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An essential role for calcium flux in phagocytes for apoptotic cell engulfment and the anti-inflammatory response

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Abstract

Cells undergo programmed cell death/apoptosis throughout the lifespan of an organism. The subsequent immunologically silent removal of apoptotic cells plays a role in the maintenance of tolerance; defects in corpse clearance have been associated with autoimmune disease. A number of receptors and signaling molecules involved in this process have been identified, but intracellular signaling downstream of corpse recognition is only being defined. Calcium plays a key role as a second messenger in many cell types, leading to the activation of downstream molecules and eventual transcription of effector genes, however, the role of calcium signaling during apoptotic cell removal is unclear. Here, using studies in cell lines and in the context of a whole organism, we demonstrate that apoptotic cell recognition induces both an acute and sustained calcium flux within phagocytes and that genes required for calcium flux are essential for engulfment. Furthermore, we provide evidence that both the release of calcium from the endoplasmic reticulum and the entry of extracellular calcium via CRAC channels into the phagocytes are important during engulfment. Moreover, knockdown in *C. elegans of stim-1* and *jph-1*, two genes linked to the entry of extracellular calcium into cells, led to increased persistence of apoptotic cells in the nematode. Loss of these genes appeared to affect early signaling events, leading to decreased enrichment of actin adjacent to the apoptotic cell during corpse removal. We also show that calcium is crucial for the secretion of TGF-β by phagocytes during engulfment of apoptotic cells. Taken together, these data point to a previously unappreciated and evolutionarily conserved role for calcium flux at two distinguishable steps: the formation of the phagocytic cup and internalization of the apoptotic cell, and the antiinflammatory signaling induced in phagocytes by contact with apoptotic cells.

Introduction

In multicellular organisms, engulfment of apoptotic cells is important for the maintenance of tissue homeostasis and prevention of autoimmunity (1). Although classically this process was believed to be performed largely by professional phagocytes such as macrophages and dendritic cells, more recent data suggests that neighboring cells often remove apoptotic cell corpses (2). Recognition of apoptotic cells occurs via receptors on the engulfing cells that recognize so called “eat-me” signals on the surface of the apoptotic cell (3). Some of the molecules involved downstream of these receptors have been identified and shown to lead to the activation of the small GTPase Rac1, resulting in the reorganization of the actin

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cytoskeleton and apoptotic cell engulfment (4). Contrary to other types of phagocytosis, recognition of apoptotic cells by phagocytes elicits the production of anti-inflammatory mediators such as TGF-β and prostaglandin E_2 (1), which are thought be part of the 'immunologically silent' clearance of apoptotic cells and tolerance to self antigens derived from apoptotic cells (3). Interestingly, the "recognition" of apoptotic cells by phagocytes without physical internalization appears to be sufficient to induce an anti-inflammatory response from macrophages (5). This suggests that signals for internalization of the corpse by phagocytes and for generation of anti-inflammatory mediators may be separate and diverge downstream of receptor activation. Identifying common versus unique signals for these engulfment events could also help define features relevant for immunological tolerance and autoimmunity.

Ca^{2+} ions also serve as important second messengers in signal transduction in many cell types, including neurons, lymphocytes, muscle cells and leukocytes. In many cells of the immune system, triggering of cell surface receptors also induces a rise in cytosolic calcium, which induces activation of a number of signaling pathways that promote activation, proliferation and various effector functions in the cell [reviewed in (6)]. Cytoplasmic levels of Ca^{2+} can increase either from release from internal calcium stores [such as the endoplasmic reticulum (ER)] or entry from outside the cell via calcium channels, leading to the rapid activation of molecules that promote activation, proliferation and other functions in a cell. Phagocytes also utilize calcium flux: activation of macrophages with LPS, for example, leads to an increase in cytosolic calcium and the eventual production of TNF-α (7). However, the role calcium flux in mammalian phagocytes during removal of apoptotic cells is not known. During the course of our work, Cuttell et al. reported that mutations in several genes linked to calcium flux can lead to defects in the proper clearance of apoptotic cells (8). It was unclear, however, whether recognition of apoptotic cells lead to calcium flux in phagocytes, at what step(s) the phagocyte calcium flux was required in the engulfment process, and whether the requirement for genes linked to calcium flux machinery was evolutionarily conserved. Here, we demonstrate that a rise in cytoplasmic Ca^{2+} occurs during recognition of apoptotic cells and that it is relevant for efficient engulfment of apoptotic cells by mammalian cells. Our studies demonstrate an evolutionarily conserved role for calcium flux during apoptotic cell clearance in *C. elegans* and that calcium flux in phagocytes regulates at least two distinct steps during apoptotic cell clearance.

Results

Intracellular calcium flux in phagocytes after contact with apoptotic cells is essential for the uptake of targets

In many cells of the immune system, triggering of cell surface receptors results in a rise in cytosolic calcium, which activates a number of signaling pathways linked to optimal activation, proliferation and downstream gene expression (6). To determine whether a calcium flux is initiated in phagocytes during recognition of apoptotic cells, we labeled LR73 cells (a phagocytic fibroblast cell line) with the calcium binding fluorescent dye Fluo-4, incubated them with apoptotic Jurkat cells and followed changes in intracellular calcium levels by real time fluorimetry. We were able to detect a steady rise in cytosolic Ca^{2+} after the addition of apoptotic cells (Fig. 1a), confirming that calcium flux occurs following stimulation of phagocytes with apoptotic cells.

Rise in cytoplasmic levels of Ca^{2+} in other contexts, such as after antigen receptor stimulation in lymphocytes, occurs as a result of release from internal calcium stores in the endoplasmic reticulum (ER), followed by entry of extracellular calcium via calcium channels in the plasma membrane (9). Entry of extracellular calcium, which is usually triggered by the sensing of the calcium release from intracellular stores, serves to replenish

the depleted stores from the endoplasmic reticulum and can also critically modulate signal transduction in other systems (10). To test whether the observed rise in calcium in phagocytes after incubation with apoptotic cells was due to the entry of calcium from outside, we added BAPTA or EGTA to chelate extracellular calcium present in the cell culture medium. Both drugs inhibited the calcium flux in phagocytes exposed to apoptotic cells (Fig 1a), suggesting a role for extracellular calcium during engulfment.

