Localization of protein kinase C ε to macrophage vacuoles perforated by *Listeria monocytogenes* cytolysin

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Summary

Three proteins secreted by *Listeria monocytogenes* facilitate escape from macrophage vacuoles: the cholesterol-dependent cytolysin listeriolysin O (LLO), a phosphoinositide-specific phospholipase C (PI-PLC) and a broad-range phospholipase C (PC-PLC). LLO and PI-PLC can activate several members of the protein kinase C (PKC) family during infection. PKCε is a novel PKC that contributes to macrophage activation, defence against bacterial infection, and phagocytosis; however, a role for PKCε in *Lm* infections has not been described. To study PKCε dynamics, PKCε-YFP chimeras were visualized in macrophages during *Lm* infection. PKCε-YFP was recruited to forming vacuoles during macrophage phagocytosis of *Lm* and again later to fully formed *Lm* vacuoles. The PKCε-YFP localization to the fully formed *Lm* vacuole was LLO-dependent but independent of PI-PLC or PC-PLC. PKCε-YFP recruitment often followed LLO perforation of the membrane, as indicated by localization of PKCε-YFP to *Lm* vacuoles after they released small fluorescent dyes into the cytoplasm. PKCε-YFP recruitment to vesicles also followed phagocytosis of LLO-containing liposomes or osmotic lysis of endocytic vesicles, indicating that vacuole perforation by LLO was the chief cause of the PKCε response. These studies implicate PKCε in a cellular mechanism for recognizing damaged membranous organelles, including the disrupted vacuoles created when *Lm* escapes into cytoplasm.

Introduction

Macrophages are essential for clearing *Listeria monocytogenes* (*Lm*) infections in mice (Mackaness, 1982; Adams and Hamilton, 1984; Kiderlen et al., 1984; Pamer, 2004). *Lm* enters macrophages by phagocytosis and then escapes from phagosomal vacuoles into the cytosol, where it can replicate and invade neighbouring cells. *Listeria* secretes a cholesterol-dependent cytolysin (CDC), listeriolysin O (LLO), which is necessary for escape from the phagosome into the cytosol (Portnoy et al., 1988; Cossart et al., 1989; Gedde et al., 2000). *Lm* also secretes two phospholipases C which have minor roles in escape (Smith et al., 1995): a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range phospholipase C (PC-PLC).

In addition to their involvement in bacterial escape, LLO and the bacterial phospholipases C (PLCs) induce signalling from the phagosome (Goldfine and Wadsworth, 2002). CDCs, including LLO, induce secretion of TNFα and IL-6 and activate macrophages by inducing iNOS expression (Park et al., 2004). In addition, Goldfine and colleagues identified a LLO-mediated activation of host PLC and phospholipase D following *Lm* infection of macrophages (Goldfine et al., 2000).

Protein kinases C (PKCs) are phospholipid-dependent, serine/threonine protein kinases whose 11 isozymes are placed into subfamilies based upon their cofactor requirements for activation (Newton, 2001). Conventional PKCs (α, β1 and II), γ] are activated by calcium (Ca2+), diacylglycerol (DAG), and phosphatidylserine (PS) or anionic phospholipids. Novel PKCs (δ, ε, η, θ) are activated by DAG and PS. Atypical PKCs (ζ, η/λ) are activated by PS. LLO and PI-PLC activate recruitment of PKCζ to plasma membranes and PKC ιII to early endosomes (Wadsworth and Goldfine, 2002). Inhibition of PKC ιII increased *Lm* phagocytosis and decreased escape from vacuoles (Wadsworth and Goldfine, 2002), indicating that *Lm* exploits host PKC ιII activity during infection.
Protein kinases C regulate a variety of cellular processes including cytoskeleton rearrangements and immune cell signalling (Tan and Parker, 2003). Signalling through the Fc-receptor induces PLC-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate, generating inositol-1,4,5 trisphosphate, which increases $[Ca^{2+}]$, and DAG, second messenger that activate conventional PKCs. PKC is recruited to IgG-opsonized particles in forming phagosomes and is necessary for FcγR-mediated phagocytosis in macrophages (Larsen et al., 2000; 2002). PKC has been implicated in innate immunity through its role in macrophage activation (Castrillo et al., 2001). PKC also upregulates the expression of iNOS and subsequent NO production (Diaz-Guerra et al., 1996). Many bacteria (Listeria, Bacillus and Staphylococcus species) also secrete phospholipases, including PI-PLC and PC-PLC, which can produce DAG and potentially recruit host PLC for subsequent signal transduction.

