Reduced Capacitative Calcium Entry Correlates with Vesicle Accumulation and Apoptosis*

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Supriya Jayadev‡, John G. Petranka, Sendhil K. Cheran, Jennifer A. Biermann, J. Carl Barrett, and Elizabeth Murphy

From the Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

A preneoplastic variant of Syrian hamster embryo cells, sup(+), exhibits decreased endoplasmic reticulum calcium levels and subsequently undergoes apoptosis in low serum conditions (Preston, G. A., Barrett, J. C., Biermann, J. A., and Murphy, E. (1997) Cancer Res. 57, 537–542). This decrease in endoplasmic reticulum calcium appears to be due, at least in part, to reduced capacitative calcium entry at the plasma membrane. Thus we investigated whether inhibition of capacitative calcium entry per se could reduce endoplasmic reticulum calcium and induce apoptosis of cells. We find that treatment with either SKF96365 (30–100 μM) or cell-impermeant, 1,2-bis(o-amino-5-bromophenoxo)ethane-N,N,N′,N″-tetraacetic acid (5–10 mM) is able to induce apoptosis of cells in conditions where apoptosis does not normally occur. Because previous work has implicated vesicular trafficking as a mechanism of regulating capacitative calcium entry, we investigated whether disruption of vesicular trafficking could lead to decreased capacitative calcium entry and subsequent apoptosis of cells. Coincident with low serum-induced apoptosis, we observed an accumulation of vesicles within the cell, suggesting deregulated vesicle trafficking. Treatment of cells with bafilomycin (30–100 nM), an inhibitor of the endosomal proton ATPase, produced an accumulation of vesicles, decreased capacitative entry, and induced apoptosis. These data suggest that deregulation of vesicular transport results in reduced capacitative calcium entry which in turn results in apoptosis.

A number of recent studies have suggested that alterations in cytoplasmic free calcium are important in apoptosis. Some groups reported that an early, sustained increase in cytoplasmic free calcium was a necessary precedent to cell death (2–4). Others found a decrease (5–7) or no detectable change (8–10) in cytoplasmic calcium associated with apoptosis. In contrast to the lack of consensus on a role for cytosolic calcium in apoptosis, a number of groups, including our own, have recently suggested that changes in compartmentalized calcium are important in the regulation of apoptosis (1, 6, 11–14). Calcium within the cell exhibits a highly compartmentalized distribution (15). Free calcium in the cytoplasm is maintained at approximately 100 nM, whereas compartments such as the ER exhibit a severalfold higher (μM to mM) concentration of calcium (16, 17). Calcium gradients are maintained in the cell by membrane transporters. In response to many hormones and growth factors, transient elevations in cytoplasmic calcium occur through the opening of channels located at the ER and plasma membranes. Cytoplasmic calcium changes are initiated through the mobilization of signaling factors such as inositol trisphosphate and cyclic ADP-ribose (18, 19). These molecules act on calcium efflux channels at the ER membrane and promote release of ER calcium stores. The subsequent depletion of ER calcium stores is hypothesized to lead to the activation of a plasma membrane calcium channel, the capacitative calcium entry (CCE) channel (20–22). The ensuing influx of calcium from the extracellular milieu serves to enhance the calcium signal and refill depleted ER stores. This coupling of ER calcium pools with capacitative entry is essential for generating transient intracellular calcium changes. The tight maintenance of calcium gradients and the generation of transient calcium changes provides a key regulatory mechanism for cellular function (23–25).