We next asked whether there was a functional requirement for this calcium flux within the phagocyte during corpse removal. Compared to control conditions where a large fraction of LR73 cells engulf targets (> 40%), blocking calcium influx with either EGTA or BAPTA greatly blocked engulfment (Fig. 1b). We did not see obvious changes in viability or morphology of the phagocytes due to the addition of either BAPTA or EGTA (data not shown). It was possible, however, that these two drugs had unintended secondary effects; to control for this, we performed the engulfment assay with or without calcium in the medium (Fig. 1b). In calcium-free medium, engulfment of apoptotic thymocytes was similarly decreased; importantly, engulfment was restored when 1mM calcium was added back to the same medium (Fig. 1b). Similar results were found with NIH/3T3 cells as phagocytes and apoptotic Jurkat cells as targets (data not shown).

Divalent cations are also important for integrin activation (11): binding domains on integrins undergo conformational changes upon cation binding (12). Thus, we tested if the inhibition of engulfment was an indirect effect of a loss of cations by assessing engulfment in media without Ca^{2+}, without Mg^{2+}, or a medium without Ca^{2+} but with a two-fold higher concentration of Mg^{2+} added to keep the number of cations the same. Inhibition of engulfment in the absence of calcium could not be rescued by the addition of extra Mg^{2+} (Fig. 1c). Although Mg^{2+}-deficient medium had a slight effect on engulfment, it did not significantly decrease apoptotic cell uptake ($p=0.065$). As a second way of addressing the potential decreased binding by phagocytes to apoptotic thymocytes, we performed the experiment at 4°C. Previous studies have suggested that any TAMRA positive staining at 4°C is the result of binding to but not internalization by phagocytes [(13) and unpublished observations]. We found no difference in the degree of binding of apoptotic targets to phagocytes when incubated without drug treatment, with EGTA, or thapsigargin (a SERCA inhibitor) at 4°C (Sup. Fig. 2). Thus, calcium flux within phagocytes appears to be important for efficient removal of apoptotic cells and this effect is not simply due to the effect of lower concentration of divalent cations on integrin activation.

Two known signaling pathways downstream of engulfment receptors converge at the small GTPase Rac1 leading actin cytoskeleton reorganization and apoptotic cell engulfment (4). Phagocytic cup formation occurs subsequent to corpse recognition by the phagocyte, which results in Rac activation and rearrangement of the actin cytoskeleton promoting membrane movement to engulf the target. To characterize the effects of calcium on cell engulfment, we addressed the actin-dependent phagocytic cup formation around apoptotic cells. Chelating calcium with BAPTA resulted in an almost complete inhibition of phagocytic cup formation, similar to the effects seen in the presence of the actin polymerization inhibitor cytochalasin D, while the vehicle control DMSO had no obvious effect on phagocytic cup formation (Fig. 2a and b). Thus, calcium flux within phagocytes appears to be essential for actin polymerization and phagocytic cup formation.

Characterization of the calcium machinery involved during engulfment

An increase in intracellular calcium levels can be a consequence of release from endoplasmic reticulum stores, influx of extracellular calcium and/or release from other organelles (e.g. mitochondria). We used a variety of compounds that have previously been used to target molecules and/or channels involved in the modulation of calcium flux to test

their involvement during engulfment of apoptotic cells. Thapsigargin is a tight binding inhibitor of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase); by blocking the ability of cells to pump calcium into the ER, thapsigargin causes these stores to be depleted. We found that thapsigargin could significantly inhibit engulfment of apoptotic thymocytes by LR73 cells (Fig. 3a), suggesting that intracellular calcium stores are important for engulfment. Ru360 binds the mitochondrial calcium uniporter in mitochondria (a relatively minor source of intracellular calcium) with high affinity and blocks calcium uptake (14). Under the conditions of our phagocytosis assay Ru360 had no detectable effect on engulfment (Fig. 3a), suggesting mitochondria are not a significant source of calcium during engulfment of apoptotic cells.

The second stage of calcium mobilization in other systems frequently involves a sustained influx of extracellular calcium across the plasma membrane via calcium channels. As seen above in the EGTA and BAPTA experiments (Fig. 1b), entry of extracellular calcium into phagocytes appears essential during engulfment of apoptotic cells. To address the role of calcium channels, we utilized Nickel, which at high concentrations blocks all voltage activated calcium channels (15;16). Addition of nickel decreased engulfment, supporting the importance of plasma membrane calcium channels in corpse removal with slightly more inhibition at higher (100 μM) than lower (1 μM) concentrations (Fig. 3a). Because Nickel is a Lewis acid and can change the pH of the medium, it was possible that low pH might inhibit engulfment, however, decreasing the pH of the medium had little effect on engulfment. Thus, calcium channels that permit entry of extracellular calcium into the cell appear important for engulfment.

Various channels on the surface of the cell allow calcium entry into the cytoplasm. Ca^{2+} release-activated Ca^{2+} (CRAC) channels are the main store-operated channels and the principal pathway for Ca^{2+} influx in developing and mature T cells (6). We used YM-58483 (BTP2), which selectively inhibits CRAC channels (17;18). Whereas lower concentrations of YM-58483 (10 μM) had only a small effect on engulfment, higher concentrations (100 μM) greatly decreased engulfment. Calcium flux during engulfment in the presence of thapsigargin, YM58483, Nickel or BAPTA is shown in Sup. Fig. 1.

To validate the drug studies above, we used siRNA mediated knockdown of the ORAI proteins ORAI1, ORAI2, and/or ORAI3, which oligomerize to form CRAC channels on the plasma membrane (19). We established that the knockdown targeting individual *orai* transcripts was specific and that the levels of other *orai* members were not greatly upregulated (to compensate for knockdown of a given *orai* member) (Fig. 3b). Knockdown of any single ORAI homologue decreased engulfment, with greater effect for knockdown of ORAI1 and ORAI2 (Fig. 3c). Knockdown of all three ORAI homologues reduced engulfment even further (Fig. 3c). Thus, CRAC channels appear to be an important pathway through which calcium enters cells during engulfment of apoptotic cells and in turn their expression and function are required for efficient engulfment.

Requirement for the calcium flux machinery is evolutionarily conserved

Recently, we identified *stim-1* as one of the ~60 genes that regulate different aspects of corpse removal in the nematode *C. elegans;* however how *stim-1* might regulate engulfment was not addressed (20). The orthologue of STIM-1 in *Drosophila* and mammals has been shown to be a component of the endoplasmic reticulum that can communicate with plasma membrane CRAC channels to promote calcium entry into cells (21;22). Orai subunits, which form the CRAC channel exist basally as a dimer until bound by Stim, resulting in tetramerization and formation of an active channel (19). Since the studies described above suggested a role for CRAC channels for optimal engulfment, we addressed the phenotype of disrupting *stim-1* in the nematode more closely. Knockdown of *stim-1* increased the

numbers of refractile cell corpses in the adult hermaphrodite gonad (Fig. 4a). However, these corpses could represent a defect in apoptosis, phagocytosis, or corpse degradation (20). To more strictly define the role of *stim-1*, we looked at 'actin halo' formation, which represents enrichment of actin in the phagocyte at sites of active apoptotic cell internalization (20;23). Consistent with phagocytic defects, we found a severe decrease in the number of actin halos in *stim-1* RNAi treated worms (Fig. 4b). This result was quite comparable to the phenotype seen with knockdown of *ced-1*, which codes for a potential phagocytic receptor (20). *Control siRNA* treated worms had very few unengulfed corpses (Fig. 4a and c), while *gla-1* deficient worms showed increased numbers of halos, consistent with an apoptotic defect (20). Taken together, these studies suggest that the importance of CRAC channel activity is evolutionarily conserved.