Upon phosphorylation at three residues in the catalytic kinase domain, PKCs become mature; a prerequisite for binding to second messengers and activation (Keranen et al., 1995; Cenni et al., 2002). Mature but unactivated PKCs are localized in the cytosol; upon activation they translocate to the membrane (Newton, 2001). Novel PKCs translocate to the plasma membrane in response to DAG formation (Stahelin et al., 2005). Once bound to DAG, novel PKCs bind to anionic phospholipids which allows the pseudosubstrate domain to be released and PKC to phosphorylate its substrate. Inside cells, compartmentalization in space or time targets the different PKC isoforms to different signalling pathways. Targeting and substrate specificity for each PKC isoform depends upon subcellular and tissue localization (Akita, 2002).

Castrillo et al. demonstrated that PKC is necessary for lipopolysaccharide-induced macrophage activation and defence against infection by Escherichia coli and Staphylococcus aureus (Castrillo et al., 2001). Macrophages from PKC−/− mice showed reduced ability to produce nitric oxide, TNF-α, and IL-1β in response to lipopolysaccharide and IFN-γ (Castrillo et al., 2001). The Toll-like receptor 4 (TLR4) adapter molecule TRAM was recently identified as a specific substrate for PKC (McGettrick et al., 2006). Phosphorylation of TRAM by PKC is necessary for TRAM-mediated TLR signalling (McGettrick et al., 2006). These observations indicate roles for PKC in TLR signalling and the early signalling events necessary for macrophage activation (Aksoy et al., 2004; McGettrick et al., 2006). There is no evidence thus far that PKC is recruited to membranes during bacterial entry.

To examine the role of PKC in macrophage responses to infection, we expressed in macrophages a fluorescent chimera of PKC, PKC-YFP, and analysed its intracellular dynamics in macrophages during Lm infection. We identified a LLO- and PLC-independent accumulation of PKC upon Lm entry into macrophages, as well as a later, LLO-dependent concentration of PKC on vacuoles following perforation of the Lm vacuole membrane. This later PKC recruitment could also be elicited by liposomes containing purified LLO or by osmotic lysis of endosomes, indicating a role for PKC in the detection of damaged membrane organelles in macrophages.

**Results**

Protein kinase C ε is recruited to Lm-associated membranes in macrophages

In resting cells, PKC-YFP was mostly distributed uniformly throughout the macrophage cytoplasm, with minor perinuclear localization (Fig. 1A). After wild-type Lm infection of macrophages, PKC-YFP robustly localized to membranes associated with the bacteria (Fig. 1B). We asked if Lm recruitment of PKC to vacuolar membranes is affected by LLO. RAW 264.7 macrophages expressing PKC-YFP were infected with wild-type Lm or hly (LLO-deficient) Lm, and localization of PKC-YFP was analysed by time-lapse microscopy of live cells. Shortly after Lm entry, PKC was recruited to both wild-type and hly Lm-associated membranes (Fig. 2A; 5 min). Time-lapse movies showed that the membranes that recruited PKC-YFP during entry were close to the bacteria, consistent with a role for PKC in Lm phagocytosis (Fig. 2A; Time 5). PKC accumulation at Lm-containing phagosomes was biphasic, occurring during phagosome formation (5 min) and again.
around the fully formed \textit{Lm} vacuoles. PKCe translocation was specific for \textit{Lm} vacuoles; it did not translocate to macropinosomes loaded with only Texas Red dextran (TRDx; 10 000 MW dextran) (Fig. 2A). This second recruitment of PKCe-YFP was dependent upon the presence of LLO, as PKCe-YFP localized to wild-type \textit{Lm} vacuoles but not \textit{hly} \textit{Lm} vacuoles (Fig. 2A, Time 30). Labelling of endogenous PKCe by immunofluorescence showed similar localization patterns: wild-type (Fig. 2B) and \textit{hly} (Fig. 2C) \textit{Lm} recruited PKCe upon entry (5 min), but at 30 min only vacuoles with wild-type \textit{Lm} contained PKCe.

\textit{Lm} PLCs do not affect PKCe recruitment to vacuolar membranes

As PKCe associates with membranes via binding to DAG as well as other lipids, we tested the hypothesis that PI-PLC or PC-PLC from \textit{Lm} generates lipids for PKCe docking to \textit{Lm} vacuoles. Specifically, we quantified the percentage of \textit{Lm} vacuoles that recruited PKCe-YFP after infection with wild-type \textit{Lm} or \textit{Lm} mutants deficient in LLO (\textit{hly}), PI-PLC (\textit{plcA}), PC-PLC (\textit{plcB}) or combinations thereof (\textit{hly plcA-plcB}, \textit{plcA-plcB}). At 5 min after infection, similar percentages were observed for wild-type (37\%), \textit{hly} (42\%), \textit{plcA} (45\%), \textit{plcB} (47\%), \textit{plcA-plcB} (47\%), and \textit{hly plcA-plcB} (42\%) \textit{Lm} phagosomes, indicating that LLO and PLCs were not necessary for PKCe recruitment during bacterial entry (Fig. 3A).

