon of homologous receptor desensitization (see Böhm *et al.*, 1997); cells were generally refractory to further stimulation with the same agonist for several minutes, but subsequent stimulation of purinergic receptors using 100 μM ATP allowed this resequestered Ca^{2+} to be released again (not shown). Very often the rate of Ca^{2+} reaccumulation following stimulation was indistinguishable in the absence and presence of the agonist, suggesting that cessation of $InsP_3$ production had occurred within a very short (1–2 min) time period. One implication of these findings is that the stimulus for capacitative Ca^{2+} entry at the plasma membrane will be terminated rapidly simply because Ca^{2+} within the ER is restored, and may account in part for the relatively small plateau phase of the cytoplasmic Ca^{2+} signal in BHK-21 cells.

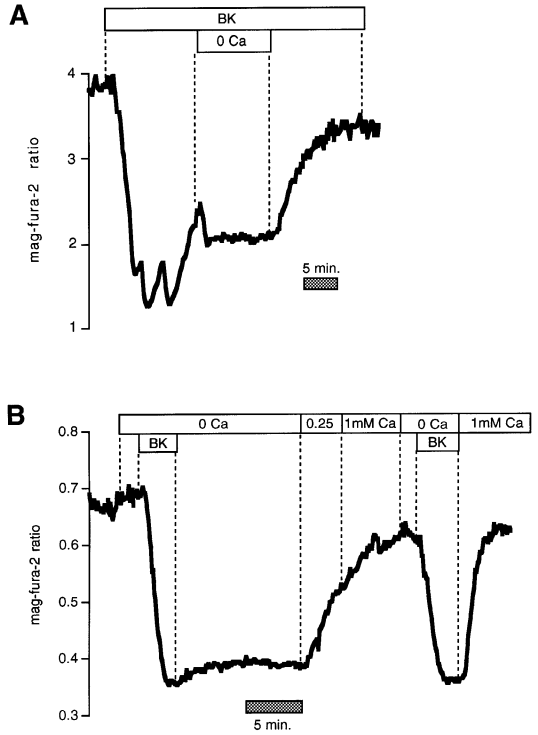

Fig. 3. (**A**) Intact mag-fura-2-loaded cells stimulated with 100 nM BK in the presence of external Ca^{2+}. Removal of external Ca^{2+} blocked the recovery. The luminal [Ca^{2+}] of this cell was oscillating initially. (**B**) Cells stimulated with BK in nominally Ca^{2+}-free solution. No recharging of Ca^{2+} stores occurred until Ca^{2+} (250 μM) was reintroduced into the bath. Following a second challenge with BK, the rate of recovery in 1 mM Ca^{2+} was enhanced.

Having established experimental conditions for measuring [Ca^{2+}] changes selectively in agonist-sensitive stores in intact cells, we could then start asking specific questions about the Ca^{2+} handling properties of cells, the first of which regards the effects of external Ca^{2+} on ER [Ca^{2+}] under resting and stimulated conditions.

Removing Ca^{2+} from the bathing solution (nominally Ca^{2+}-free solution) for up to 10 min had no effect on the resting mag-fura-2 ratio (not shown; $n = 10$), nor did increasing the bath [Ca^{2+}] from 1 to 5 mM ($n = 3$). However, as illustrated in Figure 3A and B, external Ca^{2+} was necessary in order to realize an efficient refilling of stores, either in the presence or absence of agonist. The luminal [Ca^{2+}] of the cell shown in Figure 3A was oscillating upon a general background of store refilling (probably as a result of receptor desensitization as described above). Removal of external Ca^{2+} resulted in cessation of the oscillations and an arrest of refilling that was reversed when Ca^{2+} was reintroduced into the perfusate. A similar effect of Ca^{2+}-free solution on store refilling was observed in cells which were not oscillating ($n = 3$) and for cells recovering in the absence of agonist ($n = 2$; not shown). In Figure 3B, the cell was stimulated briefly with BK in a nominally Ca^{2+}-free solution, the agonist washed off, and the cell maintained in zero Ca^{2+} for 10 min. No recovery was observed until 0.25 mM Ca^{2+} was readmitted to the bath ($n = 12$). A second stimulation in zero Ca^{2+} was performed, and this time 1 mM Ca^{2+} was readded, resulting in an enhanced rate of recovery compared with that seen in 0.25 mM Ca^{2+}.