In a previous study we investigated the role of calcium alterations during apoptosis in two immortalized cell lines derived from mutagenized Syrian hamster embryo cells (1). An early preneoplastic cell line, termed sup(+), was shown to undergo a high rate of apoptosis in low serum conditions. In contrast, a later stage preneoplastic cell line, termed sup(–), was relatively resistant to apoptosis in low serum. An increase in cytoplasmic free calcium was not associated with apoptosis in this cell model; however, consistent with studies of Baffy et al. (6) and Lam et al. (14), we found that a decrease in ER calcium was associated with apoptosis in the sup(+) model. The sup(–) cells, which were resistant to apoptosis in low serum, showed no alteration in ER calcium. Pharmacological reduction of ER calcium stores with thapsigargin resulted in apoptosis of sup(–) cells in low serum and of sup(+) cells in 10% serum (normal growth conditions). Conversely, maintaining ER calcium levels of sup(+) cells in low serum prevented the DNA fragmentation characteristic of apoptosis. Furthermore, the previous study suggested that decreased ER calcium in apoptotic sup(+) cells was due, at least in part, to decreased CCE leading to poor refilling of ER calcium stores (1).

In this study we sought to expand our understanding of the relationship between mechanisms responsible for decreased calcium entry and apoptosis. We find that preventing CCE by treating with the CCE inhibitor SKF96365 or by chelating

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‡ To whom correspondence and reprints should be addressed: NIEHS, P.O. Box 12233, MD D2–03, Research Triangle Park, NC 27709. Tel.: 919-541-4976; Fax: 919-541-3385; E-mail: jayadev@niehs.nih.gov.

1 The abbreviations used are: ER, endoplasmic reticulum; CCE, capacitative calcium entry; Baf, bafilomycin; GLUT4, glucose transporter type 4; BCECF-AM, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; GTPγS, guanosine 5′-O-[3-thiotriphosphate]; GDPβS, guanyl-5′-y-thiophosphate; BAPTA, 1,2-bis(o-amino-5-bromo-phenoxy)ethane-N,N,N′,N″-tetraacetic acid.
extracellular calcium with BAPTA could induce apoptosis of sup(+) cells. We therefore sought to understand the mechanism responsible for perturbation of CCE in apoptotic sup(+) cells. Many hypotheses have been proposed for the mechanism by which capacitative calcium entry is activated (for reviews, see Refs. 18 and 22). Studies have suggested a role for a diffusible second messenger (26–29), phosphorylation/dephosphorylation (27, 29–34), small G proteins (35–37), and, recently, a role for vesicular storage and regulated insertion of entry channels (38, 39). During apoptosis we find an accumulation of vesicles within the cell, consistent with altered vesicular trafficking. Thus we considered the possibility that perturbation of vesicle trafficking during apoptosis may lead to disrupted signaling to CCE. We find that perturbing vesicle trafficking in normally growing sup(+) cells by treatment with the macrolide antibiotic bafilomycin (Baf) results in vesicle accumulation concomitant with decreased CCE and apoptosis. Furthermore, we find that in the sup(−) population, which does not undergo apoptosis in reduced serum conditions, perturbation of either CCE (with SKF96365) or vesicle trafficking (with bafilomycin) led to apoptosis. These findings support a role for vesicle trafficking and CCE in apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials—** Fura-2 AM, BCECF-AM, and nigericin were obtained from Molecular Probes, proteinase K from Boehringer Mannheim, and RNase A from Sigma/Aldrich. Institute for Biological Research medium was obtained from Life Technologies Inc. and fetal calf serum was obtained from Summit Biotechnologies.