At later time points, PKCe translocated more frequently to phagosomes containing LLO-expressing \textit{Lm} (Fig. 3A; Time 15, 30 and 45 min). Approximately 25–35\% of LLO-expressing \textit{Lm} (wild-type, \textit{plcA}, \textit{plcB}, \textit{plcA-plcB}) recruited PKCe-YFP 15–45 min after infection, whereas only 10\% of LLO-\textit{Lm} (\textit{hly} and \textit{hly plcA-plcB}) recruited PKCe-YFP at these later times. This indicated that the bacterial PLCs were not producing the DAG that recruited PKCe to the vacuolar membrane. Interestingly, at 15 min, the measurements for \textit{plcB}, \textit{plcA}, \textit{plcA-plcB}, and \textit{hly} \textit{Lm}...
PKCε was recruited to Lm vacuoles at low levels but that LLO somehow enhanced that recruitment. Macrophages coexpressing PKCε-YFP and CFP were infected with wild-type or hly Lm. Phase-contrast, YFP and CFP images were taken of phagosomes at regular intervals. Ratio images (YFP/CFP) were calculated, and the ratio of YFP/CFP of the phagosome (Rp) was divided by the ratio of YFP/CFP in the whole cell (Rc). Rp/Rc values greater than 1.0 indicated YFP chimera recruitment to vacuoles (Fig. 3B; triangles) (Henry et al., 2004). Both wild-type and hly Lm showed similar high amounts of PKCε-YFP recruited upon entry (Time 5), with Rp/Rc values of 1.6 and 1.4 respectively. The second recruitment to the wild-type Lm phagosome (Rp/Rc values of ~1.3–1.4) was maximal at 22 min and was significantly higher than seen on hly Lm phagosomes (Rp/Rc values ~1.05) (P < 0.01).

Addition of bafilomycin A₁ to macrophages increases the pH of endocytic compartments by inhibition of the proton ATPase, and consequently inhibits LLO pore-forming activity (Beauregard et al., 1997; Shaughnessy et al., 2006). We asked if inhibiting LLO activity with bafilomycin A₁ would affect PKCε recruitment to the Lm vacuole. Treatment of macrophages with bafilomycin A₁ before and during infection reduced wild-type Lm vacuoles. Treatment of macrophages with bafilomycin A₁ showed similar high amounts of PKCε-YFP recruitment to perforated Lm vacuoles.

**Protein kinase C ε is recruited to perforated Lm vacuoles**

We previously showed by measuring the sequential release of small [Lucifer Yellow (LY); 522 MW] and large (TRDx; 10 000 average MW) fluorescent probes from Lm vacuoles that LLO-expressing (LLO⁺) Lm perforate vacuolar membranes (Shaughnessy et al., 2006). In the previous study, 50% of LLO⁺ Lm perforated the vacuole, which was characterized by the selective release of LY from the vacuole or the sequential release of LY then TRDx (Shaughnessy et al., 2006). The other 50% of LLO⁺ Lm vacuoles and 100% of LLO− Lm vacuoles did not perforate the vacuole (Shaughnessy et al., 2006). We define perforation of the Lm vacuole as the differential release of LY and TRDx; either by loss of LY only or by sequential loss of LY and then TRDx.

We adapted this method to ask if vacuoles recruiting PKCε were first perforated by LLO. Macrophages expressing PKCε-YFP were infected with wild-type Lm in the presence of LY and TRDx. The fluorescence of LY, TRDx, and PKCε-YFP was then imaged in the Lm vacuoles over time. Time-lapse fluorescence revealed that PKCε-YFP translocation to wild-type Lm vacuoles occurred after perforation of the vacuole. That is, the sequential loss of fluorescence of LY and TRDx from the

**Protein kinase C ε recruitment to the Lm vacuole membrane is dependent upon LLO and vacuolar acidification**

We quantified the levels of PKCε translocated to LLO⁺ and LLO− Lm vacuoles (Fig. 3B). LLO− Lm vacuoles were not completely devoid of PKCε recruitment, indicating that

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**Fig. 3.** Conditions affecting PKCε recruitment to Lm vacuoles.