The notion that extracellular Ca^{2+} is necessary for store refilling is well documented in the literature, although admittedly based on indirect measurements, i.e. of cytoplasmic signals. Though never questioned, this finding is in apparent contradiction to another well known property of the store, namely, that in the absence of $InsP_3$, stores are able to refill effectively at or below resting cytoplasmic [Ca^{2+}]. The question arises therefore as to why, after removal of the stimulus, no ER filling is observed until Ca^{2+} is added back to the medium. A possible clue to understanding this paradox is offered by the experiment presented in Figure 4A. In this case, cells were loaded at room temperature with fura-2 in order to monitor cytoplasmic [Ca^{2+}]. As observed previously by a number of investigators (see, for example, Muallem *et al.*, 1988, 1990), cells stimulated in the absence of external Ca^{2+} display a small (apparently ~20 nM) undershoot in Ca^{2+} below the basal level following the peak of Ca^{2+} release ($n = 3$; data from 19 cells). It is conceivable, however, that the magnitude of this decrease below the basal level might be underestimated because of the low sensitivity of fura-2 below 100 nM and because of problems of calibration (Roe *et al.*, 1990). We therefore hypothesized that the lack of refilling in Ca^{2+}-free medium could be due to this drop in cytoplasmic [Ca^{2+}] below resting, to a level which was below the K_d of the SERCA. The experiments in Figure 4B–E were thus carried out in order to test this possibility. Cells were loaded with 1,2-bis(2-aminophenoxy)ethand-*N*,*N*,*N′*,*N′*-tetraacetic acid (BAPTA)-AM, thereby providing a massive increase in cytosolic buffering capacity which prevented [Ca^{2+}] changes in the cytoplasm. Figure 4B depicts the signal from cells co-loaded with BAPTA-AM and fura-2 following stimulation with BK in Ca^{2+}-free medium. Both the cytosolic peak and the undershoot seen in Figure 4A were eliminated completely ($n = 3$). When the luminal ER [Ca^{2+}] was measured with mag-fura-2, loading with BAPTA-AM (40 μM for 20 min at 37°C) had a dramatic effect on the refilling, i.e. it abolished the requirement for extracellular Ca^{2+} in the refilling of stores, as seen in Figure 4C ($n = 14$; 25 cells). An almost complete recharging of stores occurred, even though the Ca^{2+}-free external solution contained 100 μM EGTA. The refilling was mediated by the SERCAs of the internal store. As seen in Figure 4D, following a control stimulation with BK in zero Ca^{2+}, BAPTA-AM-pre-treated cells were stimulated a second time with BK. The SERCA inhibitor 2, 5-di(*tert*-butyl)hydroquinone (tBHQ), reversibly blocked the refilling phase (always in 0 Ca^{2+}/EGTA; $n = 7$). Thus, not only does recharging of the store occur directly from the cytosol, but extended recycling of Ca^{2+} released into the cytoplasm is possible when the intracellular buffer capacity is augmented by BAPTA.

The question then arises as to whether the undershoot of cytoplasmic Ca^{2+} results in a complete blockade of the SERCA or only a major reduction in its activity. Since agonists normally release only a portion of the thapsigargin-sensitive store (see Figure 3A; Hofer *et al.*, 1995), we could test whether the SERCA pump was still active at sub-basal cytosolic Ca^{2+} by treating the cells acutely with SERCA inhibitors during the 'undershoot' phase, as shown in Figure 4E. Control cells loaded with mag-fura-2 (but not BAPTA) were stimulated briefly with BK in 0 Ca^{2+}/EGTA external solution. Five minutes later, at a

time when cytosolic [Ca^{2+}] was predicted to be well below the resting value (Figure 4A), tBHQ was administered, resulting in a small decrease in the mag-fura-2 ratio (n = 7). There was, however, no recovery of released Ca^{2+} when tBHQ was washed out. These data indicate that the SERCA was still somewhat active, but only at a level sufficient to maintain a modest recycling of the cation, and not enough to refill the store efficiently.

The effect of cytoplasmic buffering on Ca^{2+} handling by the stores was next investigated in the presence of extracellular Ca^{2+}. We measured store [Ca^{2+}] changes from a number of cells which had been pre-loaded with BAPTA-AM (20 μM; co-loaded with mag-fura-2 for the last 20 min of dye loading at 37°C). We observed that release and reloading of stores as measured by mag-fura-2 following agonist activation appeared essentially to be similar to that in control cells (not shown; n = 14). However, given the heterogeneity of responses to BK as measured at the single cell level, we decided to compare directly, in the same cell, the agonist response before and after BAPTA-AM loading.

Shown in Figure 5A are typical control recordings of luminal [Ca^{2+}] (top panel) and cytoplasmic [Ca^{2+}] (bottom panel) from different cells, each stimulated twice with BK. The rates of release and reloading, as well as the response measured in the cytoplasm following two sequential stimulations, were quite reproducible in a given cell (n = 8). The brief application of agonist (1 min) and the long interval between stimulations (at least 15 min) ensured that receptor desensitization was minimal.

