This is a postprint of an article published in
Gekara, N.O., Groebe, L., Viegas, N., Weiss, S.
Listeria monocytogenes desensitizes immune cells to subsequent Ca 2+ signaling via listeriolysin O-induced depletion of intracellular Ca2+ stores
(2008) Infection and Immunity, 76 (2), pp. 857-862.

L. monocytogenes renders cells inert to Ca^{2+} signalling via LLO induced depletion of intracellular Ca^{2+} stores

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Running title: Depletion of intracellular calcium stores by listeriolysisn O

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Abstract

Listeriolysin O (LLO), the pore-forming toxin of *L. monocytogenes* (*L.m*), is a prototype of the cholesterol dependent cytolysins secreted by several pathogenic and non-pathogenic Gram-positive bacteria. In addition to mediating escape of the bacterium into the cytosol, this toxin is generally believed to be a central player in host-pathogen interactions during *L.m* infection. LLO triggers influx of Ca^{2+} into host cells as well as release from intracellular stores. Thus, many of the cellular responses induced by LLO are related to calcium signalling. Interestingly, in this study, we report that prolonged exposure to LLO renders cells resistant to the induction of Ca^{2+} mobilization upon subsequent stimulations. Cells pre-exposed to LLO, *L.m* but not to the LLO deficient mutant *L.mΔhly* were found to be highly refractory to Ca^{2+} induction in response to receptor mediated stimulation. Such cells also exhibit diminished Ca^{2+} signals in responses to LLO and thapsigargin. The presented results suggest that this phenomenon is due to depletion of intracellular Ca^{2+} stores.

Introduction

The Gram-positive bacterium *L. monocytogenes (L.m),* is responsible for the disease listeriosis, which is mainly acquired by contaminated food. The main virulence factor of this facultative intracellular bacterial pathogen is the pore forming toxin listeriolysin O (LLO) that plays a crucial role during its complicated intracellular life cycle. First, it enables the bacterium to breach membrane barriers. Additionally, LLO acts as a pseudocytokine/chemokine by which the bacterium communicates with and influences various host cells during infection. For instance, LLO can influence the outcome of infection via modulation bacterial entry into cells (8, 28, 29), induction of apoptosis (4, 5) as well as synthesis and secretion of proinflammatory cytokines/chemokines (18, 19, 26, 27),.

Induction of calcium signals plays an important role for effector cells of the immune system. Several studies have shown that this ubiquitous signalling pathway can be hijacked by bacterial pathogens to promote their survival in the host. *L.m* is one of such pathogens. Many of the host responses triggered by LLO involve Ca^{2+} signalling (12, 28, 29). Although calcium signal induction by *L.m* is a complex process that involves more than one virulence factor, several independent studies indicate that LLO is the *sine qua non* of the Ca^{2+} mobilization (11, 22, 23, 28). This is exemplified by the fact that *L.mΔhly* a mutant strain of *L.m* that lacks LLO is incapable of eliciting any Ca^{2+} response in host cells (11, 22, 28). The mechanisms by which LLO promotes Ca^{2+} signal induction by *L.m* are manifold. LLO makes membrane pores permissible to ions and macromolecules. Thus, during infection, LLO secreted by the bacterium not only allows influx of Ca^{2+} into the host cells but it also allows listerial phosphatases PLCA and PLCB to access their intracellular substrates, hence causing Ca^{2+} release from intracellular stores (22, 28). Irrespective of that, LLO alone can directly cause Ca^{2+} release from intracellular stores via multiple mechanisms (11).

Paradoxically, in this study we show that prolonged exposure of cells to LLO or *L.m* renders them inert to Ca^{2+} induction upon subsequent stimulations. We demostrate that this phenomenon is due to the depletion of intracellular Ca^{2+} stores. A role for this phenomenon might lay in the subversion of various effector cells of the immune system during *L.m* infection.

Materials and methods

Reagents

Iscove's modified Dulbecco's medium (IMDM), Dulbecco's modified Eagle's medium (DMEM) and Ca^{2+}-free DMEM were from Gibco (Karlsruhe, Germany). LLO was purified from over-expressing *Listeria innocua* as previously described (6). Indo1-AM, Ionomycin and Thapsigargin were purchased from Sigma-Aldrich (Steinheim Germany). Anti-DNP-BSA IgE antibody and DNP-BSA were kindly provided by Pecht I (The Weizmann Institute of Science, Israel).