**Cell Lines and Cell Culture—** Two Syrian hamster embryo-derived lineages, originally immortalized via mutagenesis, were used in these studies (40, 41). The sup(+) lineage represents an early stage of tumorigenesis which has lost a senescence gene(s) but retains tumor suppressor capability; whereas the sup(−) lineage represents a later stage of tumorigenesis which has lost both senescence and tumor suppressor genes. During normal passage, sup(+) and sup(−) cells were maintained in Dulbecco’s modified Institute for Biological Research medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37 °C incubator with 10% CO2, 90% air. For experiments, cells were washed and pelleted in calcium- and magnesium-free phosphate-buffered saline (after treatment with bafilomycin). Cells were then seeded at 104 cells/30-mm plate and grown for 24 h. Cells were washed and treated for 4 h. BCECF-AM (4 μM) was introduced to each plate 15 min prior to measurements. Coverslips were then washed twice with media containing 20 mM HEPES, pH 7.4, and placed in a custom-built holder. One mL of media containing 20 μM bafilomycin, pH 7.4, was added to the chamber and the entire unit was placed on the stage of a Nikon inverted epifluorescence microscope coupled to a PTI Deltascan dual wavelength excitation spectrofluorometer. Measurements were taken in a window containing 2–6 cells. Emission was measured at 535 nm following excitation at 439 and 505 nm. Background fluorescence within an area containing no cells, was subtracted. The ratio of fluorescence intensity was obtained by dividing the emitted fluorescence for excitation at 505 nm by the emitted fluorescence for excitation at 439 nm. To calibrate dye fluorescence for each sample, the ratio of fluorescence was measured at 4–5 pH values using the high KCl-nigericin technique (44, 45).

**Electron Microscopy—** Cells were seeded in 100-mm plates at a density of 5 × 104 cells/plate and grown for 24 h. Cells were washed and incubated in calcium- and magnesium-free phosphate-buffered saline and 5 mL of the appropriate treatment media was added. Following 4–16 h (see figure legends), 5 mL of 2 × fixation buffer (4% glutaraldehyde, 0.2 M sodium cacodylate, pH 7.2) was added to the plates. Cells were scraped into the media/fx mixture and pelleted. Pellet pellets were re-suspended in 1 mL of 1 × fixative and incubated at 4 °C for 2 h. Fixed cells were washed with 0.1 M sodium cacodylate, pH 7.2, and maintained in fresh buffer at 4 °C until further processing was done. Samples were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and embedded in Poly Bed 812. The samples were cured for 3 days at 60 °C and thin sections were cut. Sections were stained with 5% uranyl acetate followed by Reynolds’ technique (44, 45). Thin sections were then examined using a Phillips 400 transmission electron microscope.

**Statistical Analyses—** Values are mean ± S.E. In comparing two groups, statistical significance was determined by Student’s t test. For multiparameter comparisons, statistical significance was determined by ANOVA, adjusting for multiple comparisons using Fisher’s test for significance. A value of p < 0.05 was considered to be significant.

**RESULTS**

**Capacitative Calcium Entry and Apoptosis in Sup(+) Cells—** Sup(+) cells, an immortalized cell line derived from mutagenesis of Syrian hamster embryo cells (40), undergo apoptosis in response to low serum conditions (41). In a previous study, a decrease in ER calcium was shown to precede apoptosis in sup(+) cells (1). The decrease in ER calcium was, in turn, found to be secondary to a decrease in the refilling pathway of CCE (1). Therefore, we were interested in testing whether inhibition of CCE per se could induce apoptosis of cells under conditions where apoptosis does not normally occur (10% serum). We found that cells with inhibition of CCE (CCE−) cells following treatment with SKF96365 (Fig. 1), an inhibitor of CCE and other calcium channels. As little as 30 μM SKF96365 was able to reduce CCE approximately 30%. Higher doses of SKF96365 decreased CCE even more significantly, from the peak control calcium entry of 1.5 ± 0.9 to 0.15 ± 0.1 μM with 100 μM SKF96365 (Fig. 1). In conjunction with CCE, we also measured ER calcium as the thapsigargin releasable calcium as described under "Experimental Procedures." Sup(+) cells treated with SKF96365 exhibited a 50% decrease in ER cal-
Capacitative Entry, Vesicles and Apoptosis

FIG. 1. SKF96365 causes a dose-dependent inhibition of capacitative calcium entry. Sup(+) cells were treated for 4 h with 0, 30, 50, or 100 μM SKF96365 in 10% serum media. During the final 30 min of treatment cells were also loaded with fura-2 (2 μM). The 100 μM SKF96365 (n = 6) treatment showed a significant decrease (p < 0.01) in CCE compared with control, untreated cells (n = 5). The 30 μM (n = 2) and 50 μM (n = 3) SKF96365 treatments also follow a trend of decreased CCE compared with controls.