Figure 5B depicts the responses to a brief control stimulation with agonist, followed by a loading period with 40 μM BAPTA-AM for 10–15 min at 37°C on the microscope stage. Cells were then washed for 3–5 min in normal Ringer's solution, and then restimulated with agonist. The two applications of BK yielded responses which were quite similar in spite of the BAPTA-induced increase in cytoplasmic buffering. The rate of release during the control stimulation with a supramaximal dose of BK (100 nM) was 97 ± 12% (SEM) of that following BAPTA treatment, while the rate of the control recovery was 147 ± 17% (SEM) of that observed after BAPTA loading (n = 14); neither were significantly different. Subtle kinetic differences in the release phase similar to those reported by Montero *et al.* (1997) (i.e. a more abrupt termination of the release) were sometimes observed. This aspect was not investigated in any detail.

As a control that the BAPTA-AM loading protocol had indeed reduced the cytosolic [Ca^{2+}] changes, the same protocol was repeated in fura-2-loaded cells (Figure 5B, bottom panel). The response to a second challenge with BK was highly attenuated (on average 16 ± 3% of control, with 58% of cells giving no measurable response).

The tendency of BHK-21 cells to sequester AM-ester

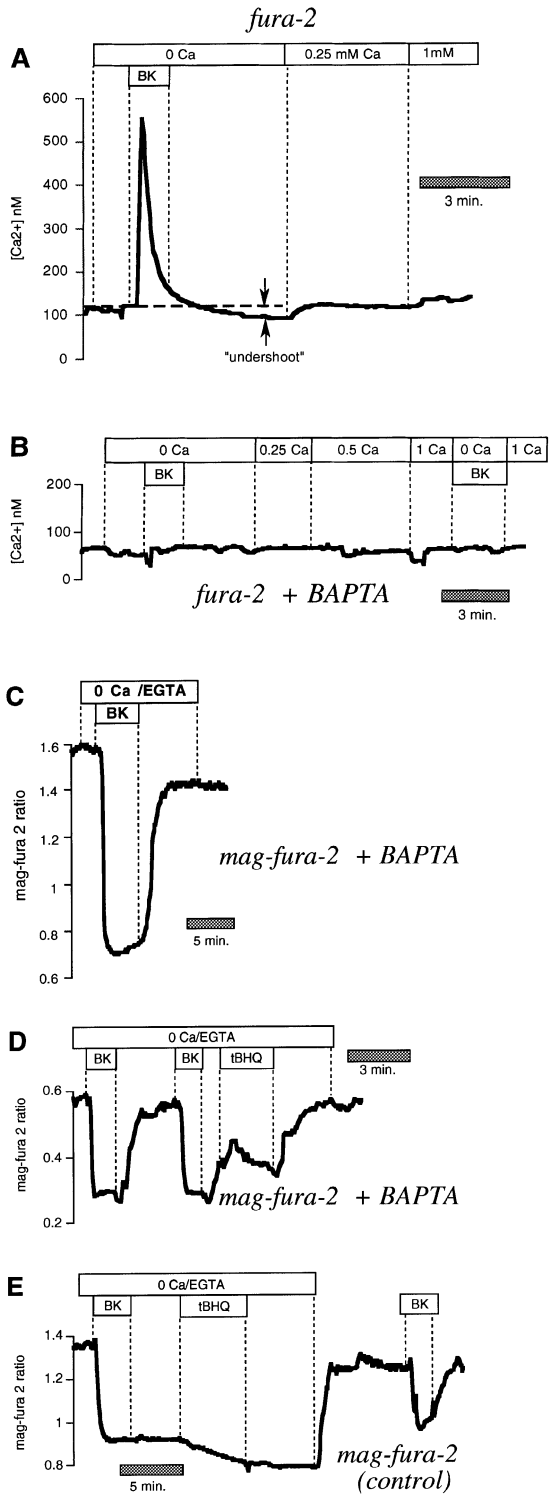

Fig. 4. (**A**) 'Undershoot' in cytoplasmic [Ca^{2+}] as measured by fura-2 was observed following stimulation with 100 nM BK in nominally Ca^{2+}-free solution. Readmission of 250 μM Ca^{2+} resulted in a small increase in cytoplasmic [Ca^{2+}]. (**B**) BAPTA-AM (co-loaded with fura-2) abolished both the peak and the undershoot following stimulation in Ca^{2+}-free solutions containing 100 μM EGTA. (**C**) Intact mag-fura-2-loaded cells pre-loaded with 40 μM BAPTA-AM for 20 min. Complete recovery in store [Ca^{2+}] following BK challenge in Ca^{2+}-free external solution containing 100 μM EGTA. (**D**) Intact mag-fura-2-loaded cells pre-loaded with 40 μM BAPTA-AM for 20 min. Store refilling in zero Ca^{2+} is mediated by SERCA pumps. Cells were maintained in Ca^{2+}-free solutions plus 100 μM EGTA throughout the experiment, and 100 nM BK given where indicated. During the recovery phase following the second BK stimulation, application of 20 μM tBHQ reversibly blocked the refilling. (**E**) Control mag-fura-2-loaded cells (no BAPTA) stimulated with BK in zero Ca^{2+} (100 μM EGTA). tBHQ (20 μM) was added at the time indicated, resulting in a slight drop in the mag-fura-2 ratio, but no recovery when tBHQ was washed out. Readdition of extracellular Ca^{2+} resulted in complete refilling. Control response to BK in the presence of external Ca^{2+}.

