Ca²⁺ Homeostasis in the Agonist-sensitive Internal Store: Functional Interactions Between Mitochondria and the ER Measured In Situ in Intact Cells

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Abstract. Mitochondria have a well-established capacity to detect cytoplasmic Ca^{2+} signals resulting from the discharge of ER Ca^{2+} stores. Conversely, both the buffering of released Ca^{2+} and ATP production by mitochondria are predicted to influence ER Ca^{2+} handling, but this complex exchange has been difficult to assess in situ using conventional measurement techniques. Here we have examined this interaction in single intact BHK-21 cells by monitoring intraluminal ER $[Ca^{2+}]$ directly using trapped fluorescent low-affinity Ca^{2+} indicators. Treatment with mitochondrial inhibitors (FCCP, antimycin A, oligomycin, and rotenone) dramatically prolonged the refilling of stores after release with bradykinin. This effect was largely due to inhibition of Ca^{2+} entry pathways at the plasma membrane, but a significant component appears to arise from reduction of SERCA-mediated Ca^{2+} uptake, possibly as a consequence of ATP depletions in a localized subcellular domain. The rate of bradykinin-induced Ca^{2+} release was reduced to 51% of control by FCCP. This effect was largely overcome by loading cells with BAPTA-AM, highlighting the importance of mitochondrial Ca^{2+} buffering in shaping the release kinetics. However, mitochondria-specific ATP production was also a significant determinant of the release dynamic. Our data emphasize the localized nature of the interaction between these organelles, and show that competent mitochondria are essential for generating explosive Ca^{2+} signals.

Key words: metabolic microdomains • calcium homeostasis • organelle interactions • mitochondrial uncouplers • signal transduction

CELLULAR Ca^{2+} homeostasis reflects an intricate balance of many factors (pumps, leaks, release channels, and buffer systems; Berridge, 1993; Clapham, 1995) that are expected to directly or indirectly influence Ca^{2+} handling by internal stores. Among these are interactions with other organelles, including mitochondria. By now it has been firmly established using a variety of techniques (for example, using targeted recombinant aequorin; Rizzuto et al., 1992) that mitochondria sense Ca^{2+} released into the cytoplasm during Ca^{2+} signaling events (Rizzuto et al., 1993; Sparagna et al., 1995; Babcock et al., 1997), and that this uptake of Ca^{2+} constitutes a physiologically important mechanism for activating mitochondrial metabolism (Denton and McCormack, 1990; Rutter et al., 1996; Hajnóczky et al., 1995).

Thus far this problem has been analyzed from the point of view of the mitochondria or of the cytoplasm. But how do mitochondrial metabolism and Ca^{2+} uptake influence Ca^{2+} release from the ER? In fact, interactions between these two organelles may be expected to occur on multiple levels.

First, with respect to the refilling of internal stores with Ca^{2+}, it is obvious that ATP (derived from mitochondrial and glycolytic sources) is required to fuel accumulation of Ca^{2+} by SERCAs, the Ca^{2+}-ATPases resident on the ER membrane. Second, previous studies have demonstrated that store-operated Ca^{2+} entry pathways (those that are activated by store depletion; Putney, 1990) are influenced by mitochondrial metabolism (Gamberucci et al., 1994; Hoth et al., 1997; Marriot and Mason, 1995). Since the refilling of internal stores after agonist-induced Ca^{2+} release relies principally on this pathway (see for example Hofer et al., 1998), mitochondrial inhibition will also be expected to impede the recharging of internal stores.

Regarding the mobilization of Ca^{2+} from stores, ATP is known to modulate Ca^{2+} release channels in the ER,

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including inositol 1,4,5-trisphosphate (InsP3) receptors (Bezprozvanny and Ehrlich, 1995; Ferris et al., 1990; Smith et al., 1985), as well as Ca^{2+} leaks (Hofer et al., 1996). The allosteric modulation of InsP3 receptors by ATP shows a bell-shaped dependence on [ATP], with progressive reduction in open probability of the channel at ATP concentrations below 500 μM and above 4 mM (Bezprozvanny and Ehrlich, 1993). It has been questioned in the past whether local [ATP] can vary sufficiently under physiological conditions to effect any sort of regulation on the release channels in situ.

The ability of mitochondria to buffer cytoplasmic Ca^{2+} transients is expected to influence InsP3 receptor opening further, since both this release channel and the ryanodine receptor (that mediates Ca^{2+}-induced Ca^{2+} release) have also been shown to exhibit a bell-shaped dependence of open probability on cytosolic $[Ca^{2+}]$ (Bezprozvanny et al., 1991; Finch et al., 1991). In support of this view is the recent demonstration by Jouaville et al. (1995) that mitochondrial substrates alter the propagation of Ca^{2+} waves in oocytes, a finding confirmed by another study using blockers of mitochondrial respiration in cultured oligodendrocytes (Simpson and Russell, 1996). These phenomena have been attributed to local alterations in Ca^{2+} buffering as a consequence of Ca^{2+} uptake into energized mitochondria, resulting in modulation of InsP3 receptor opening.

Here we have addressed the integrated actions of the above factors from the perspective of the ER, using a fluorescence technique that permits selective measurement of free $[Ca^{2+}]$ changes in the agonist-sensitive store of single intact cells. Our work demonstrates that not only is the ability of mitochondria to buffer Ca^{2+} transients important, but also that mitochondrial ATP production alters the dynamics of Ca^{2+} release, the two factors acting in concert to permit explosive discharge of Ca^{2+} from the store under conditions where mitochondria are actively respiring. Our results also indicate that the effects of mitochondrial inhibitors on the Ca^{2+} entry pathways at the plasma membrane result in considerable inhibition of store refilling. At the same time, SERCAs in BHK-21 cells appear to depend on mitochondrial ATP production for Ca^{2+} sequestration to a surprising extent, a result best explained by the existence of subcellular metabolic microdomains. Our findings suggest that there is an intimate functional (and possibly physical) relationship between these two organelles.