CCE within 4 h following treatment. In control cells (10% serum) thapsigargin released 116 ± 15 nM ER calcium into the cytosol versus 42 ± 11 nM in SKF96365 (100 μM) treated cells.

The same doses of SKF96365 which effected CCE were also able to induce apoptosis (Fig. 2). Within 16 h of treatment with 30 μM SKF96365, sup(+) cells grown in 10% serum showed the classic DNA laddering pattern indicative of apoptosis (lane 2). Similar to the dose-dependent inhibition of CCE, higher doses of SKF96365 were able to induce more prominent laddering of sup(+) cells (lanes 3 and 4). Since SKF96365 is a non-selective inhibitor of CCE, we further substantiated the relationship between CCE and apoptosis using cell-impermeant BAPTA to chelate extracellular calcium. BAPTA chelates calcium with one to one binding, therefore millimolar concentrations of BAPTA are required to chelate the 1.8 mM calcium concentration present in the treatment media. Thus, lower doses of BAPTA were unable to reduce extracellular calcium beyond the high micromolar range (e.g. 1 mM BAPTA only decreased the calcium concentration to ~800 μM), and correspondingly were unable to induce apoptosis (Fig. 3, lanes 2–5). In contrast, doses of BAPTA which significantly chelated extracellular calcium (5 mM BAPTA reduced the calcium concentration to ~390 nM and 10 mM BAPTA reduced the calcium concentration to ~150 nM), were able to induce DNA ladders in sup(+) cells (Fig. 3, lanes 6 and 7, for comparison, lane 8 shows low serum-induced apoptosis).

Capacitative Entry and Vesicle Accumulation in Sup(+) Cells—Many hypotheses have been proposed for the mechanism responsible for CCE (for reviews, see Refs. 18, 22, 37, and 46). One model which has been suggested is the control of calcium entry through regulated insertion of CCE channels (38, 39). In this model, signals from a store-depleted ER would “activate” an endomembrane compartment enabling it to fuse with the plasma membrane, inserting more entry channels into the plasma membrane. A prediction of this model would be that inhibiting vesicle movement to and/or fusion with the plasma membrane would inhibit CCE. Intriguingly, in many morphological discussions of cell death, the apoptotic process is associated with accumulation of vesicles within the cell (47–52).

Similar to many other systems that undergo apoptosis, we found that apoptotic sup(+) cells accumulate vesicles in the cytoplasm. Sup(+) cells maintained in low serum conditions for 16 h show a marked increase in the number of vesicles present per cell as well as an increase in the number of cells with vesicles (compare Fig. 4A (10%, 16 h) to Fig. 4B (low serum, 16 h)). The increase in vesiculation in low serum-treated sup(+) cells occurs early, within 4 h an increase in vesicles is readily apparent (Fig. 4C), preceding other known morphological markers of apoptosis such as nuclear and cytoplasmic compaction (49, 51).

It is interesting to note that not all apoptotic treatments lead to vesicle accumulation. Whereas low serum treatment can lead to vesicle accumulation (Fig. 4, B and C) and decreased CCE (Ref. 1 and Fig. 7), SKF96365 treatment only decreases CCE (Fig. 1) without affecting vesicle accumulation (Fig. 4D). This suggests that the sequence of events is one in which vesicle accumulation precedes decreased CCE. Thus, pharmacological alteration of CCE (e.g. with SKF96365) would not necessarily require the involvement of vesicles.