In an independent screen for regulators of engulfment in *Drosophila*, a recent study identified a junctophilin related molecule and further demonstrated a role for dStim in apoptotic cell clearance (8). Junctophilins mediate the apposition between the ER and the plasma membrane and are thought to link emptying of intracellular calcium stores to the opening of plasma membrane calcium channels allowing calcium entry into cells (24). RNAi mediated knockdown of the *junctophilin* homologue *jph-1* in *C. elegans* resulted in defective engulfment with an accumulation of a large number of uninternalized refractive corpses (Fig. 4a and c). We used RNAi for all of the *C. elegans* experiments, since a Stim-1 mutant is not available, and the *Jph-1* mutant worms have severe developmental defects that precluded the analysis of normal engulfment of dying cells in this strain. Collectively, these data suggest a role for components of the calcium flux machinery in apoptotic cell clearance in the context of a whole organism. These data also suggest that the requirement for calcium during early stages of engulfment is evolutionarily conserved.

Anti-inflammatory cytokine production induced by apoptotic cells requires intracellular calcium flux in phagocytes

Phagocytes engulfing apoptotic cells secrete anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) (25). Previous studies have suggested that secretion of TGF-β is separable from the physical engulfment of the corpse, such that “recognition” of apoptotic cells by the phagocyte induces TGF-β. For example, treatment of phagocytes with the actin-disrupting drug cytochalasin D inhibits internalization of the corpse, but minimally affects TGF-β production (5); similarly, “recognition” of phosphatidyl serine alone is sufficient to induce cytokine secretion by the phagocyte (26). Thus, we asked whether calcium flux in phagocytes, besides its role in phagocytic cup formation, would also affect the generation of TGF-β (5). We incubated murine J774 macrophages with apoptotic thymocytes in medium with or without calcium and measured the amount of TGF-β secretion. Addition of apoptotic cells to phagocytes stimulated high levels of TGF-β secretion (>100 pg/ml) after 18 hrs of initial encounter with apoptotic cells; however, in the absence of extracellular Ca^{2+} in the medium, the production of TGF-β was severely reduced (Fig. 5a). It is noteworthy that this defect is likely not due to defects in recognition of targets by macrophages, since binding between apoptotic cells and phagocytes appears to occur comparably in medium with or without calcium (Sup. Fig. 2).

Furthermore, we tested the effect of inhibitors of Ca^{2+} release on TGF-β production. Both extracellular and intracellular calcium stores appear important for TGF-β secretion by macrophages, as addition of either BAPTA or thapsigargin resulted in decreased TGF-β production (Fig. 5a). It is noteworthy that under the same conditions, cytochalasin D, an actin polymerization inhibitor (27) that inhibits corpse internalization (28), did not have an effect on TGF-β production ($p=0.14$), confirming that engulfment/internalization of the corpse *per se* is not required for macrophage TGF-β production. Intriguingly, knockdown of all three ORAI homologues in NIH3T3 cells (Fig. 3b) severely reduced levels of TGF-β

secretion following incubation with apoptotic thymocytes (Fig. 5b) (compared to the non-specific siRNA treated control condition).

Discussion

In this report, we identify the existence of an early calcium flux in phagocytes during recognition of apoptotic cells and show that this calcium flux is necessary for engulfment of apoptotic targets. Based on *in vivo* studies in *C. elegans*, where downregulation of *stim-1* or *jph-1* through RNAi inhibits the phagocytosis of apoptotic cells upstream of actin reorganization, coupled with the mammalian studies on the phagocytic cup formation, we propose that the rise in intracellular calcium during apoptotic cell recognition plays a key role in steps leading to remodeling of the actin cytoskeleton and apoptotic cell internalization. These data also suggest that one or more of the engulfment receptors engaged during apoptotic cell recognition may lead to the activation of intracellular molecules that promote early and sustained calcium release (29–32). We also identify some of the important players involved in calcium flux downstream of apoptotic cell recognition, which is likely to have implications for corpse ingestion, as well as potential downstream post-engulfment consequences.

In our studies, thapsigargin severely inhibited engulfment but not calcium flux. Thapsigargin has previously been shown to specifically inhibit the SR/ER calcium ATPase (34–37), supporting the notion that thapsigargin affects the calcium flux rather than some unrelated effect on engulfment. The “small” contribution of the ER stores to cytoplasmic calcium levels (as measured by fluorimetry) is also consistent with previously published literature (33). Addition of thapsigargin releases intracellular calcium stores into the cytoplasm, but the net change in calcium levels is barely detectable even in T cells (33), which are known to have large calcium fluxes. However, on a cellular level, this change is significant in that it induces a rapid cellular response/downstream signal. Hence, it is not surprising, that we see a significant effect on engulfment when treating cells with thapsigargin, yet little to no effect on total calcium flux.

Recently, genetic studies in *Drosophila* proposed roles in engulfment for *Drosophila* homologues of mammalian STIM, Orai and the Ryanodine Receptor, all genes previously shown to have a role in Ca^{2+} flux (8). Furthermore, they proposed roles for Draper, a CED1 homologue and the adaptor molecule Ced-6, the orthologue of worm CED6 and mouse GULP in calcium flux. Using STIM1 or STIM2 deficient MEFs (33) we were unable to show defects in uptake of apoptotic cells (data not shown), potentially due to compensatory function by one STIM homologue when the other was missing. Futhermore, Ryanodine, a specific Ryanodine Receptor inhibitor did not inhibit engulfment in our model (data not shown). Using $LRP1^{-/-}$ CHO or GULP knockdown fibroblasts we also found no essential role for these proteins in calcium flux following recognition of apoptotic cells (data not shown). Thus, these studies suggest that although a role for calcium flux in corpse removal is evolutionarily conserved, the signal transduction activating this process may be different in mammals and flies.