Materials and Methods

Cell Culture and Dye Loading

BHK-21 cells (purchased from Consorzio. Gest. Biotec. Avanzate, Genova, Italy) were grown in Earle's MEM medium containing 10% FBS, and were maintained in a humidified incubator at 37°C in the presence of 5% CO2/95% 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 (Grynkiewicz et al., 1985), or mag-fura-2-AM (Raju et al., 1989), respectively. Cells were loaded in tissue culture medium 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). Subcultures were prepared by trypsinization, and the cells used for not more than 10 passages after 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-msec duration) at 340 nm 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. The filter exchange system and data sampling software were designed by Giuseppe and Antonio Troccoli (Bari, Italy). All measurements were automatically corrected for background. The ratio of emitted light from the two excitation wavelengths (340/380) of fura-2 or mag-fura-2 provide a measure of ionized cytoplasmic $[Ca^{2+}]$ (Grynkiewicz et al., 1985) or intra-store $[Ca^{2+}]$ (Hofer and Machen, 1993; Hofer et al., 1998), respectively. Mag-fura-2 data are presented as uncalibrated ratio changes and not free $[Ca^{2+}]$ due to uncertainties in the calibration procedure as described previously by Hofer and Schulz (1996).

Ratio Imaging Experiments

Some measurements in this study were made using a commercial imaging system (Georgia Instruments, Roswell, Georgia) 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 AG (Buchs, Switzerland) or Sigma Chemical Co. (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. Bradykinin and ionomycin were from Calbiochem-Novabiochem Corp. (La Jolla, CA); fura-2-AM and mag-fura-2-AM were obtained from Molecular Probes (Eugene, OR); InsP3 was from L.C. Services (Woburn, MA). When 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

As described previously (Scheenen et al., 1998; Hofer et al., 1995), dye-loaded cells were rinsed briefly in a high K^+ solution (in mM: 125 KCl, 25 NaCl, 10 Hepes, pH 7.25, 0.1 $MgCl_2$), and 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 mg/ml digitonin. After plasma membrane permeabilization, cells were continuously superfused with intracellular buffer (without digitonin). Imaging measurements were performed as above for intact cells.

Results

Mitochondria are known to take up Ca^{2+} released into the cytoplasm during agonist stimulation via an electrogenic Ca^{2+} uniporter, a process dependent on the membrane potential across the inner membrane (Rizzuto et al. 1992, 1993; Pozzan et al., 1994). Carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP)[1] is a protonophore

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1. *Abbreviations used in this paper*: BK, bradykinin; FCCP, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; InsP3, inositol 1,4,5-trisphosphate; tBHQ, 2, 5-Di(tert-butyl)hydroquinone.

that uncouples mitochondrial respiration and ATP production by dissipating the proton gradient across the inner mitochondrial membrane. As it abolishes the membrane potential normally maintained by oxidative phosphorylation, FCCP also completely prevents mitochondrial Ca^{2+} uptake (Gunter and Pfeiffer, 1990; Gunter et al., 1994; Rizzuto et al., 1994).

Mitochondrial Inhibitors Impede Ca^{2+} Uptake into Internal Stores by at Least Two Mechanisms

We tested the effects of FCCP and other mitochondrial inhibitors on Ca^{2+} handling by the ER of BHK-21 cells loaded with the AM-ester of the fluorescent Ca^{2+} dye mag-fura-2 (Fig. 1). We have shown previously in this cell type that prolonged loading with this indicator results in preferential accumulation of dye into organelles (Hofer et al., 1998; see also Golovina and Blaustein, 1997). The low affinity of the dye for Ca^{2+} allows for sensitive measurements almost exclusively in organelles where Ca^{2+} is high, i.e., the ER (Hofer and Schulz, 1996; Hofer et al., 1995). Adding 1 μM of FCCP to resting cells (in the presence of glucose) frequently caused a slow, persistent, and reversible decrease in the ratio (Fig. 1 *A*; $n = 8$ out of 13 cells) comparable to that elicited by treatments that block SERCA-mediated Ca^{2+} uptake (Hofer et al., 1995; Hofer et al., 1996). A similar, although poorly reversible decrease in the ratio was observed upon acute treatment with 1 μM oligomycin, which blocks the ATP synthetase responsible for coupling the proton gradient to ATP production ($n = 3$; not shown).

As further illustrated by Fig. 1 *A*, when intact cells were stimulated in the presence of FCCP with 100 nM bradykinin (BK) to release stored Ca^{2+}, the rapid recovery of Ca^{2+} that was normally seen when the agonist was removed (see for example Fig. 3) was not observed until FCCP was washed out. Adding FCCP during the recovery phase in BK-stimulated cells prevented store refilling (Fig. 1 *B*). When SERCA activity was abolished with 2,5-Di(tert-butyl)hydroquinone (tBHQ), however, (resulting in a relatively slow leak of Ca^{2+} out of the store) FCCP did not further reduce the mag-fura-2 ratio, showing that tBHQ and FCCP act on the same Ca^{2+} pool (Fig. 1 *B*; $n = 6$). In fact, FCCP caused a slight reversal of the downward trend in the signal, a result we attribute to effects of ATP depletion on the passive Ca^{2+} leak of internal Ca^{2+} stores (Hofer et al. 1996).