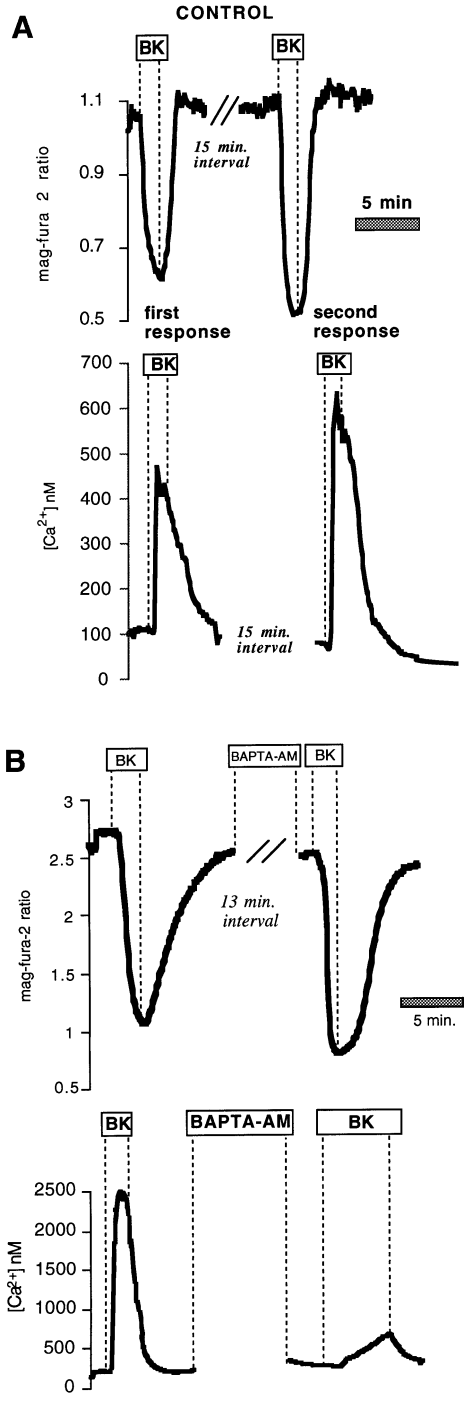

Fig. 5. The upper panels of both parts of the figure depict the responses from intact mag-fura-2-loaded cells, while the corresponding measurement of cytosolic [Ca^{2+}] from a different coverslip of fura-2 loaded cells is illustrated in the bottom panels. (**A**) Control responses to two sequential stimulations with 100 nM BK with a 15 min interval between agonist applications. (**B**) Brief control stimulation with 100 nM BK followed by superfusion with 20 μM BAPTA-AM. The response to the second BK challenge following BAPTA-AM loading was largely unchanged in the ER lumen compared with the control response, but highly attenuated in the cytoplasm.

derivatives efficiently suggested that it should be possible to manipulate the [Ca^{2+}] within the stores during acute loading with BAPTA-AM. In fact, when mag-fura-2-loaded cells were exposed to BAPTA-AM, a clear decrease in the store [Ca^{2+}] was observed (Figure 6A; $n = 14$). This decrease was more profound when BAPTA-AM was applied in Ca^{2+}-free medium (Figure 6B; $n = 3$). This lowering of store [Ca^{2+}] should activate capacitative influx, and therefore a paradoxical effect of treating cells with the Ca^{2+} chelator is expected in the cytosol, i.e. a transient increase in intracellular [Ca^{2+}] during BAPTA-AM loading. This was indeed the case, as seen in Figure 6C and D. When BAPTA-AM was added to fura-2-loaded cells maintained in 1 mM external Ca^{2+} (Figure 6C), there was an increase in cytosolic [Ca^{2+}] that was quite variable ($n = 50$; see figure legend for details). In contrast, when the loading was allowed to proceed for a few minutes in Ca^{2+}-free external medium, and the cells then reperfused with 1 mM Ca^{2+}, a large and reproducible increase in cytoplasmic [Ca^{2+}] was observed ($n = 7$).