Bafilomycin Increases Vesiculation and Induces Apoptosis of Sup(+)/Cells—If vesicle accumulation is involved in the regulation of apoptosis as opposed to a result of apoptosis, then pharmacological inhibition of vesicular trafficking should induce apoptosis. Bafilomycin A₁ (Baf), a potent selective inhibitor of vacuolar H⁺-ATPase (53, 54) has previously been shown to inhibit late Golgi and post-Golgi trafficking of vesicles to the plasma membrane (55–57). We find that treatment of sup(+) cells in 10% serum with 50 nM Baf for 16 h resulted in the accumulation of large vacuoles within the cell (Fig. 4E). As with low serum (in the absence of Baf), with Baf treatment vesiculation was apparent early (within 4 h, Fig. 4F), preceding other morphological markers of apoptosis such as nuclear/cytoplasmic compaction, rounding up of cells, and decreased cell size, apparent by 16 h of treatment (Fig. 4E). Interestingly, treating sup(+) cells with Baf in low serum served to speed up apoptosis, such that morphological changes such as nuclear and cy-
Capacitative Entry, Vesicles and Apoptosis
toplastic condensation were seen as early as 4 h following treatment (Fig. 4G).

We next evaluated whether the increase in vesiculation induced by Baf resulted in induction of apoptosis. As with SKF96365 treatment, Baf treatment caused apoptosis in sup(+) cells under conditions where apoptosis does not normally occur, e.g., in 10% serum. As shown in Fig. 5, 16 h of treatment with 50 nM Baf in 10% serum (lane 2), conditions in which Baf induced intracellular vesicle accumulation, resulted in apoptosis similar to that observed with low serum treatment (lane 3).

Bafilomycin Does Not Affect Cytoplasmic pH of Sup(+) Cells—Baf acts to dissipate endosomal pH gradients and in some cells this perturbation of vesicular pH causes a change in the cytoplasmic pH (58). In addition, there have been reports that a decrease in cytoplasmic pH is involved in initiating apoptosis (59–62). To ensure that the results with Baf were due to changes in vesiculation and not to changes in pH, we investigated whether changes in cytoplasmic pH occur with the addition of Baf or during low serum-induced apoptosis in sup(+) cells. By 4 h of treatment with Baf, when vesicle accumulation was evident, there was no significant effect on cytoplasmic pH of sup(+) cells (Fig. 6). Sup(+) cells grown in 10% serum had a pH of 7.43 ± 0.04 and cells grown in 10% serum plus Baf had a pH of 7.46 ± 0.02. Similar to Baf treatment, low serum did not significantly change the cytoplasmic pH of sup(+) cells. Thus, we conclude that Baf induces apoptosis and vesiculation without effecting cytoplasmic pH.

Bafilomycin Decreases Capacitative Entry—We next examined whether Baf treatment causes a decrease in CCE. If CCE is regulated by insertion of calcium entry channels into the plasma membrane, we would expect that perturbing vesicular insertion into the plasma membrane by addition of Baf would decrease CCE. Typical traces of CCE measurements are shown in Fig. 7A. In 10% serum CCE is markedly higher than in low serum or Baf-treated cells. Fig. 7B shows similar data summing up observations from 8 to 11 separate experiments. At 4 h of Baf treatment in 10% serum, CCE is reduced approximately 30% compared with control, 10% serum levels (similar to the reduction seen with low serum treatment). Interestingly, Baf treatment in low serum conditions did not produce an additive effect on CCE at 4 h (Fig. 7B). Although co-treatment may have sped up the apoptotic process (Fig. 4G) the non-additive effect of Baf and low serum on CCE at 4 h suggests that early events which lead to apoptosis may be the same with both treatments.

Since Baf is known to inhibit the vacuolar proton ATPase within minutes, we also investigated the effect of Baf on CCE at early time points. Acute treatment with Baf had no effect on CCE and even following 30 min of treatment, Baf had no significant effect on CCE (remaining at 82% ± 36 of control (n = 6)). It was only after prolonged treatment with Baf that reduced CCE could be observed, with 2 h of treatment reducing CCE by 45% (n = 4) and 4 h treatment reducing CCE by 32%.