Collectively, the data of Fig. 1 strongly suggest that uncoupling mitochondria grossly affects Ca^{2+} uptake into the ER. However, mitochondrial inhibitors, particularly protonophores such as FCCP, are subject to several nonspecific effects (e.g., membrane potential and pH changes) that could influence our measurements. It should be stressed that in previous experiments on permeabilized BHK-21 cells where [ATP] in the superfusion was kept constant at 3 mM, there was no effect of 1 μM FCCP on the mag-fura-2 ratio (Hofer et al., 1995), nor was there any consequence of adding ruthenium red, valinomycin, or oligomycin + azide (Hofer et al., 1995), agents that either prevent mitochondrial Ca^{2+} uptake and/or induce Ca^{2+} release from that organelle. Thus, these drugs have no effect per se on the fluorescence of mag-fura-2, and do not cause
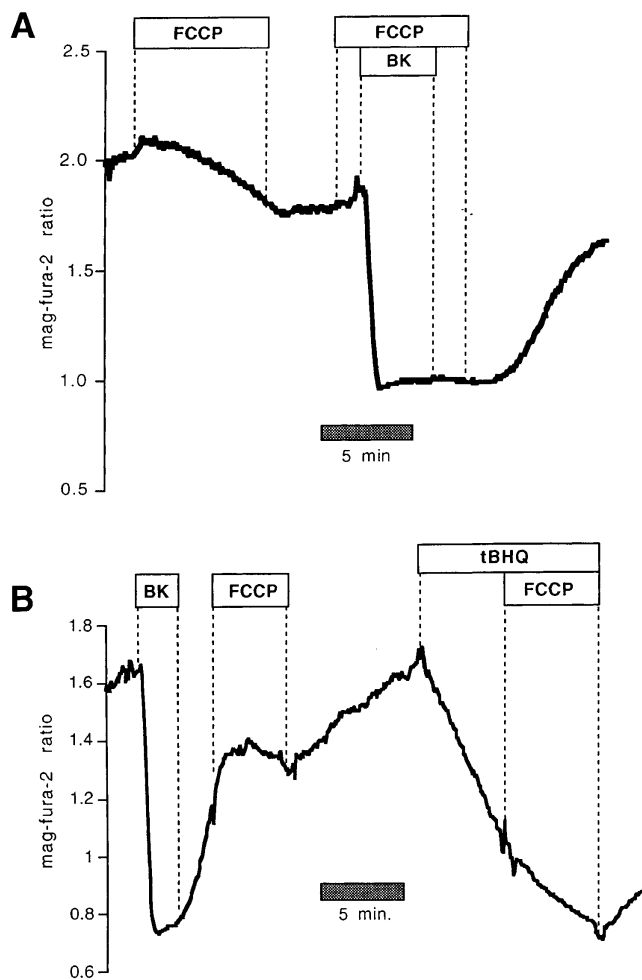

Figure 1. FCCP inhibits ER Ca^{2+} uptake. Mag-fura-2 measurements of [Ca^{2+}] in agonist-sensitive internal stores in intact cells. (*A*) Resting intact cell treated with FCCP (1 μM) followed by FCCP 1 BK (100 nM). (*B*) FCCP administered during store refilling subsequent to BK-induced release inhibited Ca^{2+} sequestration, but had little effect on the mag-fura-2 ratio when given after treatment with a SERCA blocker, tBHQ (10 μM).

release of Ca^{2+} from mitochondria that can be detected by this low-affinity fluorophore in permeabilized BHK-21 cells. Furthermore, as shown in Fig. 2, InsP3-induced release and reloading of internal stores of permeabilized cells was unaffected by FCCP. The response to a supramaximal dose of InsP3 (6 μM) was similar before and after treatment with 1 μM FCCP under conditions where [Ca^{2+}] and [ATP] of the intracellular-like buffer were clamped to 170 nM and 0.5 mM, respectively. When FCCP was given to cells during the recovery phase after InsP3 washout, there was no consequence on the mag-fura-2 ratio (Fig. 2).

In contrast to the data of Fig. 2, measurements of store [Ca^{2+}] in intact cells revealed that metabolic poisons dramatically prolonged the time course of store refilling (Fig. 3). Direct comparison of the rates of recovery after BK stimulation between control and FCCP-treated cells (Fig. 3 *A*) showed that refilling rates were on average only 18.3 ± 2.5% of control values in the same cell ($n = 23$), with some
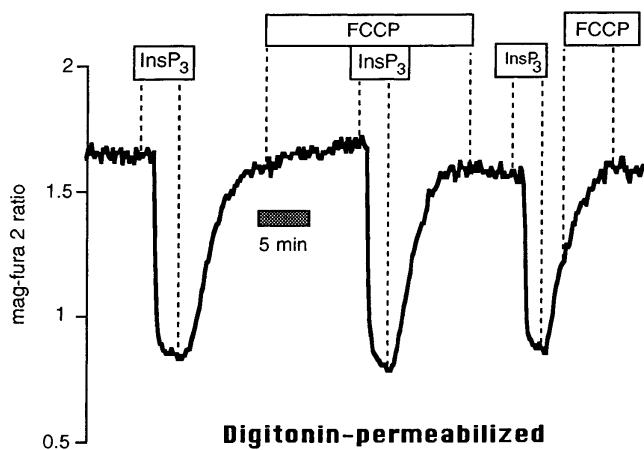
Figure 2. Effects of FCCP on Ca^{2+} homeostasis in internal stores of digitonin-permeabilized mag-fura-2-loaded BHK-21 cells. Cells were continuously superfused with intracellular buffer. [Ca^{2+}] was clamped with EGTA buffers to 170 nM, and a constant [ATP] of 0.5 mM was maintained. FCCP had no effect on the mag-fura-2 ratio or InsP3-induced release and reloading of internal stores under these conditions.

variability at the single-cell level. Similar experiments using oligomycin to block ATP synthesis revealed that the inhibitor reduced the rate of recovery to $25 \pm 4\%$ of control (Fig. 3 B). Comparable results were obtained using 4 μM rotenone (not shown; blocks oxidative phosphorylation at complex I; $n = 3$), oligomycin plus rotenone ($n = 3$), or combinations of rotenone, oligomycin, and 1 μM atractiloside (inhibits ATP/ADP exchange across the inner mitochondrial membrane; $n = 4$). Taken together, these data indicate that a specific effect on mitochondrial function accounts for the inhibition of store refilling in the intact cell.