Discussion

Agonist-induced Ca^{2+} signals generally consist of two well-defined components, the 'spike' of Ca^{2+} release, which is followed by a 'plateau' phase, a sustained elevation of Ca^{2+} resulting from Ca^{2+} entry (Tsien and Tsien, 1990). As external Ca^{2+} has been established in virtually all cell systems to be obligatory for the recharging of internal stores, it was natural to associate the plateau with the period of store refilling, and it was therefore assumed that the elevation was required for replenishment of stores. The seemingly mysterious observation that under particular experimental conditions internal stores could refill in the absence of any Ca^{2+} elevation in the cytoplasm led to the proposal of the 'privileged pathway' for Ca^{2+} entry, a special conduit connecting the lumen of the ER to the exterior of the cell which mediated passive refilling of the store (Putney, 1986; see also Cabello and Schilling, 1993). This notion was difficult to reconcile, however, with a number of later experimental observations which indirectly suggested that Ca^{2+} must actually enter the cytoplasm before being taken up by SERCAs of the internal store (see, for example, Negulescu and Machen, 1988; Muallem *et al.*, 1990) and, furthermore, seemed to make the existence of SERCAs themselves redundant. More recently, Petersen and collaborators (Mogami *et al.*, 1997) revived a modification of the privileged pathway hypothesis, and proposed the existence of an 'operational Ca^{2+} tunnel' which could permit refilling of Ca^{2+} stores in the apical part of a polarized epithelial cell when Ca^{2+} was applied focally to a very limited region in the basolateral aspect of the cell.

Here we have re-examined the fundamental question of why external Ca^{2+} is necessary for store refilling, and how cytosolic [Ca^{2+}] affects this process, by using the highly selective Ca^{2+} chelator BAPTA to clamp cytosolic [Ca^{2+}]. Indispensable to this study was the establishment of a dye-loading protocol which, by inducing the preferential loading of mag-fura-2 into organelles in intact BHK-21 fibroblasts, allowed us to monitor directly the release and reloading of store Ca^{2+} at the single cell level. Although the exact distribution of dye is not known, it is clear that even in intact cells, the high K_d of mag-fura-2 permits sensitive and selective measurements of free [Ca^{2+}] changes in agonist-releasable internal stores. That the interference from residual cytoplasmic indicator or from dye retained in mitochondria has minimal effects on

the measurement of luminal Ca^{2+} changes is evidenced by the experiment shown in Figure 2A, which demonstrates that cytosolic Ca^{2+} elevations and Ca^{2+} release from organelles, other than those mobilized by thapsigargin and agonist, cannot be detected by the dye.

An interesting corollary to the dye-loading protocol employed in this study is that acute treatment with the AM-ester of a strong Ca^{2+} chelator often lowered the free $[Ca^{2+}]$ in internal stores as measured by mag-fura-2, and, even more striking was that this resulted in the activation of capacitative Ca^{2+} entry at the plasma membrane (Figure 6). This finding further demonstrates that the cleavage of the AM-ester group inside the ER lumen is very efficient, as only the hydrolyzed form of the chelator is expected to bind Ca^{2+}, and at least two AM-groups must be removed for any binding whatsoever of Ca^{2+} to take place. As expected, BAPTA-AM was more potent in lowering $[Ca^{2+}]$ within the store in the absence of extracellular Ca^{2+} than in its presence (Figure 6B). The fact that BAPTA-AM sometimes elicited a very robust Ca^{2+} entry, even though the degree of internal store depletion was quite modest (as assessed in parallel experiments using mag-fura-2), may be due to the ability of cytosolic BAPTA to suppress locally the negative feedback by Ca^{2+} ions themselves at the mouth of the store-operated channels (Louzao *et al.*, 1996; Skutella and Ruegg, 1996). The results presented here further support the idea that the lowering of Ca^{2+} in the ER *per se* is sufficient to activate store-operated Ca^{2+} entry, and present a new tool for investigating the entry phenomenon (Hofer *et al.*, 1998).

The fact that recharging of internal stores is strictly dependent on external $[Ca^{2+}]$ is a well established, yet poorly explained, experimental observation. Our data suggest that there are two interrelated factors which account for the inability of stores to refill in zero external Ca^{2+}: (i) the undershoot of cytosolic Ca^{2+} below the resting level compromises the activity of the SERCAs; and (ii) the endogenous buffering capacity for Ca^{2+} of non-ER compartments (including mitochondria and the cytoplasm) is inadequate to provide sufficient Ca^{2+} for recharging the store in the absence of an external Ca^{2+} source. Loading cells with BAPTA-AM both clamped the cytoplasmic Ca^{2+} at resting levels and augmented the Ca^{2+} buffering capacity of the cytoplasm, so that Ca^{2+} released from the ER was captured by the chelator, and then recycled into the store upon termination of $InsP_3$ production. As illustrated by the model in Figure 7, this implies that the large majority of the Ca^{2+} released during activation of the $InsP_3$ receptor (the spike) ordinarily is extruded from the cell through the action of the plasma membrane Ca^{2+}-ATPase. Unless this occurred, there would be no undershoot, and refilling of stores could take place without the requirement for external Ca^{2+}. This is