Cells

Bone marrow derived mast cells (BMMCs) were matured by culturing bone marrow cells in the presence of IL-3 for 4-8 weeks as described (11), while T cells were freshly isolated from spleens of HA-TCR $RAG1^{-/-}$ mice.

Pre-exposure of cells to bacteria or LLO

Cells were cultured with *L.m* or *Δhly* (multiplicity of infection: 100) for various time periods 3 – 48 hrs, washed, then labeled with Indo1-AM as described below.

Ca^{2+} measurements by flow cytometry

5 x 10^6 BMMCs in 500 µl DMEM were incubated with 50 µM Indo 1-AM in complete medium at 37°C. After 45 min, cells were washed once in Ca^{2+}-free medium supplemented with 10mM EGTA to ensure removal of any residual extracellular calcium from cells, then twice in unsupplemented Ca^{2+}-free medium to remove residual EGTA. This second step was necessary since influx of residual EGTA into the cell via the LLO pore could chelate intracellular Ca^{2+} hence marring the Ca^{2+} signals due to release from intracellular store. Therefore to evaluate the relative contribution of extracellular and intracellular Ca^{2+} pools in the overall Ca^{2+} signals triggered by LLO, after this washing procedure, cells were resuspended in either normal or Ca^{2+}-free medium then kept on ice until ready for measurement. Cells were warmed up to 37°C before start of Ca^{2+} measurements. Measurements were carried out in a MoFlo high-speed cell sorter (DakoCytomation) equipped with an UV argon ion laser (351-363 nm). Indo 1-AM emissions were detected with 405/30 (Ca^{2+} -bound Indo 1-AM) and 515/30 (Ca^{2+}-free Indo 1-AM) fluorescence filters. First the ratio of the fluorescence emitted at the two Indo 1-AM excitation wavelengths (*F405/30*/*F515/30*) was calculated then calibrated into arbitrary fluorescence units (a.f.u) by the equation *F405/30*/*F515/30 X128*, where the constant *128* is the median of the instrument´s fluorescence spectrum. The data were subsequently normalized for fluctuations in the initial baseline measurements in Excel software, by dividing all the a.f.u with the average a.fu of the initial 30 sec baseline period. Therefore, the arbitrary ratiometric units represent the fold increase in *F405/30*/*F515/30* over that of the initial baseline.

Results

L.m and LLO render mast cells unresponsive to antigen induced calcium signalling

In the present study, we mainly used mast cells as a model target cell type for *L.m*. Mast cells have a wide tissue distribution, especially at the host-environment interfaces such as the skin, airways and gastrointestinal tract, where pathogens and other environmental agents are frequently encountered. As such, they represent the first host cell type that most likely encounters pathogens when they cross epithelia like the intestinal barrier. Indeed the role of mast cells in listeriosis is now well established (9, 10).

Classical activation of mast cells occurs via IgE dependent cross-linking of the high affinity FcεRI, when antigen is encountered. One of the early events triggered via antigen receptor in such cells is Ca^{2+} mobilization. This involves an initial release from the intracellular stores which then in turn triggers influx via the plasma membrane channels. Interestingly, when mast cells were pre-exposed to *L.m,* they appeared to be highly resistant to calcium induction via cross-linking of the FcεRI (Figure 1A). This phenomenon was also observed in cells pretreated with LLO alone. As shown in Figure 1B, Ca^{2+} mobilization following stimulation with IgE plus antigen was significantly diminished in LLO pretreated cells as compared to untreated controls). Consistently, no signal was elicited in cells pretreated with thapsigargin a compound that depletes intracellular Ca^{2+} stores.