Vesciculation, CCE, and Apoptosis in Sup(−) Cells—Finally, if vesicle trafficking and CCE are involved in the apoptotic process in general, then the same relationships should hold true in other cell systems. Thus, to further substantiate our findings we expanded our study to sup(−) cells. Sup(−) cells do not exhibit decreased ER calcium or undergo apoptosis under low serum conditions; however, our previous study established that pharmacological reduction of ER calcium during low serum treatment of sup(−) cells resulted in apoptosis (1). Thus, we investigated whether decreasing CCE or altering vesicle trafficking could induce apoptosis in sup(−) cells. We found that sup(−) cells treated with SKF96365 showed decreased CCE and underwent apoptosis (Fig. 8). Unlike sup(+) cells, sup(−) cells did not show decreased CCE with low serum treatment alone (Fig. 8A). Consistently, sup(−) cells treated with low serum did not exhibit the classic laddering pattern indicative of apoptosis but instead showed a smeared DNA pattern, indicative of necrotic death (Fig. 8B, lane 2). In contrast, sup(−) cells treated with SKF96365 in conjunction with low serum showed both decreased CCE (Fig. 8A) and apoptosis (Fig. 8B, lanes 3–6). As with sup(+) cells, reduction in CCE temporally preceded apoptosis, with over an 80% decrease in CCE apparent within 4 h and DNA ladders apparent only after 16 h.

Since we hypothesize that vesicle trafficking affects CCE and, in turn, apoptosis, we next determined the effect of perturbing

**Fig. 4.** Electron micrographs of sup(+) cells undergoing apoptosis. Sup(+) cells were treated for 4 h (panels C, F and G) or 16 h (panels A, B, D, and E) with; panel A, 10% serum; panels B and C, low serum; panel D, 10% serum + 100 μM SKF96365; panels E and F, 10% serum + 50 nM Baf; panel G, low serum + 50 nM Baf. Control cells (A) show "normal" morphology of elongated cells containing intact organelles and few vesicles. Under low serum conditions (B and C) and with SKF96365 (D) or Baf (E-G) treatment, cells exhibit the classic morphological changes which accompany apoptosis: nuclear condensation, rounded morphology, and decreased cell size. Only with low serum or Baf treatment is increased vesicle accumulation observed (B, C, and E-G). The increase in vesicle accumulation occurs early, preceding many of the other markers of apoptosis (C and F). Combined treatment with low serum and Baf appears to speed up the apoptotic process such that by 4 h apoptotic morphology can be seen in the cell population (G). Magnifications: A, × 6,200; B, × 14,190; C, × 8,580; D, × 11,000; E, × 14,190; F, × 6,200; G, × 11,000.

**Fig. 5.** Bafilomycin induces apoptosis in sup(+) cells in 10% serum. Sup(+) cells were treated for 16 h with 10% serum (lane 1), 10% serum + 50 nM Baf (lane 2), or low serum (lane 3). A representative agarose gel of extracted DNA is shown. Both low serum treatment and Baf treatment resulted in DNA fragmentation.

**Fig. 6.** Bafilomycin does not alter cytoplasmic pH. Sup(+) cells were treated for 4 h with 10% serum, low serum, or 10% serum + 50 nM Baf. During the final 15 min of treatment cells were loaded with BCECF. Values are means ± S.E. from five to nine separate experiments. Neither low serum (7.39 ± 0.1) nor Baf treatment (7.46 ± 0.02) produced significant changes in cytoplasmic pH compared with control levels (7.43 ± 0.04).
vesicle trafficking in sup(−) cells. As with the sup(+) population, sup(−) cells maintained in 10% serum conditions show a minimal number of vesicles present within the cell (Fig. 9a). Sup(−) cells grown in low serum did not exhibit an accumulation of vesicles (Fig. 9b), consistent with our suggestion that aberrant vesicle trafficking is part of (and is upstream of) the signal for decreased CCE and apoptosis. Since sup(−) cells do not exhibit reduced CCE or undergo apoptosis in low serum, they should not show vesicle accumulation in this condition. Conversely, we would hypothesize that altering vesicle trafficking with an agent such as Baf should result in the same effects as was observed in the sup(+) population, decreased CCE and apoptosis. Indeed we find that Baf treatment of sup(−) cells in low serum results in a marked accumulation of vesicles (Fig. 9c) and approximately 60% reduction in CCE within 4 h (Fig. 10a). Furthermore, Baf treatment of sup(−) cells was able to change necrotic death (evidenced in low serum as smeared DNA, Fig. 10b, lane 1) to apoptotic death (evidenced by the classic DNA ladders, Fig. 10B, lane 2).