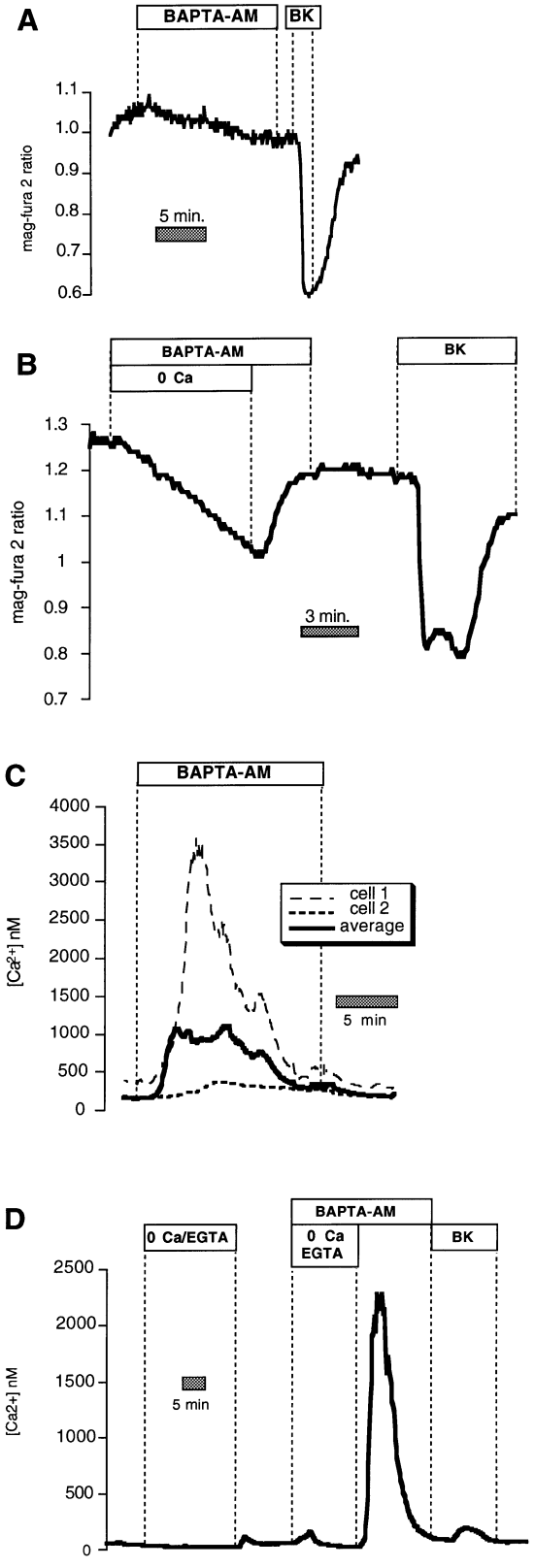

Fig. 6. Effects of acute treatment of BAPTA-AM on luminal ER and cytoplasmic $[Ca^{2+}]$. (**A**) In the presence of external Ca^{2+}, addition of BAPTA-AM caused a small decrease in the mag-fura-2 ratio. (**B**) BAPTA-AM applied in Ca^{2+}-free medium resulted in more profound lowering of the mag-fura-2 ratio. Shown for comparison in both (A) and (B) is the response to 100 nM BK. (**C**) In fura-2-loaded cells, the addition of BAPTA-AM alone caused a relatively slow but significant increase in the cytoplasmic $[Ca^{2+}]$, the magnitude of which averaged 39 ± 6% of the first BK response in the same cell. The response to BAPTA-AM in the presence of external Ca^{2+} was, however, highly variable, with 20% of cells showing no elevation of $[Ca^{2+}]$, and 18% showing a $[Ca^{2+}]$ rise that was actually greater than the peak elicited by agonist. Superimposed are the average from 15 cells (solid line) and traces from two individual cells (dotted lines). (**D**) Fura-2-loaded cells. A larger and more consistent influx of Ca^{2+} upon Ca^{2+} readdition was observed when cells were treated with BAPTA-AM in Ca-free medium. The subsequent response to BK is nearly abolished following loading with the chelator.