L.m or LLO pretreated cells exhibit resistance to calcium induction by LLO

Since LLO itself induces calcium signals (11, 22), we also tested whether pretreatment with *L.m* also affects LLO induced calcium signals. BMMCs were pre-exposed to *L.m* or the LLO deficient mutant *L.mΔhly* for 4 hrs before analyzing induction of calcium fluxes by LLO. As shown in Figure 2, cells pre-exposed to *L.m* but not *L.mΔhly* became highly refractory to calcium signals induced by LLO. Thus the reduced calcium signalling of host cells pre-exposed to *L.m* is not just specific for cross-linkage of the FcεRI but also extend to other Ca^{2+} mobilization agonists.

Resistance to calcium induction in LLO pretreated cells is not due to diminished influx of extracellular Ca^{2+}

The overall amplitude of calcium signals induced via FcεRI receptor or LLO is a product of Ca^{2+} release from intracellular Ca^{2+} stores as well as influx from the extracellular melieu (11, 15, 17). From the above experiments, it was not certain whether the refractory Ca^{2+} responses in *L.m*/LLO pretreated cells was due to diminished Ca^{2+} influx or to absence of release from intracellular stores.

Pore-forming agents have been reported to render cells resistant to subsequent membrane perforation (21). Since, LLO-mediated Ca^{2+} influx is due to the membrane perforating activity of the toxin, the possibility of resistance of host cells to membrane perforation was considered. To test the above idea, control and LLO pretreated cells were evaluated for their capacity to take up propidium iodide (PI). PI is a DNA binding fluorescent dye, which permeates the cell only in the event of membrane damage. Compared to the control, LLO pretreated cells were not diminished in PI uptake upon subsequent treatment with LLO (data not shown). Thus, resistance to perforation was ruled out as the cause of the diminished Ca^{2+} responses in *L.*m or LLO pre-exposed cells.

To independently confirm the potential role of Ca^{2+} influx in the refractory phenomenon observed after pretreatment of cells with LLO, ionony cin-mediated influx of extracellular Ca^{2+} was assessed. Whereas cells pretreated with LLO showed a profound impairment in LLO induced intracellular Ca^{2+} increase, such cells were highly responsive to ionomycin (Figure 3). Thus, the LLO pretreatment does not effect ionophore-mediated Ca^{2+} mobilization.

L.m and LLO cause depletion of intracellular Ca^{2+} stores

To determine whether the refractory Ca^{2+} responses was due to a diminished Ca^{2+} release from intracellular stores, cells were pretreated with LLO and tested under Ca^{2+}-free conditions. As shown in Figure 4, LLO pretreated cells also exhibited diminished calcium responses under Ca^{2+}-free conditions. Together, these data suggest that *L.m* or LLO pretreatment mainly affects the intracellular release of Ca^{2+}, but not its influx from extracellular medium.

To confirm this possibility, we opted to employ thapsigargin - the endoplasmic reticular Ca^{2+}-ATPase inhibitor, which depletes intracellular calcium stores by causing unregulated efflux of Ca^{2+} from intracellular stores. Treatment of cells with LLO led to a strong elevation of cytosolic Ca^{2+} that rapidly dropped to near basal level. When such cells were immediately re-exposed to thapsigargin to evaluate the Ca^{2+} level in intracellular stores, hardly any elevation in Ca^{2+} was induced (Figure 5A, purple trace). This indicates that LLO had depleted intracellular Ca^{2+} stores.

Then the order was reversed and the cells were treated with thapsigargin to deplete intracellular stores before stimulation with LLO. Thapsigargin evoked a strong elevation in cytosolic Ca^{2+} in untreated cells (Figure 5A, green trace).Such thapsigargin pretreated cells were highly refractory to calcium induction upon re-exposure to LLO (compare first peak of purple trace and second peak of green trace in Figure 5A). Since LLO causes Ca^{2+} influx from the extracellular pool in addition to release from intracellular stores, it should be noted that, as expected, LLO still caused cytosolic Ca^{2+} elevation, albeit at low levels, in thapsigargin pretreated cells. Depletion of intracellular Ca^{2+} stores was also tested following exposure of cells to live bacteria. Figure 5B shows Ca^{2+} signals elicited by thapsigargin in cells pre-incubated for 4 hrs with *L.m* or *L.mΔhly*. As depicted, Ca^{2+} release in cells pre-exposed to *L.m* was significantly diminished as compared to *L.mΔhly* pre-exposed cells. Together, these data show that due to unregulated release of Ca^{2+}, exposure of cells to *L.m* or its toxin LLO causes

depletion of intracellular Ca^{2+} stores hence rendering them refractory to intracellular Ca^{2+} release agonists.