A. Quantitative analysis of the timing of PKCε localization to Lm. Macrophages were infected for 3 min with the indicated strains of bacteria, then were washed and fixed at 5, 15, 30 and 45 min after infection. Bacteria were labelled with DAPI and scored for colocalization with PKCε-YFP. Fifty bacteria were counted in triplicate in three different experiments. B. Macrophages were transfected with plasmids for PKCε-YFP and CFP (or YFP and CFP for the negative control; n = 10). Transfected macrophages, untreated or treated with 500 nM bafilomycin A₁ (BAF; n = 10), were infected with either wild-type (n = 18) or hly Lm (n = 11) for 3 min. Time-lapse phase-contrast, CFP and YFP images were taken of Lm phagosomes every 30 s for 30 min, and ratio images were prepared (YFP/CFP). The ratio of YFP/CFP in the phagosome (Rp) was divided by the ratio of YFP/CFP in the entire cell (Rc) to measure recruitment of PKCε-YFP to the phagosome (Rp/Rc).

were significantly different from that of wild-type Lm (P < 0.05). This suggests the PLCs may modulate the PKCε response.

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phagosome was apparent 10–15 min after \textit{Lm} addition whereas translocation of PKCe was not detected until approximately 23 min (Fig. 4). As in previous studies (Shaughnessy \textit{et al.}, 2006), half of wild-type \textit{Lm} vacuoles (6 of 11 recorded events) showed perforation. Of the six phagosomes that perforated, five later recruited PKCe-YFP (Fig. 4). Of the five events in which \textit{Lm} did not lose LY and TRDx, only one showed PKCe-YFP translocation to the vacuole. No vacuoles perforated after recruitment of PKCe-YFP. A Pearson’s chi-squared test applied to these results indicated that the correlation between perforation and PKCe recruitment was significant ($P = 0.0356$). These results indicated that LLO pore-forming activity is necessary for accumulation of PKCe.

\textit{Protein kinase C \varepsilon is recruited by vacuolar LLO}

We next asked if LLO activity in the vacuole was sufficient to recruit PKCe. Macrophages were allowed to phagocytose pH-sensitive liposomes containing LLO. These liposomes (phosphatidylethanolamine–cholesterol–hemisuccinate) become unstable at pH < 6.0 and release their contents, allowing delivery of encapsulated molecules, including LLO, into the endocytic compartment (Lee \textit{et al.}, 1996). Macrophages expressing PKCe-YFP were fed pH-sensitive liposomes in which LLO was co-encapsulated with a small fluorescent dye, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). Delivery of LLO (Fig. 5A), but not heat-inactivated LLO (hiLLO) (Fig. 5B) or HPTS alone (Fig. 5C), into the endocytic compartment caused PKCe-YFP translocation to the phagosomes. LLO and HPTS liposomes recruited PKCe-YFP to 5% of the vacuoles, whereas liposomes containing HPTS alone or hiLLO plus HPTS never recruited PKCe-YFP (50 vacuoles were counted for three separate experiments). This indicated that endosomal LLO was sufficient to recruit PKCe to compartments and suggests that PKCe recruitment signals vacuole perforation.

\textit{Protein kinase C \varepsilon is recruited to osmotically lysed endosomes}

The results to this point are consistent with two models: (i) LLO is required for PKCe recruitment or (ii) LLO is not required and other signals that induce membrane damage will serve equally for the same task. To distinguish between these possibilities, we determined the effect of osmotic lysis of endosomes on PKCe translocation. Endosomes in RAW macrophages expressing PKCe-YFP were ruptured by osmotic lysis, a commonly used method to deliver antigens from endosomes into the cytosol (Okada and Rechsteiner, 1982; Moore \textit{et al.}, 1988). Briefly, macrophages were allowed to endocytose hypertonic medium, and then hypotonic medium was added, causing endosome rupture and release of their contents into cytoplasm. Finally an isotonic solution was added to allow macrophage recovery. When this procedure was performed with macrophages expressing PKCe-YFP, multiple
vacuoles recruited PKCe-YFP to their membranes (Fig. 6A). Omission of 10% polyethylene glycol 1000 from the hypertonic medium does not allow cytoplasmic release of macromolecules (Okada and Rechsteiner, 1982; Moore et al., 1988). In macrophages exposed to this control condition, PKCe-YFP no longer translocated to endocytic membranes (Fig. 6B). Moreover, isotonic medium and hypotonic medium alone also did not cause PKCe-YFP to translocate to endocytic membranes (data not shown). This indicates that PKCe recruitment to Lm vacuoles is part of a cytoplasmic signalling mechanism that recognizes damaged organelles.