Our data also suggest that calcium is required for post-engulfment responses of phagocytes such as the anti-inflammatory response that normally accompanies engulfment of apoptotic cells. The results presented here suggest that an impaired calcium flux in phagocytes could predispose an individual to potential autoimmune responses from two sides: reduced clearance of apoptotic cells has been linked to autoimmune phenotypes and a failure of the anti-inflammatory response would further contribute to inflammation and/or possible autoimmunity. Furthermore, the finding that downstream signaling components for

engulfment and the anti-inflammatory response may have both overlapping and distinct players should help further study of the divergent downstream signaling.

Materials and Methods

General

Immortalized cell lines and primary cells were maintained as described previously (38). All calcium inhibitory drugs were obtained from Calbiochem. Drugs were used at the following concentrations: BAPTA 10 μM, EGTA 10 mM, thapsigargin 1 or 10 μM, YM-58483 (BTP-2) 10 or 100 μM, $NiCl_2$ 1–100 μM, Ru360 10 μM, Cytochalasin D 0.1 μM. All protocols were consistent with accepted National Institutes of Health guidelines for the care and use of laboratory animals and approved by the University of Virginia Institutional Animal Care and Use Committee. Statistical significance was assessed using the t-test in Graphpad Prism. Significance is indicated in figures in the following way: p>0.05 – (ns), 0.01–0.05 – (*), 0.001–0.01 – (**), <0.001 – (***).

In vitro phagocytosis assay

Cell lines (LR73 Chinese hamster ovary cells and NIH 3T3 cells) were plated overnight in 24 well plates. The targets were either apoptotic thymocytes or apoptotic Jurkat or SCI cells labeled with TAMRA-SE (Invitrogen) as described previously (29) and added to the plated cells for 2 hrs. Apoptotic Jurkat and SCI cells were generated by incubating them in 5 μM Camptothecin (Sigma) overnight, followed by washing. Apoptosis in thymocytes was induced by incubation in 5 μM dexamethasone for 4 hours. The flow cytometry based engulfment assay was performed as described previously (39).

Calcium measurement assay

LR73 cells were plated on 96 well white opaque plates overnight. The cells were labeled with 1 μM Fluo-4 (Invitrogen) in Fluorimetry Media (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Glucose and 1% FBS) for 1 hour. Apoptotic Jurkats were prepared as explained above. Drugs targeting the calcium machinery in phagocytes were added to the plate 5 min. before the apoptotic cells. A small volume (10 μL) of apoptotic cells was added to prevent any changes in the temperature of the medium of LR73 cells. Changes in intracellular calcium levels were measured using Synergy HT microplate reader (Bio-Tek).

C. elegans studies

Feeding RNAi was performed as described (40) with the following modifications: NGM-agarose plates containing 2mM IPTG (Eppendorf) and 100μg/mL carbenocillin (Teknova) were inoculated with ~150 μL of appropriate bacterial cultures (transformed with constructs for generation of double stranded RNA under the control of the T7 promoter) were incubated for 8–12 hours before addition of 30–60 Bristol N2 wild type or *opIs110* [$P_{lim\text{-}7}$*:yfp:actin]* (23) worms. Hypochlorite synchronized L1 stage worms were placed on each RNAi plate and left for ~72h at 20°C. Worms fed with HT115(DH3) bacteria transformed with the original L4440 RNAi vector containing no insert were used as a control strain. *jph-1* RNAi construct was obtained from the Ahringer RNAi library and was confirmed by sequencing; a ~600bp *stim-1* fragment was amplified from N2 genomic DNA (gcatggtacccaattgaaattccttcaaaaaccatacc and gcatgcggccgcggcaaatgcgctctatcggca) adding KpnI and NotI restriction sites and then subcloned into L4440LB (23) and transformed into HT115(DE3) bacteria. To score germ cell death, worms were placed on 2% agarose pads, anaesthetized with 3–5mM levamisole (Sigma) and mounted under a cover slip for observation using a Zeiss Axiovert 200 microscope equipped with DIC (Nomarski) optics

and standard epifluorescence filter sets appropriate for visualization of YFP. The number of germ-cell corpses and/or actin fluorescence "halos" were then scored by DIC or fluorescence microscopy as previously described (23). Bacteria transformed with the original *L4440*(RNAi) vector containing no insert were used as a reference strain.

Immunofluorescence microscopy of mammalian cells

NIH/3T3 fibroblasts were plated on LabTekII culture chambers (Fisher) in DMEM 10% FBS overnight, then incubated with 1×10^6 apoptotic SCI cells for 30 minutes. Images were acquired using a Zeiss 510-UV laser scanning confocal microscope with 488 and 543 lasers (Zeiss AG). Cells were then fixed with 3% paraformaldehyde (Sigma) in PBS for 30 min, permeabilized with 0.1% Triton X-100 (Sigma) and blocked with 5% skim milk that had been clarified by high speed centrifugation. Cells were subsequently stained with Alexa 488-phalloidin as previously described (41).

Knockdown

NIH3T3 cells were transfected with ~1.2 μg siRNA (Dharmacon cat#: *orai1* M-056431-00, *orai2* M-057985-00, *orai3* M-054417-00, control siRNA M-001206-13-05) using an Amaxa nucleofector (Amaxa Biosystems) according to the manufacturer's specifications (kit R, program U-30) [as previously described (20)]. Knockdown was confirmed using Real Time PCR. First, RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using Superscript III First Strand Kit (Invitrogen). Real Time PCR was done using the Step One Plus RT-PCR System using Taqman probes (both from Applied Biosystems). 18S and GAPDH served as endogenous controls. Analysis was done using StepOne v2.0 software (Applied Biosystems).