Refractory Ca^{2+} signals wanes with longer periods of toxin exposure

To determine whether depletion of intracellular Ca^{2+} stores by LLO is a property that can be generalized to other cell types, primary T cells were also tested. Untreated as well as LLO pretreated T cells were re-stimulated with LLO in Ca^{2+}- free medium to only assess Ca^{2+} release from intracellular stores. As shown before, intracellular Ca^{2+} release in cells pretreated with LLO was remarkably lower than in untreated cells. Interestingly, almost no measurable calcium release was obtained with T cells that were pretreated with LLO for 4 hrs while cells pretreated for 18 hrs showed a low but definitive Ca^{2+} signal (Figure 6). Cells pretreated with LLO for 48 hrs exhibited normal response to induction of Ca^{2+} by LLO (data not shown). This suggests that calcium depletion by LLO is reversible and that with time cells recover from the toxin effects and restock their intracellular Ca^{2+} stores.

Discussion

Modulation of calcium signals is a very important mechanism by which many pathogenic bacteria influence host cells. Alterations of metabolism, activation of apoptosis, induction of proinflammatory mediators as well as cytoskeletal reorganization have been reported in this context (12, 24, 26, 28, 29). A prominent example of bacterial factors known to participate in bacteria-induced Ca^{2+} signalling are pore-forming toxins. LLO, a family member of the cholesterol dependent pore-forming toxins is well described for its role in the various Ca^{2+} dependent host responses during *L. monocytogenes* infection (8, 28, 29). In the present work we show that mobilization of Ca^{2+} from intracellular stores by *L.m* and LLO goes beyond just triggering Ca^{2+} dependent responses. LLO depletes intracellular Ca^{2+} stores hence rendering pre-exposed cells inert to subsequent Ca^{2+} inductions by various intracellular Ca^{2+} release

agonists. This is most compellingly demonstrated by the fact that cells pretreated with *L.m* or LLO exhibit highly diminished intracellular Ca^{2+} mobilization in response to thapsigargin. Such cells also exhibit diminished Ca^{2+} levels when restimulated with LLO. The possibility that LLO pretreatment increases membrane rigidity hence a diminish in Ca^{2+} influx from the extracellular milieu was exluded. This was demonstrated by the fact membrane perforation by LLO and hence cellular uptake of propidium control and pretreated was comparable. Additionally, unlike for the intracellular Ca^{2+} release agonists, Ca^{2+} influx caused by via the Ca^{2+} ionophore ionomycin was found to be unaffected by the LLO pretreatment. The consequence of intracellular Ca^{2+} depletion by *L.m* and LLO is that pre-exposed cells become highly resistant to Ca^{2+} signals induced via membrane receptors such as the FcεRI.

Resistance of cells to stimulation consequent to pre-exposure to LLO is most likely attributable to its ability to cause Ca^{2+} release from intracellular stores via multiple mechanisms. LLO triggers Ca^{2+} release via G - protein and protein tyrosine kinase activation of the PLC-IP_3R regulated Ca^{2+} channels. Additionally it causes reversible injury to the intracellular stores such as the endoplasmic reticulum and the lysosomes (11). All these combined effects may therefore lead to unregulated efflux of Ca^{2+} and hence depletion of intracellular stores.

That LLO triggers Ca^{2+}mobilization in cells and yet render them inert to subsequent stimuli is an important finding.

Although it is not clear at the moment how this might benefit the pathogen, we are tempted to speculate that refractory induction of calcium signals in host cells may have severe physiological significance in the context of listeriosis. One such possibility is the interference with various Ca^{2+} dependent cytokines/chemokines or antigen induced effector functions during *L.m* infection.