**FIG. 7. Bafilomycin inhibits capacitative calcium entry.** Sup(+) cells were treated as indicated for 4 h. During the final 30 min of treatment cells were also loaded with 2 μM fura-2. Data are presented as 340/380 ratios. A, a representative trace is shown for: 1, 10% serum; 2, low serum; 3, 50 nM Baf + 10% serum; B, data from eight to 11 separate measurements were averaged and averages ± S.E. are shown. All three treatment groups (low serum, 10% serum; 3, 50 nM Baf + 10% serum) showed a significant increase in CCE compared with control, untreated cells as assessed by analysis of variance for repeated measurements followed by the Fisher’s test.

**FIG. 8. SKF96365 inhibits capacitative calcium entry and induces apoptosis of sup(−) cells.** Panel A, sup(−) cells were treated for 4 h with 10% serum (n = 7), low serum (n = 6), or low serum + 50 μM SKF96365 (n = 3). During the final 30 min of treatment cells were also loaded with 2 μM fura 2-AM. Data are presented as 340/380 ratios ± S.E. Unlike the sup(+) cell line, sup(−) cells do not exhibit a significant decrease in CCE with low serum treatment. However, combined treatment with SKF96365 and low serum results in a significant (p < 0.01 versus low serum or 10% treatment) inhibition of CCE. Panel B, sup(−) cells were treated for 16 h with: lane 1, 10% serum; lane 2, low serum; lane 3, low serum + 1 μM SKF96365; lane 4, low serum + 5 μM SKF96365; lane 5, low serum + 10 μM SKF96365; and lane 6, low serum + 50 μM SKF96365. Low serum shows a smeared pattern of DNA indicative of necrotic death of the sup(−) population. In contrast, SKF96365 treatment shows laddering, indicative of apoptotic death, higher doses show more prominent ladders with 50 μM exhibiting the most prominent ladders.

**DISCUSSION**

Calcium regulation of apoptosis has been studied in a myriad of systems (63–65). As more studies have focused on calcium and apoptosis, there has been increasing controversy as to the exact role that calcium plays. Our work and that of others, have suggested that decreased ER calcium stores are important in activation of apoptosis (1, 6, 12, 13). We have shown previously that decreased ER calcium correlated with decreased CCE (1). However, it was not clear from the previous study whether the decrease in CCE was a cause or an effect of apoptosis. To address this question, we inhibited CCE by addition of SKF96365, a nonspecific inhibitor of the CCE channel (66). We find in the sup(+) and sup(−) cell lines that SKF96365 inhibits CCE within 4 h of treatment. We therefore examined whether SKF96365 inhibition of CCE per se could induce apoptosis. As shown in Figs. 2 and 8, at doses which inhibit CCE, SKF96365 also induces apoptosis. Since SKF96365 is reported to inhibit other calcium entry pathways (66), we confirmed the interconnection between CCE and apoptosis using an extracellular calcium chelator-BAPTA. Similar to low serum or SKF96365 treatment, chelating extracellular calcium with cell-impermeant BAPTA resulted in DNA fragmentation. Thus, inhibiting the ER refilling pathway seems sufficient to signal apoptosis of sup(+) and sup(−) cells.