TGF-β secretion assay

J774 macrophages or siRNA transfected NIH3T3 were plated in a 12-well plate at 37°C. One or two days later (respectively), 2×10^6 apoptotic thymocytes (generated as described above) were co-incubated for 18 hours with the J774 cells in culture medium (X-VIVO 10) with or without Ca^{2+} or the indicated Ca^{2+} specific inhibitors. The culture supernatants were then collected, centrifuged, and analyzed for total TGF-β using the TGF-β ELISA/assay kit (R&D Systems) following the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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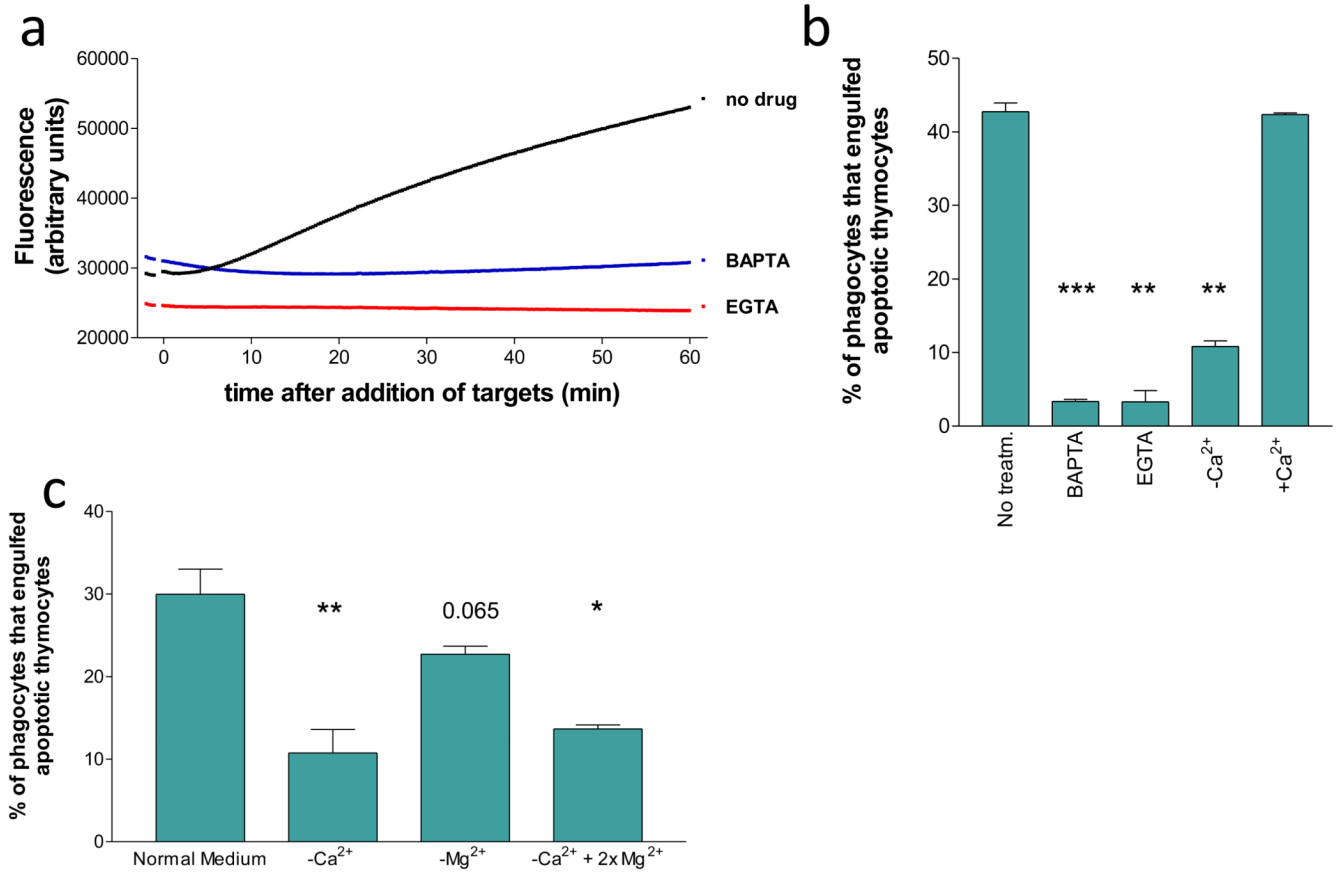


Figure 1. Functional requirement of calcium flux during engulfment of apoptotic cells

a) Apoptotic Jurkat cells were added (time 0) to Fluo-4 labeled LR73 phagocytic cells and cytoplasmic calcium levels were assessed over time using a fluorescent microplate reader. BAPTA and EGTA were added to LR73 cells 5 min prior to addition of apoptotic cells.

b) Labeled apoptotic thymocytes were incubated with LR73 cells in the presence or absence of BAPTA or EGTA, as well as in medium with or without calcium, and engulfment was assessed by a flow cytometry-based phagocytosis assay.

c) Labeled apoptotic thymocytes were incubated with LR73 cells in complete fluorimetry medium, medium without Ca^{2+}, medium without Mg^{2+}, or medium without Ca^{2+} but with 2-fold higher Mg^{2+} concentration so that the total divalent cation concentration in the medium was as in complete medium and engulfment was assessed by flow cytometry. Significance is indicated as follows: p = 0.01−0.05 (*), 0.001−0.01 (**), <0.001 (***).

All of these experiments were repeated a minimum of three times.