For instance, the productive activation of lymphocytes requires a balanced integration of Ca^{2+} and other signalling pathways. Stimulation of the antigen receptor in the absence of Ca^{2+}

signals leads to a state of anergy or antigen unresponsiveness (1, 2, 20). On the other hand, sustained calcium signalling in the absence of antigen receptor stimulation causes the same (13, 14, 16). Indeed, when lymphocytes are subjected to a sustained exposure to ionomycin in absence of antigen stimulation, they become unresponsive to subsequent antigen induced Ca^{2+} signals, and exhibit an anergic state (13, 14, 16). Thus, it is imaginable that during *Listeria* infection, a prior exposure of host lymphocytes to LLO could render such cells unresponsive to antigen stimulation, which would undermine the host's ability to mount an effective immune response, much to the pathogen's advantage. A similar scenarion could be invisioned for responsiveness to cytokines or other mediators involving Ca^{2+} signals.

The use of toxins as an immunosuppressive tool by bacteria to evade the adaptive immune responses is not uncommon. Inhibition of T lymphocyte activation and proliferation by the *Helicobacter pylori* vacuolating toxin VacA is a well established phenomenon (3, 25). Although the mechanisms by which this toxin suppresses T cell activation is different from that proposed for LLO herein, it is interesting to note that Ca^{2+} signal induction is a common feature shared by both toxins (7). Additional studies can now be performed to establish whether the ability of LLO to render host cells inert to Ca^{2+} mobilization is indeed involved in the process by which *L.m* evades the innate and the adaptive immune response.

Our data might therefore have important implication in the understanding of how *L.m* as well as the other pathogenic Gram-positive bacteria which secrete the analogous CDCs circumvent the host defences in order to establish a niche in the host.

Acknowledgment

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG) und the Deutsche Krebshilfe. The authors declare no conflict of interest.

Abbreviations

LLO, listeriolysisn O; *L.m, Listeria monocytogenes*; *L.mΔhly, L.m* mutant deficient i LLO; BMMCs, Bone marrow derived mast cells; Thaps, Thapsigargin

Figure legends

Figure 1. *L.m* and LLO renders BMMCs inert to antigen induced Ca^{2+} mobilization. A, BMMCs were exposed with *L.m* and loaded with Indo1-AM then incubated on ice with an IgE antibody specific for DNP. After washing off the unbound antibody, cells were stored on ice. When ready for calcium measurements cells were warmed up to 37° C before adding DNP-BSA to cross-link the FcεRI. Stimulation was done in Ca^{2+}-containing medium. **B.** BMMCs were left untreated or pretreated with LLO or thapsigargin for 4 hrs, loaded with Indo 1-AM then stimulated via FcεRI as described in **A**. Each trace represents the average of intracellular Ca^{2+} levels in the cells during the time of acquisition. A 30 sec baseline was recorded each time before stimulation. The arrow indicates the time point of stimulation. The experiment was repeated at least 3 times with similar results.

Figure 2. Pre-incubation with *L. m* renders cells resistant to LLO induced calcium signals. After incubation with (or without) *L.m* or Δ*hly* (MOI: 100) for 3 hrs, BMMCs were washed then loaded with Indo1-AM in penicillin/streptomycin supplemented medium for 45 min (to kill all the bacteria). Cells were again washed then suspended in Ca^{2+} containing medium and analyzed by flow cytometry for intracellular Ca^{2+} mobilization following stimulation with LLO (0.25 μg/ml). The experiment was repeated at least 3 times with similar results.

Figure 3. LLO pretreated cells are not resistant to Ca^{2+} mobilization by ionomycin. BMMCs were pretreated (or not) with LLO for 3 hrs, loaded with Indo1-AM, resuspended in Ca^{2+}-containing medium. LLO was added to such cells and intracellular Ca^{2+} was measured. At the time point indicated by the arrow , 1 μM ionomycin was added.

Figure 4. Refractory Ca^{2+} signals in LLO pretreated cells is in part due to diminished release from the intracellular stores. A. BMMCs were incubated with (or without) LLO (0.25μg/ml) for 4 hrs, then loaded with Indo1-AM. To evaluate only Ca^{2+} release from intracellular stores, cells were thoroughly washed in Ca^{2+}-free medium, then resuspended in Ca^{2+}-free before stimulation with LLO.