How do mitochondrial inhibitors impede internal store refilling? While the simplest explanation for the effects of such pharmacologically diverse compounds as FCCP, oligomycin, rotenone, and atractiloside in the intact cell (Figs. 1 and 3) is that the energy supply for Ca^{2+} uptake had been rapidly compromised upon mitochondrial poisoning, another important consideration is the effect of these agents on capacitative Ca^{2+} entry at the plasma membrane. Since store refilling depends strictly on Ca^{2+} supplied from the extracellular milieu in many cell types, including BHK-21 cells (Hofer et al., 1998), inhibition of this pathway will have important consequences on Ca^{2+} reuptake. To bypass the requirement for Ca^{2+} entry, we took advantage of a phenomenon that we have described recently, permitting internal store refilling in Ca^{2+}-free external medium, provided that the cells have been preloaded with BAPTA-AM (Hofer et al., 1998). As seen in Fig. 3 C (typical of $n = 14$), the rate of recovery in the presence of FCCP after agonist washout was considerably faster in the Ca^{2+}-free solution/BAPTA-AM pretreated condition, where Ca^{2+} entry pathways had been bypassed (FCCP alone was $71 \pm 7\%$ of the BAPTA/FCCP recovery rate). However, the rate of refilling was even faster in

zero Ca^{2+}/BAPTA conditions in the absence of FCCP ($147 \pm 6\%$ of the FCCP + BAPTA recovery rate).

A similar protocol using BAPTA preloading to circumvent the plasma membrane Ca^{2+} entry pathway showed that oligomycin reduced the rate of recovery in BAPTA/0 Ca^{2+} to $47 \pm 7\%$ of that of the control ($n = 19$; Fig. 3 D). It should be noted that somewhat higher concentrations of oligomycin have been reported to inhibit store-operated channels by a means independent of cellular ATP depletion (Cho et al., 1997).

These data imply that a major action of mitochondrial inhibitors on store refilling operates via an indirect mechanism as a consequence of inhibition of capacitative Ca^{2+} entry pathways at the plasma membrane. However, Fig. 3, C and D also indicates that mitochondrial ATP production (or alterations in the ATP/ADP ratio) likely influence Ca^{2+} reaccumulation by the ER to a significant degree. This result was quite surprising, as the available literature suggests that rather dramatic reductions in [ATP] would be required to block SERCA-mediated Ca^{2+} uptake (see Discussion). We therefore checked this point in permeabilized BHK-21 cells by comparing the rates of Ca^{2+} reaccumulation after InsP3-induced release while clamping [ATP] (and [ADP]) in the perfusate to various levels. As shown in Fig. 4 A, rapid recovery was observed even when [ATP] in the bath was reduced to 50 μM. Stores reaccumulated Ca^{2+} at near maximal speed with [ATP] fixed at 200 μM (not shown). Thus, profound cellular [ATP] depletions are necessary to impede Ca^{2+} reuptake.

A further consideration, however, is that metabolic inhibition may alter not only [ATP], but also the ATP/ADP ratio (which is the true index of the driving force for Ca^{2+} reaccumulation). There was very little effect on the recovery when the ATP/ADP ratio was set to one (Fig. 4 B; 0.5 mM ATP, and 0.5 mM ADP or 0.05 ATP and 0.05 ADP; $n = 6$). In contrast, the recovery after InsP3-induced Ca^{2+} release was almost completely blocked when [ATP] was 0.5 mM and [ADP] was 4 mM ($n = 4$; Fig. 4 C).

Mitochondrial Inhibitors Impede Ca^{2+} Release from Internal Stores by at Least Two Mechanisms

In addition to interfering with the refilling process, mitochondrial inhibitors also affect the kinetics of Ca^{2+} release in intact cells. These results can be better appreciated when the time scale of the data presented in the inset of Fig. 3 is expanded, as shown in Fig. 5.

As seen in Fig. 5 A after a 1-min pretreatment with FCCP, cells were stimulated briefly (to minimize receptor desensitization), consistently resulting in a marked slowing of the rate and magnitude of Ca^{2+} release compared with the control BK response in the same cell (rate $51.6 + 4.5\%$ of control; $n = 23$). This result was observed irrespective of whether the control stimulation was performed before or after the stimulation plus FCCP. On the other hand, the rates of release for two successive control challenges with BK were not significantly different from one another (not shown; $n = 16$; see also additional data in Hofer et al., 1998).