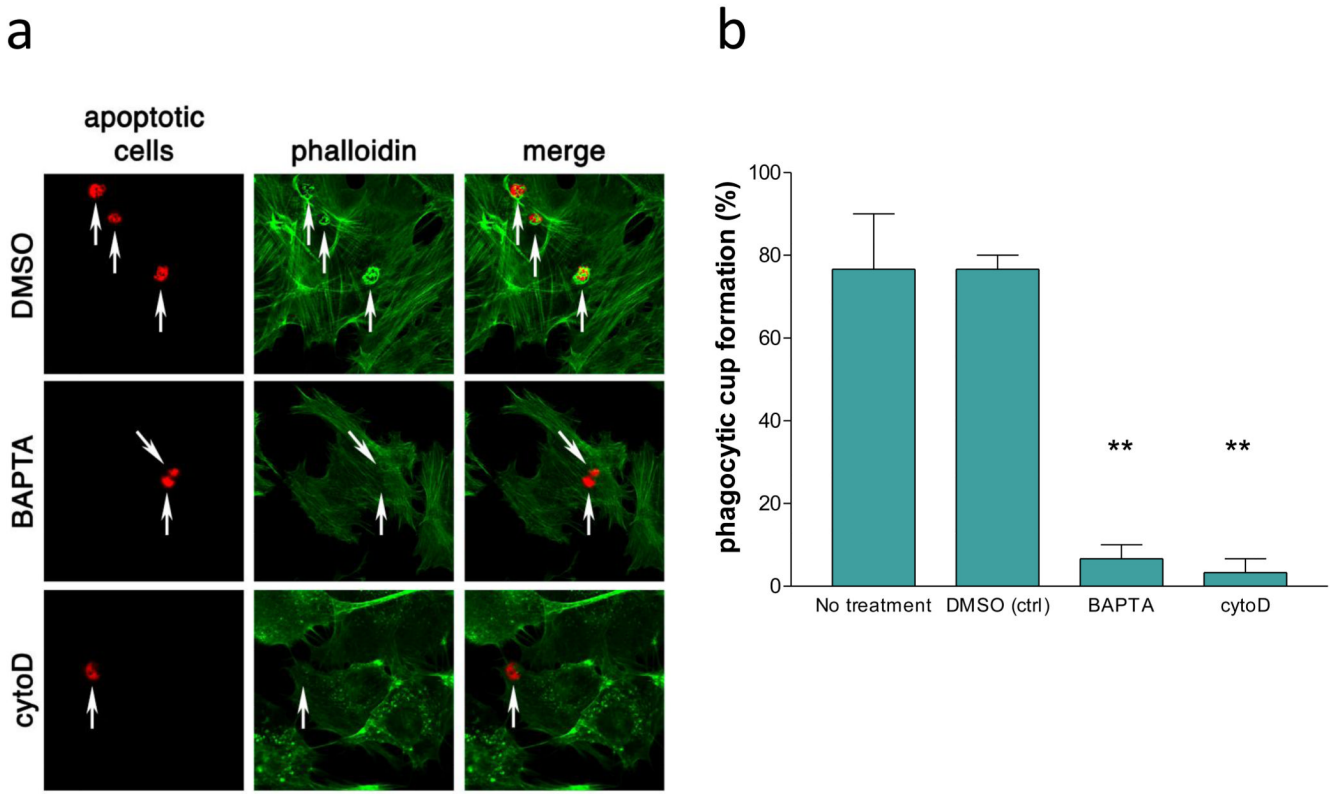
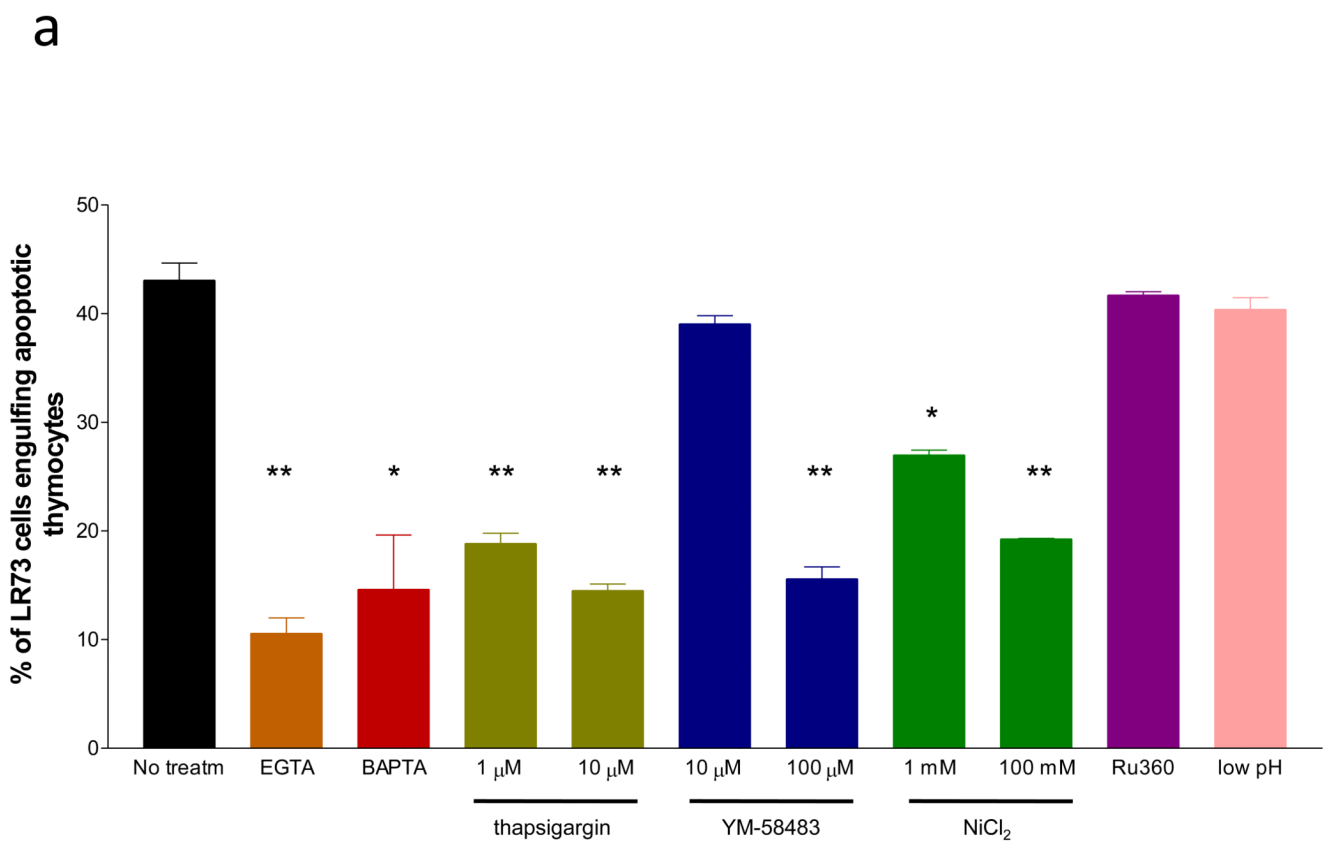


Figure 2. Calcium is important for actin polymerization and phagocytic cup formation

a) Representative photographs of actin polymerization during apoptotic cell engulfment. Quantitation of the percentage of apoptotic cells that displayed phagocytic cup formation. Apoptotic cells were labeled with TAMRA and incubated with NIH/3T3 cells and cells treated with nothing (WT), vehicle (DMSO), BAPTA, or the actin polymerization inhibitor cytochalasin D. Actin was stained with Alexa 488-phalloidin. Significance is indicated as follows: p>0.05 – (ns), 0.01–0.05 – (*), 0.001–0.01 – (**), <0.001 – (***). n = 10.