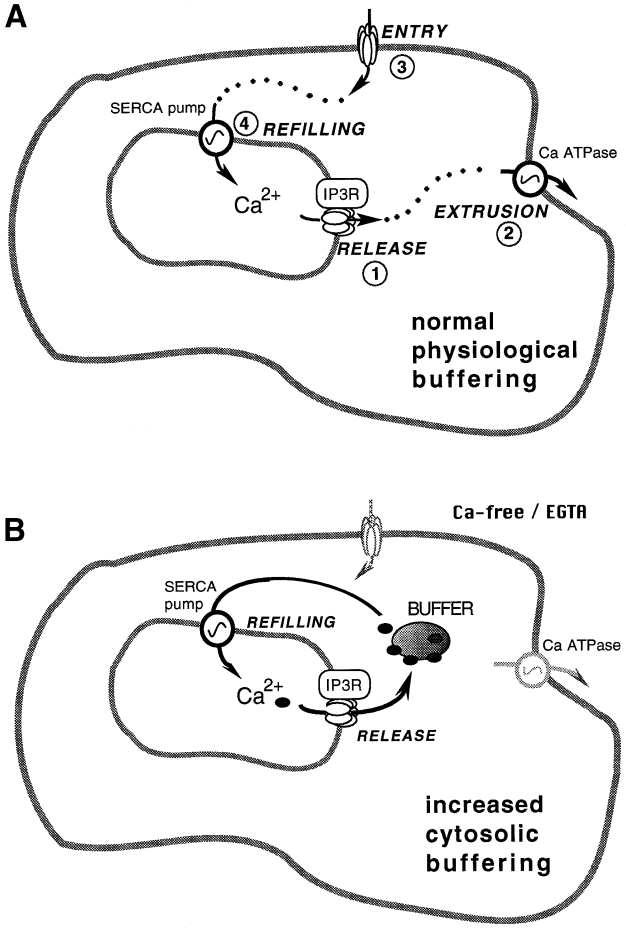

Fig. 7. Model of Ca^{2+} homeostatic mechanisms in the intact cell. Normally Ca^{2+} released from the ER during the spike is exported effectively to the exterior of the cell by the plasma membrane pump (**A**). This precludes the recycling of liberated Ca^{2+} back into the ER, hence the requirement for Ca^{2+} entry from the outside to provide adequate amounts of the cation for store recharging. In the case where the endogenous buffer capacity is supplemented with BAPTA (**B**), Ca^{2+} released from the ER is captured by the chelator; without the Ca^{2+} spike, the activation of the plasma membrane ATPase is reduced. The Ca^{2+} which was released can be salvaged from the cytoplasm to refill the stores.

consistent with data of Tepikin *et al*. (1992) demonstrating that following a supramaximal stimulation with agonist, virtually the entire content of the store is ejected rapidly to the exterior of the cell. Furthermore, their data showed that while the plasma membrane pump is nearly silent at resting levels of cytoplasmic Ca^{2+}, it is activated dramatically when [Ca^{2+}] is between 100 and 450 nM (Camello *et al.*, 1996). This is in accordance with our data regarding the refilling of stores of BAPTA-loaded cells in the absence of extracellular Ca^{2+} (Figure 4C and D); under conditions where the spike of Ca^{2+} release is prevented, discharged Ca^{2+} remains available within the cell for re-accumulation into stores. A second ramification of these data is that refilling of stores must take place directly from the cytosol, as external Ca^{2+} was not present. Therefore, a privileged pathway is not required for the recharging of Ca^{2+} stores in BHK-21 cells. These data are not inconsistent with those of Mogami *et al.* (1997) regarding the proposed existence of an operational Ca^{2+} tunnel. One of the key features of the refilling process in their system was that reaccumulation of store Ca^{2+} occurred in the absence of any detectable elevation in cytoplasmic [Ca^{2+}]. However, the authors concluded that Ca^{2+} probably traversed the cytoplasm in a very localized (silent) domain before being sequestered by SERCAs at the basolateral aspect of the cell.

The fact that the endogenous Ca^{2+} buffering of the cytosol is insufficient to allow refilling of stores at resting [Ca^{2+}] is not surprising if one considers the following arguments. The basal free [Ca^{2+}] in the cytoplasm is ~100 nM and, assuming the native buffering in this compartment to be ~50-fold (Zhou and Neher, 1993), this means that at most there are 5 μM total Ca^{2+} available for reuptake into the stores. Assuming that the ER occupies 10% of the cell volume, this 5 μM of Ca^{2+} could be concentrated to 50 μM in the store. However, the resting free [Ca^{2+}] in the lumen of the ER is ~400 μM, and it is also buffered (perhaps as much as 10-fold). It is apparent that once Ca^{2+} is released and extruded to the exterior of the cells by the plasma membrane Ca^{2+} pump, the intrinsic supply of Ca^{2+} in the cytoplasm is nowhere near enough to allow recharging of the ER. Accordingly, the free [Ca^{2+}] in the cytoplasm will drop as the SERCA scavenges remaining intracellular Ca^{2+}, until finally the threshold for SERCA pumping is approached. A logical consequence is that, in such a system, entry of Ca^{2+} from outside the cell will be obligatory for refilling of stores. The data presented here highlight the exquisite temporal coordination that exists between Ca^{2+} release from the internal store and disposal of the cation to the exterior of the cell through the action of the plasma membrane pump, resulting in the controlled termination of the spike during Ca^{2+} signaling events. These elements must, in turn, be integrated carefully with the entry of Ca^{2+} and its reuptake into internal stores, the scrupulous matching of which permits refilling even in the absence of elevations in the cytoplasmic [Ca^{2+}] when the stimulus is removed.