Using the same experimental protocol, oligomycin was also shown to alter the kinetics of Ca^{2+} release (Fig. 5 B), although to a lesser extent ($73 \pm 4\%$ of control, $n = 23$)
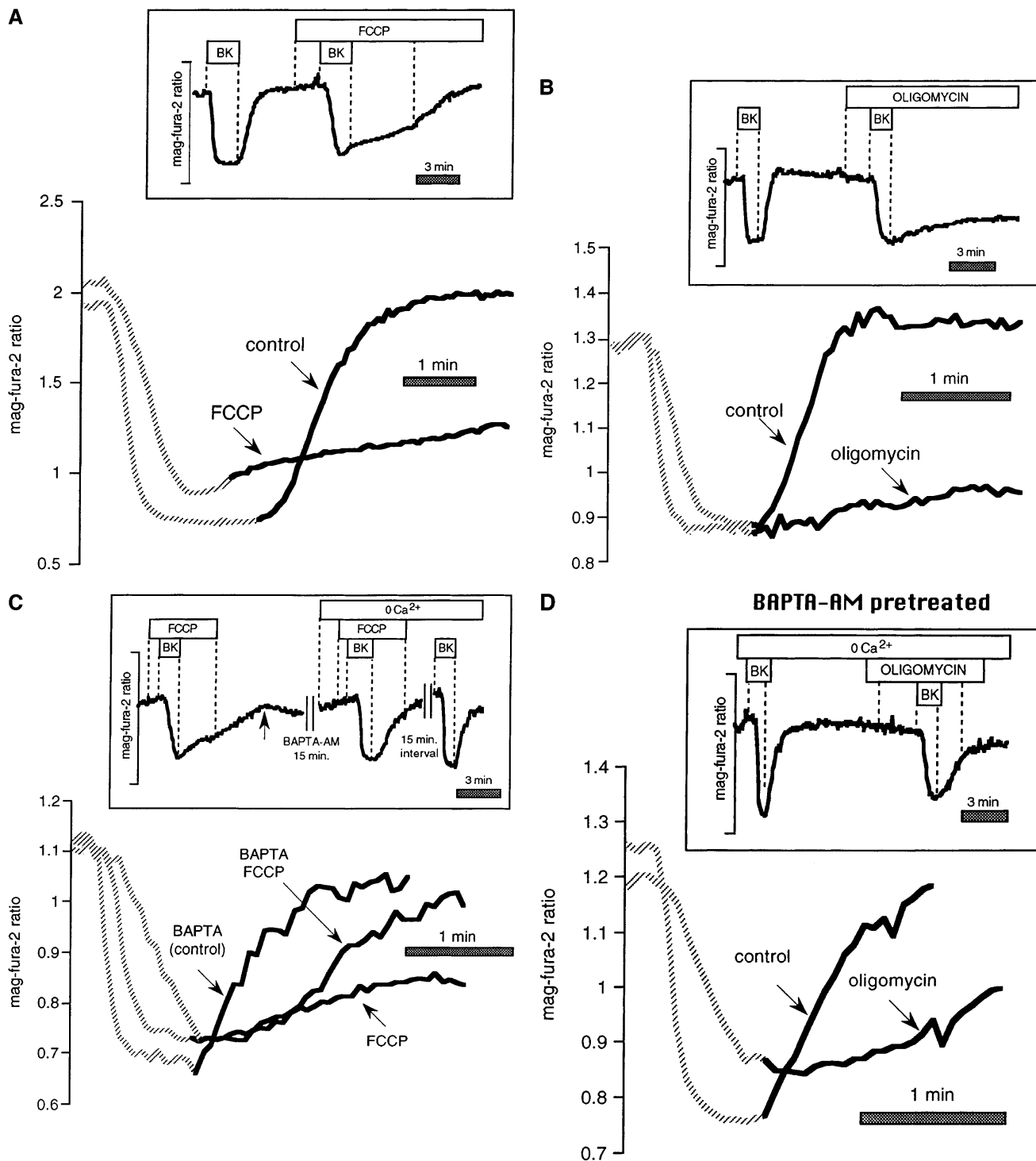
Figure 3. FCCP and oligomycin impede store refilling after BK-induced Ca^{2+} release in intact mag-fura-2-loaded cells. (*A*) Control recovery and response in the same cell to hormone after brief pretreatment with FCCP. (*Inset*) Entire experimental record. (*Bottom*) Enlarged overlay emphasizing the recovery phases with and without FCCP. (*B*) As above, profile of store reloading before and after treatment with 1 μM oligomycin. (*C*) Comparison of store refilling after three separate stimulations with 100 nM BK: first in the presence of 1 μM FCCP in Ca^{2+}-containing medium; second after a 15-min incubation with 40 μM BAPTA-AM (added at *arrow*); cells were washed with normal Ringer's for 5 min, and were then stimulated with BK in Ca^{2+}-free medium containing 1 μM FCCP. Finally, cells were stimulated in the absence of FCCP, still in Ca^{2+}-free external medium. (*D*) Cells were loaded with 40 μM BAPTA-AM for 15 min before the start of experiment. (*Inset*) Responses to 100 nM BK in Ca^{2+}-free medium before and after treatment with 1 μM oligomycin. (*Bottom*) Enlarged overlay of intraluminal [Ca^{2+}] changes, oligomycin vs. control.
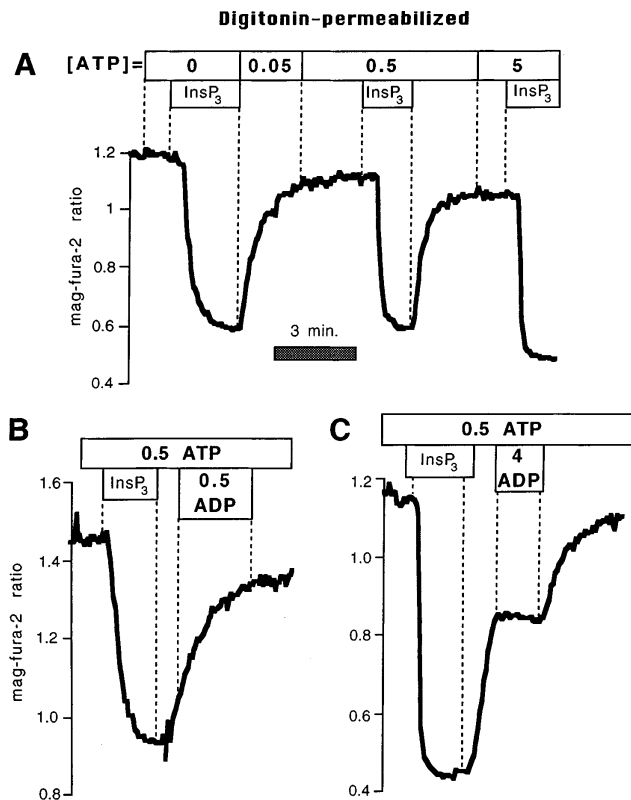
Digitonin-permeabilized