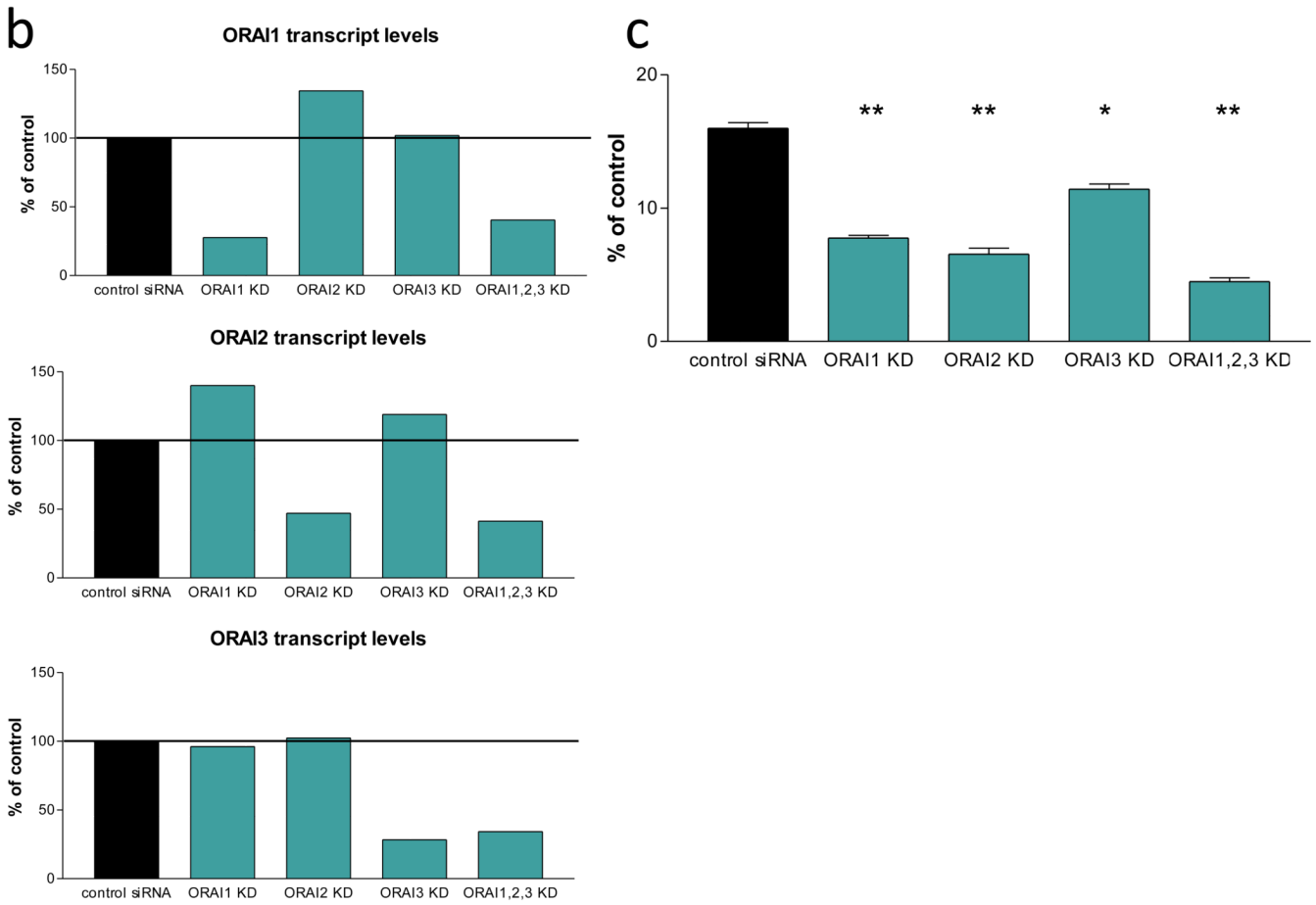


Figure 3. Both internal calcium stores and cell membrane calcium channels like the CRAC channels are important for the engulfment of apoptotic cells

a) LR73 cells were incubated with labeled apoptotic thymocytes in the presence or absence of the indicated drugs and scored in the engulfment assay. Drugs shown were added at the same time as apoptotic thymocytes to minimize gross effects on the phagocytes. This is a representative of three independent experiments.

b) ORAI 1, 2 and 3 transcript levels in NIH3T3 cells transfected with siRNA specific for the genes indicated on the x-axis as measured by real-time PCR.

c) NIH3T3 that were transfected with the indicated siRNA were incubated with labeled apoptotic thymocytes and analyzed for engulfment. b&c are representative of two independent experiments.

Significance is indicated as follows: $p>0.05$ – (ns), 0.01–0.05 – (*), 0.001–0.01 – (**), <0.001 – (***).