Figure 5. LLO depletes intracellular Ca^{2+} stores. LLO was added to Indo1-AM loaded BMMCs and intracellular Ca^{2+} was measured. Then, thapsigargin (1 μM) was added to evaluate remaining Ca^{2+} release from intracellular stores (purple trace). Vice versa, thapsigargin was first added to cells to achieve maximum Ca^{2+} release from intracellular stores before adding LLO (green trace). Note that unlike in untreated cells, hardly any Ca^{2+} was released from intracellular stores by thapsigargin after LLO pretreatment. Ca^{2+} mobilization induced by LLO was also greatly diminished in thapsigargin pretreated cells. Since the experiment was done in Ca^{2+}-containing medium, note that the minor peak elicited by LLO in thapsigargin pretreated cells is most likely due to Ca^{2+} influx via the toxin pores. **B.** Depletion of intracellular Ca^{2+} stores by *L.m.*, BMMCs were incubated for 3 hrs with *L.m* or *L.mΔhly* (MOI: 100) then loaded with Indo1-AM and treated with thapsigargin (1 μM) to evaluate maximum Ca^{2+} release from intracellular stores.

Figure 6. Refractory Ca^{2+} signalling wanes with longer periods of toxin exposure. Primary T cells were pretreated (or untreated) with LLO for 4 hrs or 18 hrs. To evaluate intracellular Ca^{2+} stored in such cells, cells were labeled with Indo-1-AM and washed thoroughly as described before then resuspended in Ca^{2+}-free medium before restimulation with LLO.

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Figures

Figure 1

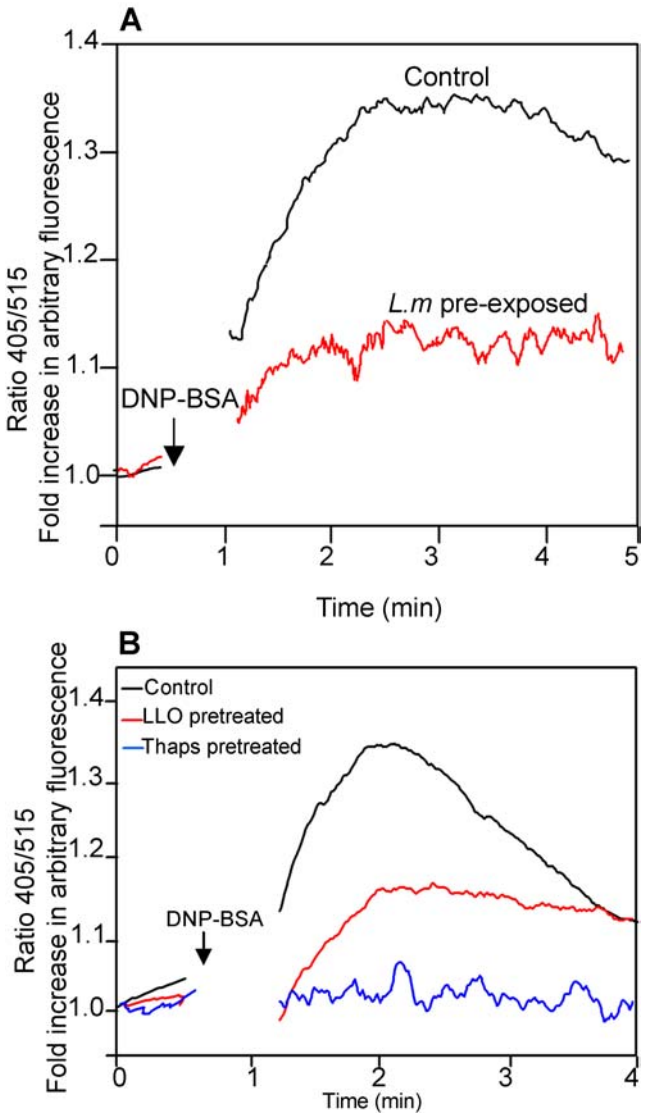
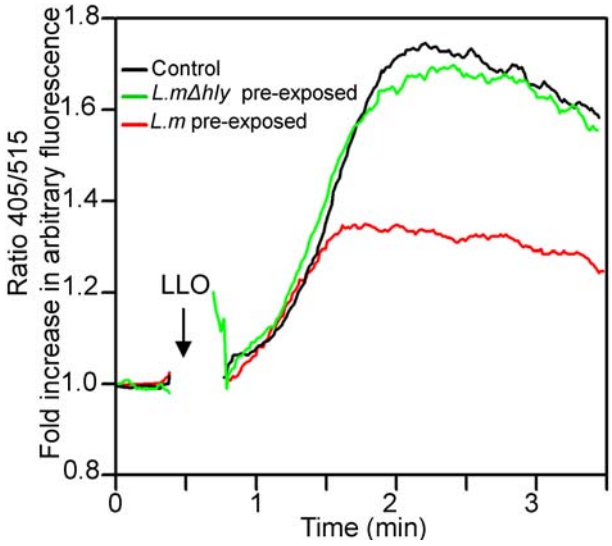