Materials and methods

Cell culture and dye loading

BHK-21 cells (purchased from Consorzio, Gest. Biotec., Avanzate, Genova, Italy) were grown in Earle's minimal essential medium containing 10% fetal bovine serum and were maintained in a humidified incubator at 37°C in the presence of 5% CO_2/95% air. Cells were seeded at low density on glass coverslips and used the following day for microspectrofluorimetric or ratio imaging measurements of cytoplasmic or intraorganellar free [Ca^{2+}] with fura-2-AM or mag-fura-2-AM, respectively. Cells were loaded for 20–30 min at room temperature with fura-2-AM (5 μM) or at 37°C for 45–60 min with mag-fura-2-AM (5–10 μM). When cells were pre-loaded with BAPTA-AM, the chelator was solubilized in an equal volume of 2% Pluronic F-127, and was added only during the last 20 min of dye loading to ensure cytosolic retention (final concentration 40 μM). Subcultures were prepared by trypsinization and the cells used for not more than 10 passages following receipt from the distributor.

Microspectrofluorimetric measurements

Coverslips with dye-loaded cells were mounted into a heated metal flow-through perfusion chamber described previously (Negulescu and Machen, 1990) placed on the stage of an inverted Zeiss IM 35 microscope and perfused by gravity feed at a rate of 1.5–2 ml/min. Change of solutions was made by a remote-controlled electronic manifold. Emitted fluorescence from single cells was measured in response to alternate pulses of excitation light (5 ms duration) at 340 and 380 nm, using a computer-controlled four-place sliding filter holder manufactured in-house. The emitted fluorescence (510 nm) was focused on a photomultiplier tube,

amplified, digitally converted and sampled on an IBM-compatible computer. All measurements were corrected automatically for background. The ratio of emitted light from the two excitation wavelengths (340/380) of fura-2 or mag-fura-2 (Raju *et al.*, 1989) provides a measure of ionized cytoplasmic [Ca^{2+}] (Grynkiewicz *et al.*, 1985) or intrastore [Ca^{2+}] (Hofer and Machen, 1993), respectively.

Ratio imaging experiments

Some measurements in this study were made using a commercial imaging system (Georgia Instruments, Roswell, GA) described previously in more detail (Gamberucci *et al.*, 1994). The 345/375 nm excitation ratio (emission 450 nm) was acquired from individual cells within the microscope field every 4 s. Cells were superfused continuously on the heated microscope stage in an open Leiden chamber equipped with gravity feed inlets and vacuum outlets for solution changes.

Solutions and materials

Unless otherwise stated, all chemicals were purchased from Farmitalia Carlo Erba (Milano, Italy), Fluka Chemie (Switzerland) or Sigma (St. Louis, MO). Experiments were performed with a Ringer's solution containing (in mM) 121 NaCl, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5.5 glucose, 10 HEPES/NaOH pH 7.20. BK and ionomycin were from Calbiochem-Novabiochem (Switzerland); BAPTA-AM, fura-2-AM and mag-fura-2-AM were obtained from Molecular Probes (Eugene, OR); thapsigargin and $InsP_3$ were from L.C.Services (Woburn, MA). When dimethylsulfoxide (DMSO) or ethanol were used as a solvent, their final concentration never exceeded 0.01 or 0.1%, respectively. Where applicable, data are expressed as means ± SEM, with *n* equal to the number of experimental runs.

Cell permeabilization

Dye-loaded cells were rinsed briefly in a high K^+ solution (in mM: 125 KCl, 25 NaCl, 10 HEPES, pH 7.25, 0.1 $MgCl_2$), then exposed for 2–3 min to an 'intracellular buffer' at 37°C (the same solution supplemented with 0.5 mM MgATP, pH 7.25 and Ca^{2+}/EGTA buffers, 0.1 total [EGTA], 200 nM free Ca^{2+}, calculated according to the computer program described in Bers *et al.*, 1994) also containing 5 μg/ml digitonin. After plasma membrane permeabilization, cells were superfused continuously with intracellular buffer (without digitonin). Measurements of mag-fura-2 fluorescence were performed as above for intact cells.

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