Figure 4. (*A*) Effects of changing [ATP] on rates of refilling in digitonin-permeabilized BHK-21 cells loaded with mag-fura-2. Rates of refilling after stimulation with 6 μM InsP3 in the presence of 50 μM ATP is compared with that in 0.5 mM. Maximal recovery velocity was obtained when [ATP] was 200 μM (not shown). (*B*) Rates of recovery after release with 6 μM InsP3 were barely affected by adding 0.5 mM ADP during the recovery phase. (*C*) 4 mM ADP (in the presence of 0.5 mM ATP) blocked refilling.

than FCCP. Direct comparison in the same cells further confirmed that the uncoupler was more effective in slowing the release of Ca^{2+} than was oligomycin (the rate in FCCP was $68 \pm 6\%$ of that in oligomycin; $n = 22$).

The question then arises as to whether the effects of FCCP or oligomycin in intact cells are due to impairment of ATP production, inhibition of Ca^{2+} uptake by mitochondria, or both. It should be stressed that oligomycin does not block Ca^{2+} uptake by mitochondria, but only inhibits ATP synthesis. Thus, the most plausible explanation for inhibition of Ca^{2+} release by oligomycin alone is that in the absence of mitochondrial ATP production, the release channels in the store become susceptible to reductions in [ATP] (possibly in a localized domain), and to an extent that can compromise the Ca^{2+} release process. The fact that FCCP was more effective in slowing the discharge from internal stores (Fig. 3, *A* and *C*) could therefore be explained by the fact that FCCP produces a more profound depletion of cytoplasmic [ATP] than does oligomycin (see for example Budd and Nicholls, 1996). Alternatively, there may be an additional effect of FCCP because of the failure of mitochondria to buffer cytoplasmic Ca^{2+} changes near the InsP3 receptor, resulting in a reduced

rate of Ca^{2+} release from the stores. To distinguish between these possibilities, we compared the rates of release after FCCP alone with those after treatment with a combination of FCCP and oligomycin (not shown). The rationale for these experiments is as follows: with oligomycin present, the ATP consumption by the mitochondrial F0-F1 ATPase that normally occurs upon FCCP addition will be prevented so that the degree of ATP depletion with the combination of FCCP and oligomycin will be the same as with oligomycin alone. Therefore, if the effect that we observe on Ca^{2+} release is due only to ATP depletion, then FCCP alone should slow down the response to BK much more than the combination of FCCP and oligomycin. However, we observed that the rate of Ca^{2+} release under these two conditions was essentially identical in paired experiments conducted on the same cells ($n = 44$ cells). This result indirectly suggests that Ca^{2+} uptake by mitochondria (abolished by FCCP) also plays a role in modulating Ca^{2+} release from the stores.

We next undertook a series of experiments to more directly assess the role of mitochondrial Ca^{2+} buffering in
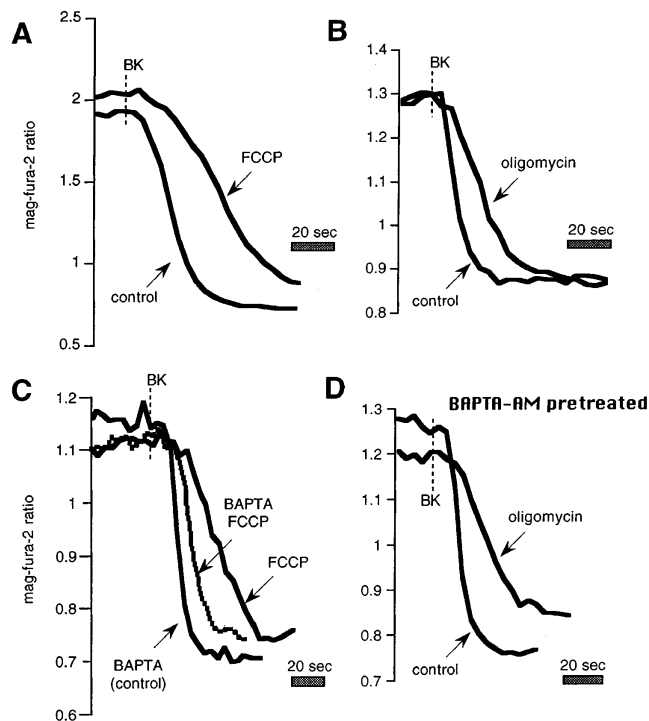

Figure 5. Effects of mitochondrial inhibitors on rates of release in intact mag-fura-2–loaded cells. Data are taken from the records shown in Fig. 3, and are represented on an expanded time scale to allow better appreciation of the release kinetics. (*A*) FCCP vs. control, (*B*) oligomycin vs. control, (*C*) as in Fig. 3 *C*, comparison in the same cell between rates of release: (*a*) in the presence of 1 μM FCCP (FCCP); (*b*) after a 15-min incubation with 40 μM BAPTA-AM, after which cells were washed with normal Ringer's for 5 min and then stimulated with BK in Ca^{2+}-free medium containing 1 μM FCCP (BAPTA/ FCCP); (*c*) cells stimulated in the absence of FCCP, still in Ca^{2+}-free external medium (BAPTA control). (*D*) Data from Fig. 3 *D*: rates of release after BAPTA-AM pretreatment in the presence and absence of oligomycin.