Figure 2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Fold increase in arbitrary fluorescence
1.8
1.6
1.4
1.2
1.0
0.8
Control
L.mΔhly pre-exposed
L.m pre-exposed
LLO
0
1
2
3
Time (min)

Figure 3

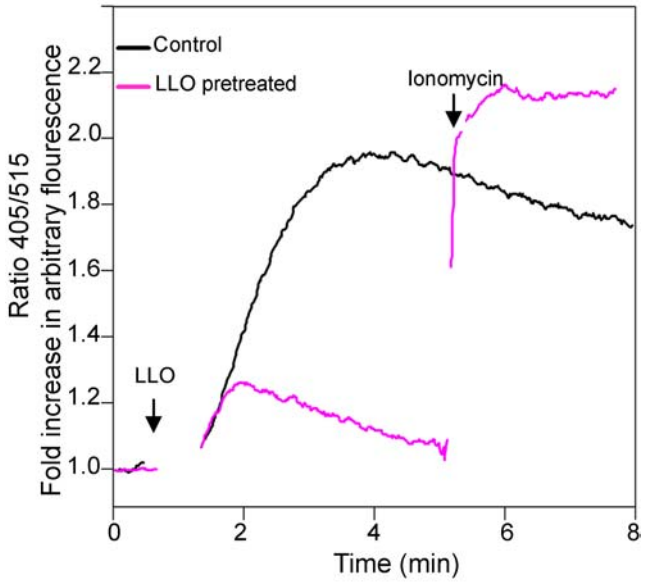

Control
LLO pretreated
Ionomycin
LLO
Ratio 405/515
Fold increase in arbitrary flourescence
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
2
4
6
8
Time (min)

Figure 4

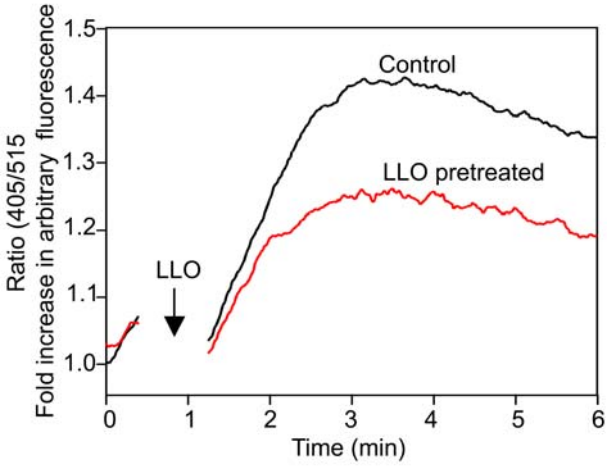

Ratio (405/515
Fold increase in arbitrary fluorescence
1.5
1.4
1.3
1.2
1.1
1.0
Control
LLO pretreated
LLO
0
1
2
3
4
5
6
Time (min)