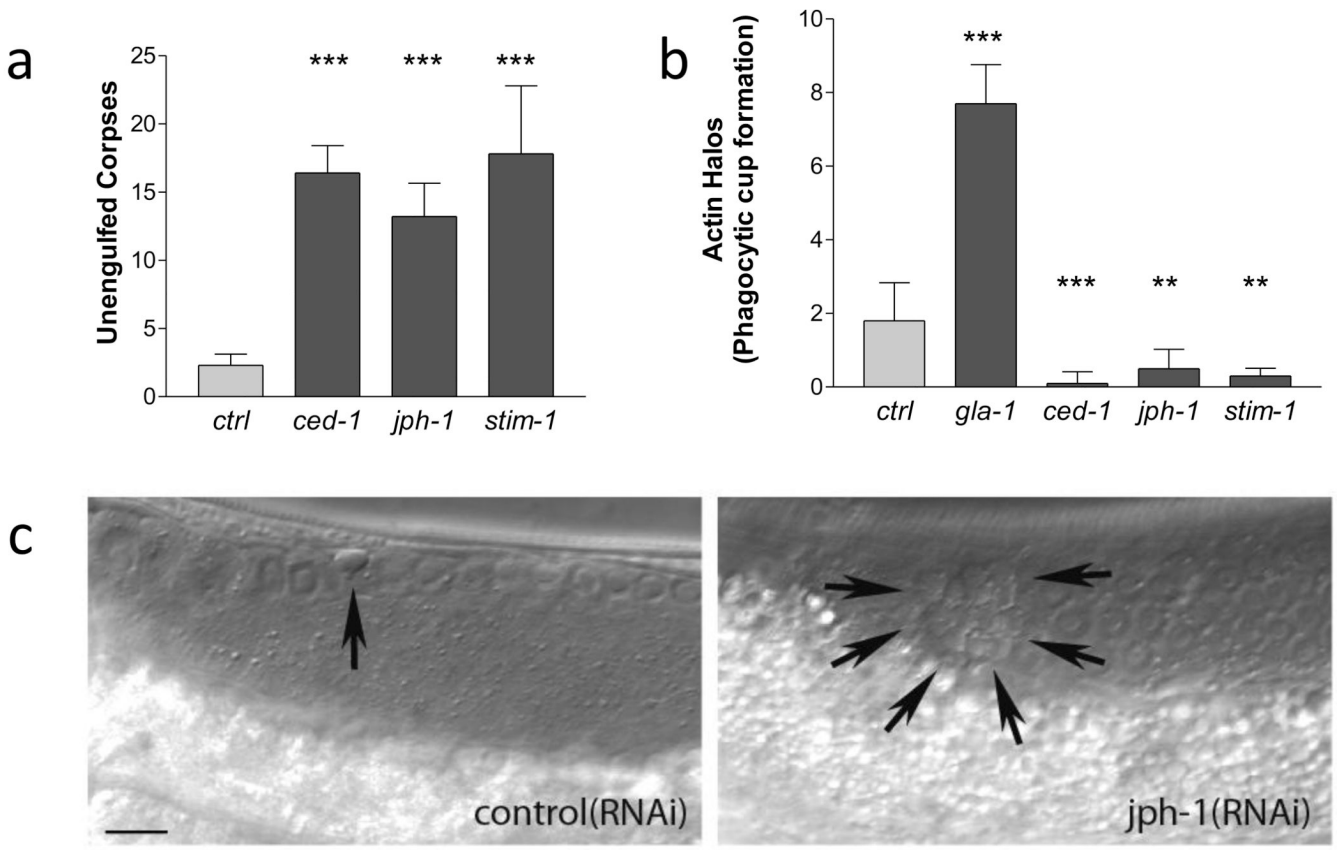


Figure 4. jph-1 and stim-1 are important for corpse clearance in C. elegans

a) Quantitation of the number of undegraded refractile corpses in the gonad of worms after RNAi knockdown of the indicated genes. Control worms were maintained on bacteria transformed with an RNAi vector containing no insert.

b) Defective corpse internalization in *jph-1(RNAi)* and *stim1(RNAi)* worms was determined by scoring the number of actin halos in worms with RNAi knockdown of the indicated genes. Lack of halos in *ced-1*(RNAi) worms served as the positive control for defects in corpse internalization. Significance is indicated as follows: p>0.05 – (ns), 0.01–0.05 – (*), 0.001–0.01 – (**), <0.001 – (***).

c) DIC micrographs showing the *C. elegans* adult hermaphrodite gonad in control (left) or the increased number of corpses in *jph-1* deficient animals. Arrows point to refractile cell corpses.

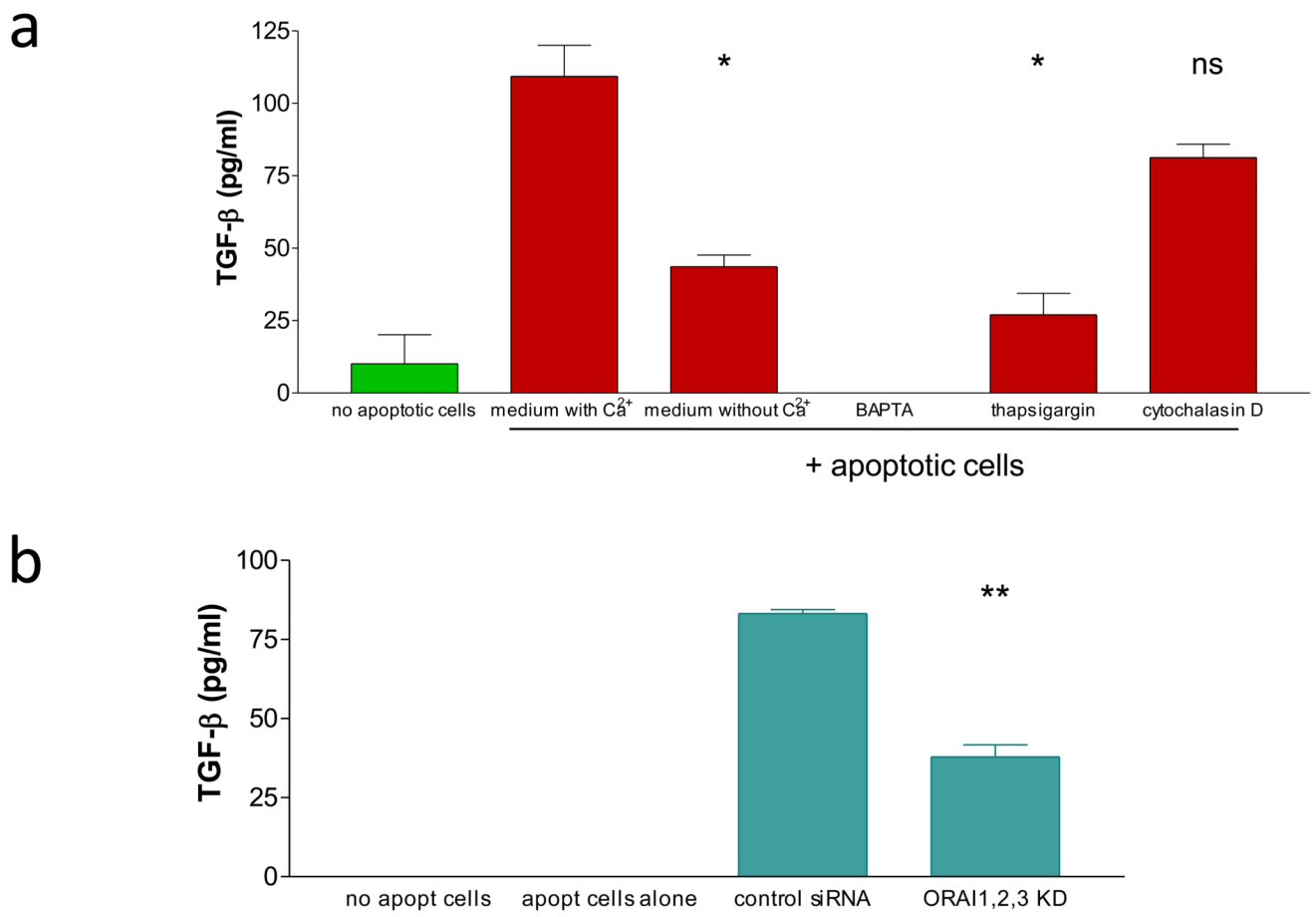


Figure 5. Both intracellular and extracellular Ca^{2+} is crucial for TGF-β secretion by phagocytes encountering apoptotic cells

a) J774 phagocytes cells were incubated with apoptotic thymocytes in the presence of absence of calcium in the medium, or in the presence of calcium but with calcium inhibitor drugs. The supernatants were collected and analyzed for TGF-β levels by ELISA. Drugs were added at the same time as the apoptotic cells.

b) NIH3T3 cells transfected with control or *orai1*, *orai2* and orai*3* specific siRNA were incubated with apoptotic thymocytes and secretion of TGF-β was measured in the medium 24 hrs later. No apopt cells shows the amount of TGF-β secreted by NIH3T3 cells without addition of apoptotic thymocytes, while apopt cells alone is the amount of TGF-β in wells with apoptotic thymocytes without any phagocytes.

Each figure is representative of at least three independent experiments. Significance is indicated as follows: p>0.05 – (ns), 0.01–0.05 – (*), 0.001–0.01 – (**), <0.001 – (***).