shaping the profile of Ca^{2+} discharge by comparing the rates of release in the presence of FCCP before and after loading of the cytoplasm with the rapid Ca^{2+} buffer BAPTA-AM. The exogenous Ca^{2+} buffer should prevent local increases in $[Ca^{2+}]cyt$ near the InsP3 receptor, in effect rescuing the physiological Ca^{2+}-buffering activity that we predict to be furnished by the mitochondria. Fig. 5 *C* shows that the inhibitory effect of FCCP on BK-induced Ca^{2+} release was largely overcome when the same cells were loaded on the microscope stage for 15 min with 40 μM BAPTA-AM. The rates of release for the first stimulation in the presence of FCCP alone were $59 \pm 7\%$ ($n = 51$) of those with FCCP and BAPTA. This rate was in turn generally slightly slower than the BK-induced Ca^{2+} release measured in the BAPTA-loaded cells in the absence of FCCP, and as illustrated by Fig. 5 *C*, with an altered kinetic profile.

Fig. 5 *D* shows that the rates of release were also attenuated by oligomycin under these conditions; noteworthy were the kinetic differences between the two conditions.

The results of Fig. 5 *C* attest to the importance of mitochondrial Ca^{2+} buffering in modulating the kinetics of ER Ca^{2+} discharge. However, the data of Fig. 5, *B* and *D* also strongly suggest that [ATP] depletion alters the dynamics of Ca^{2+} mobilization, possibly through a direct interaction with the InsP3 receptor. We again checked this point in permeabilized cells. Alterations in [ATP] over a wide range of concentrations had clear effects on the kinetics of Ca^{2+} release. As shown in Figs. 4 and 6 *A*, while the initial rates of release were somewhat slower when [ATP] was reduced from 5 to 0.5 to 0 mM, the most striking action was on the later phases of the response ($n = 18$). The drop in luminal $[Ca^{2+}]$ was consistently less abrupt (less quantal; Meyer and Stryer, 1990) as [ATP] was lowered, as further illustrated by Fig. 6 *B*. Our results in BHK-21 cells differ in part from those of Bezprozvanny and Ehrlich (1993) regarding the type I InsP3 receptor, which was found to have a reduced open probability at [ATP] above 4 mM. No effect of ADP (used at concentrations of 0.5 to 4 mM) was seen (not shown; $n = 6$). These results in permeabilized cells confirm that alterations in intracellular [ATP] can potentially influence the profile of Ca^{2+} release after agonist activation.

Discussion

A large literature exists concerning the effects of mitochondrial inhibitors on Ca^{2+} homeostasis in intact cells (see for example Mohr and Fewtrell, 1990; Babcock et al., 1997; Drummond and Fay, 1996; Friel and Tsien, 1994). These studies relied principally on measurements of Ca^{2+} changes in the cytoplasm (for example, using fluorescent indicators), or used $^{45}Ca^{2+}$ to make population measurements of the total cellular Ca^{2+} content. While some investigators concluded that poisoning of mitochondrial metabolism could compromise Ca^{2+} pumping by the SERCAs (resulting in loss of Ca^{2+} from the ER; Fasolato et al., 1991; al-Baldawi et al., 1993), many other studies have promoted the idea that the mitochondria themselves serve as a large reservoir of Ca^{2+} that can be released into the cytoplasm after treatment with inhibitors (Babcock et al., 1979; Fulceri et al., 1991).
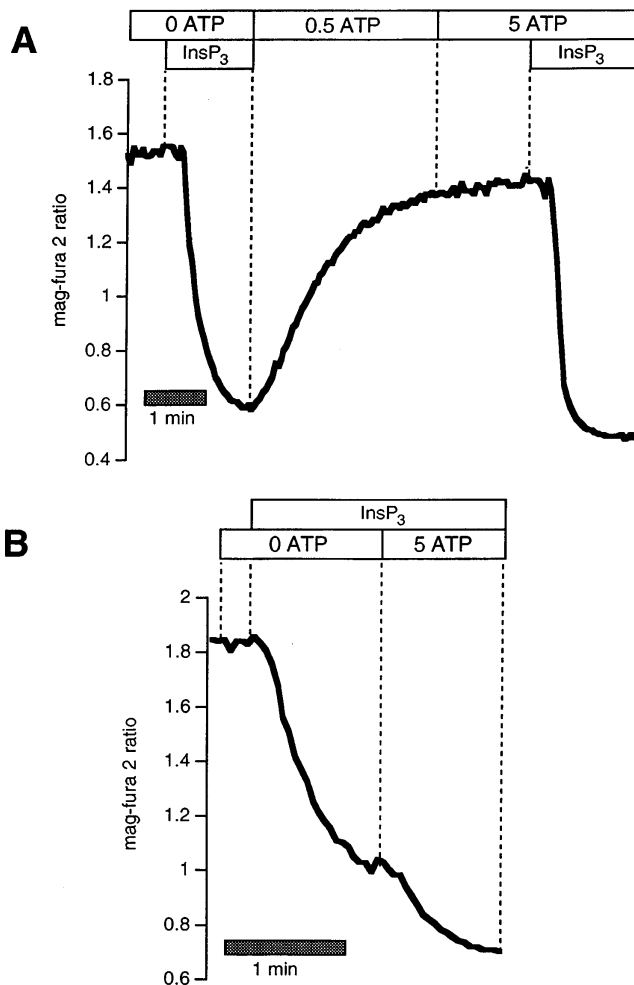

Figure 6. ATP modulates InsP3-induced Ca^{2+} release in digitonin-permeablized cells. (*A*) Comparison of two sequential stimulations with 6 μM InsP3 in the absence of ATP and in the presence of 5 mM ATP. (*B*) Increasing [ATP] to 5 mM during the release phase enhances the rate of InsP3-induced Ca^{2+} discharge.