Figure 5

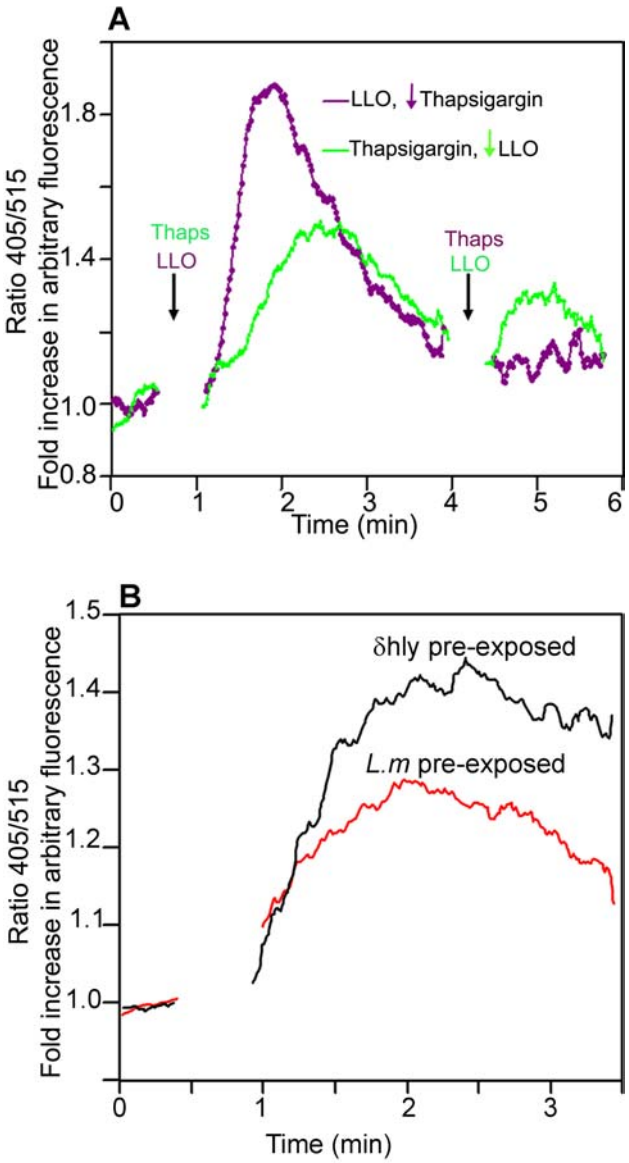
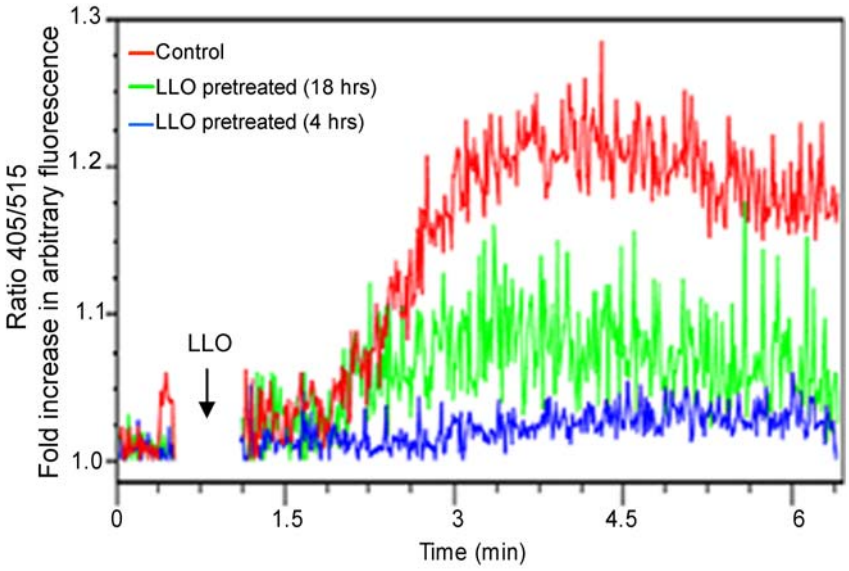

A
Ratio 405/515
Fold increase in arbitrary fluorescence
1.8
1.4
1.0
0.8
LLO, Thapsigargin
Thapsigargin, LLO
Thaps
LLO
Thaps
LLO
0
1
2
3
4
5
6
Time (min)
B
1.5
1.4
1.3
1.2
1.1
1.0
δhly pre-exposed
L.m pre-exposed
0
1
2
3
Time (min)

Figure 6


Control
LLO pretreated (18 hrs)
LLO pretreated (4 hrs)
LLO
Ratio 405/515
Fold increase in arbitrary fluorescence
1.3
1.2
1.1
1.0
0
1.5
3
4.5
6
Time (min)