The signal connecting a decrease in ER calcium to CCE has been studied by a number of investigators and continues to be a very active area of investigation (22, 46, 67, 68). At this point...
the precise mechanism of CCE regulation remains largely unknown, despite intense investigation. It has been reported that a soluble messenger is responsible for stimulating CCE (26–29), although others have questioned this mechanism (37). Alterations in phosphorylation have also been proposed to be important in CCE (27, 29–34), although again there are data to the contrary (22). One consistent finding across laboratories is that addition of GTP\(_{\text{gS}}\) or GDP\(_{\text{bS}}\) can inhibit CCE (35, 36, 69, 70). The heterotrimeric G proteins and most small G proteins are activated by GTP\(_{\text{gS}}\) and inhibited by GDP\(_{\text{bS}}\) (71). Inhibition by both GTP\(_{\text{gS}}\) and GDP\(_{\text{bS}}\) is consistent with the involvement of the Rab proteins (71–73), which are small G proteins important in vesicle trafficking (72, 73). Further support suggesting that vesicle transport is involved in the regulation of CCE has come from: 1) studies showing that CCE exhibits cold sensitivity (39), and, 2) studies showing that CCE is inhibited by the lysosomotropic inhibitor primaquine (38). It is intriguing to speculate that perhaps CCE is regulated in a manner similar to GLUT4 regulation. In the GLUT4 model, insulin stimulation of cells leads to the fusion of vesicles containing the GLUT4 transporter with the plasma membrane (74–76). Thus, increased glucose uptake results from increased transporters at the plasma membrane. Whether regulated insertion of CCE channels represents an important and universal mechanism for modulating calcium entry following store depletion remains to be determined. Nonetheless, studies from a number of groups as well as the current study suggest a role for vesicle trafficking and insertion of CCE channels as a means of modulating CCE.

In light of a possible role for vesicle trafficking in CCE, we were struck by the accumulation of vesicles that occurs in sup(+) and other cells undergoing apoptosis. Indeed, in many morphological discussions of apoptosis, accumulation of vesicles has been defined as one of the hallmarks of apoptosis (47–52). The accumulation of vesicles in early apoptosis is consistent with altered vesicle trafficking and might be related to inhibition of CCE. To test further this hypothesis, we inhibited vesicular transport by addition of bafilomycin. Bafilomycin, a known specific inhibitor of the vacuolar H\(^+\)-ATPase (53,
54), has been previously shown to inhibit late Golgi and post-Golgi trafficking of vesicles to the plasma membrane (55–57). As expected from such an effect, Baf was able to induce the accumulation of vesicles within the cytoplasm of sup(+) and sup(−) cells. More importantly, we found that Baf treatment of sup(+) cells in 10% serum and sup(−) cells in low serum, significantly decreased CCE and induced apoptosis of cells. The current study not only establishes a connection between vesicle trafficking and CCE but also between these two events and apoptosis. Although a number of studies have suggested a connection between CCE and vesicle trafficking, this is the first study to address the interconnection of these events with apoptosis.

Mechanistically, it appears that the means by which signal- ing to CCE becomes disrupted during apoptosis involves vesicular trafficking. One of the classical morphological characteristics which defines apoptosis is vesicle accumulation near the plasma membrane (47–52). In the sup(+) population undergoing apoptosis we also observe vesicle accumulation. Further- more, in cells exhibiting vesicle accumulation associated with low serum or Baf, we observe decreased capacitative entry and apoptosis. The converse does not appear to be true, however, cells treated with SKF96365 have decreased CCE but they do not show accumulation of vesicles (Fig. 4D). These findings serve to order events into a scheme whereby vesicle accumulation precedes aberrant CCE and decreased CCE precedes apoptosis. Thus, as shown with SKF96365, inhibition of CCE entry without affecting vesicular trafficking appears to be sufficient to induce apoptosis of cells. The universality of this mechanism of regulating CCE and apoptosis to be determined.

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