Here we have reexamined this complex issue by measuring Ca^{2+} in the ER directly using a low-affinity fluorescent indicator in intact cells. Our results show that brief pretreatment with a wide range of mitochondrial inhibitors (atractiloside, rotenone, oligomycin, and FCCP) all resulted in a dramatic inhibition in store refilling after release of stored Ca^{2+} with agonists (Fig. 2). In addition, FCCP or oligomycin added acutely to resting cells frequently caused a slow but measurable loss of stored Ca^{2+} that cannot be attributed to release of the cation from the mitochondria themselves, as the effect was not additive to that obtained by blocking SERCAs with tBHQ. Furthermore, the lack of effect of FCCP in the permeabilized cell preparation, where the $[Ca^{2+}]$ (buffered by EGTA) and the [ATP] of the extraorganellar milieu are fixed (Fig. 2), are consistent with the idea that physiological interactions between mitochondria and other cellular components (the ER and plasma membrane) account for the actions of these drugs in the intact cell.

The data of Fig. 3 *C*, in which stores were refilled in the intact BAPTA-loaded cells in the absence of external

Ca^{2+}, indicate that a significant mechanism by which mitochondrial inhibitors block store refilling is through an indirect effect on plasma membrane Ca^{2+} entry pathways. Consistent with this idea is the finding that FCCP significantly inhibited the plateau phase of Ca^{2+} entry as measured by fura-2 (data not shown; see also Hoth et al., 1997; Marriot and Mason, 1995; Gamberucci et al., 1994). These data illustrate that the entry pathway was potently inhibited in spite of the fact that stores remained relatively empty after treatment with the uncoupler (Fig. 3 *A*).

The results of Fig. 3, *C* and *D* also show, however, that mitochondrial inhibitors influence Ca^{2+} reuptake by an additional mechanism besides that at the plasma membrane, as the rate of refilling in BAPTA-pretreated cells was significantly slower in the presence of oligomycin or FCCP compared with the control condition. We attribute this difference to effects on mitochondrial ATP production. Interpreted in this way, however, these results are somewhat surprising. In the presence of glucose in the bathing medium (such as was the case here), mitochondrial inhibitors are known to cause only a relatively slow and partial (50%) depletion of intracellular ATP in BHK-21 cells (Burkhardt and Argon, 1989), and in a variety of other cell types as well (see for example Mohr and Fewtrell, 1990; Fulceri et al., 1991; Luo et al., 1997). Meanwhile, SERCA pumps, like other ion-motive ATPases, are known to function even when [ATP] falls to quite low levels. In fact, our data from permeabilized BHK-21 cells (Fig. 6) show that stores are able to recover Ca^{2+} at maximal speed when the [ATP] in the bath is clamped to just 200 µM. Reducing the [ATP] in the bath to 50 µM only slowed the rate of refilling to ~65% ($n = 37$ cells), and still allowed complete recovery. Our data may therefore suggest that local ATP depletions (and/or extreme local alterations in ATP/ADP) near the SERCAs that result from the increased pumping activity after agonist activation occur before global changes in cellular [ATP] (or the ATP/ADP ratio) are evident, making the Ca^{2+} uptake process more susceptible to mitochondrial inhibition than might be expected from measurements of total cellular adenine nucleotide content. A wide variety of morphological evidence showing close physical apposition between mitochondria and ER lends support to this idea (McGraw et al., 1980; Nixon et al., 1994; Takei et al., 1992; Rizeuto et al., 1998).

Ca^{2+} buffers have well-established effects on the dynamics of Ca^{2+} release from internal stores (Hajnóczky and Thomas, 1997; Montero et al., 1997). A number of other studies have stressed the importance of mitochondrial Ca^{2+} uptake and release in modulating cytosolic Ca^{2+} signals (Mohr and Fewtrell, 1990; Babcock et al., 1997; Drummond and Fay, 1996; Loew et al., 1994; Jouaville et al., 1995; Simpson and Russel, 1996; Hoth et al., 1997), and our direct measurements of intraluminal ER Ca^{2+} dynamics (Fig. 5, *A* and *C*) also support this view. In addition, however, our data using oligomycin alone to inhibit ATP synthesis also indicates that local ATP availability can subtly alter the responsiveness of stores to agonists. This effect is most readily explained by the ATP dependence of the InsP3 receptor (Smith et al., 1985; Ferris et al., 1990; Bezprozvanny and Ehrlich, 1993), although the possibility that ATP depletion can impede InsP3 production must

also be seriously considered. The data of Figs. 4 and 6 show that altering [ATP] in the intracellular buffer in permeabilized BHK-21 cells had clear effects on the kinetics of InsP3-induced release, which were largely compatible with the observed effects of mitochondrial inhibitors on ER [Ca^{2+}] in intact cells. Again, it is possible that local [ATP] changes may be responsible for the observed differences.

In any event, our results clearly demonstrate that active mitochondrial respiration modulates the dynamics of Ca^{2+} release, allowing for explosive efflux of Ca^{2+} from internal stores. How these kinetic differences influence cytosolic or intraluminal Ca^{2+}-binding effectors, thereby influencing the signal transduction potential of intracellular [Ca^{2+}] changes, remains to be established. These findings prompt the interesting possibility that subtle alterations in the release dynamics may somehow provide a signal to the rest of the cell, conveying information about the cellular metabolic status.

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