Discussion

This work demonstrates that PKCe localizes twice to Lm-associated membranes during Lm infection of macrophages. PKCe localization during Lm entry is independent of LLO and the bacterial PLCs. Later, PKCe localizes to the Lm-containing vacuole after perforation by LLO. This indicates a role for PKCe in recognizing damaged Lm vacuoles.

A role for PKCe in Lm pathogenesis

Protein kinase Cε localized to Lm upon entry into macrophages, independently of LLO or the bacterial PLCs. This localization is likely related to that described for FcγR-mediated phagocytosis of IgG-opsonized particles (Diaz-Guerra et al., 1996; Larsen et al., 2000; 2002). Inhibition of PKCe slowed the rate of FcγR-mediated phagocytosis and decreased NO production (Larsen et al., 2000; 2002). NO production is important for the clearance of Lm in activated macrophages. It is possible that PKCe recruitment during Lm entry signals the induction of NO production near the vacuole.

The activity of LLO inside Lm vacuoles stimulated a second localization of PKCe. The recruitment of PKCe to wild-type Lm vacuoles followed LLO-mediated vacuole perforation and required LLO activity. This LLO-dependent recruitment of PKCe-YFP suggests either that Lm exploits host cell signalling mechanisms or that the host uses PKCe as a means to signal for the presence of a damaged vacuole. The low level of PKCe recruitment to vacuoles without LLO may indicate the normal level of PKCe that is recruited to phagosomes which is necessary for defence against bacterial infection.
Our hypothesis at the outset of these studies was that Lm PLCs recruit PKCε to the vacuolar membrane. Both host cells and Lm produce PLCs that hydrolyse phospholipids, releasing DAG on membranes. Therefore, both the host and Lm are equipped to activate PKCε. It was previously reported that Lm PLCs activate host PKCs (Goldfine and Wadsworth, 2002; Wadsworth and Goldfine, 2002). However, as we did not see a decrease in PKCε recruitment relative to wild-type Lm after infection with Lm mutants plcA-, plcB- or the double mutant plcA-plcB-, we conclude that they have no role in activating PKCε. These results therefore implicate host PLCs for PKCε activation (Goldfine et al., 2000). DAG, localized in macrophages expressing the DAG-binding domain from PKCε (C1δ-GFP), appeared on Lm-associated membranes both during entry and later on the vacuole membrane (data not shown).

Cholesterol-dependent cytolysins could form pores that specifically translocate proteins into the host cell. This is supported by studies of another CDC, streptolysin O, which secretes an effector molecule through its pore into the host cell (Madden et al., 2001). Likewise, it was hypothesized that LLO is a specific translocator for the Lm-secreted proteins, PI-PLC and PC-PLC (Goldfine and Wadsworth, 2002). Our studies indicate no role for the bacterial PLCs in the activation of PKCε, despite the fact that PKCε recruitment occurs after membranes have been permeabilized by LLO.

**Listeriolyisin O: an activator of signalling events from the phagosome**

The substrate of PKCε on the Lm vacuole is not yet known, so it is unclear what downstream molecules are involved. LLO and other CDCs have previously been shown to mediate host signalling pathways. CDCs activate TLR4 signalling events, including induction of TNFα, IL-6 and iNOS (Park et al., 2004). PKCε has also been implicated in TLR signalling (Aksoy et al., 2004; McGettrick et al., 2006). Recently, the TLR4 adaptor molecule TRAM was identified as a specific substrate for PKCε; phosphorylation of TRAM by PKCε was necessary for downstream TRAM-mediated TLR4 signalling (McGettrick et al., 2006). It is unknown whether TLR4 is involved in recognition of Lm; however, activation of MyD88 (another TLR4 adaptor molecule) is essential for the innate immune defence against Lm (Edelson and Unanue, 2002; Seki et al., 2002; Pamer, 2004). Therefore, it is possible that Lm is recognized by TLR4 and signals through TRAM and PKCε on the Lm vacuoles. Future studies should determine if PKCε recruitment to the Lm vacuole enhances signalling by TLRs or other pattern-recognition receptors.

**The role of PKCε in cellular responses to membrane damage**

It is unknown what happens to damaged compartments and how the host cell either repairs or recycles the membrane. Damaged organelles could be sealed and ready to use again or, if the membrane is beyond repair, the membrane may be sequestered and degraded by autophagy. PKCε signalling may be involved in the breakdown and recycling of damaged membrane.

The PKCε recruitment to Lm vacuoles may indicate a general mechanism for recognizing damaged organelles. What could be the consequences of recruiting PKCε to a damaged organelle? Many viruses and intracellular pathogens reach cytoplasm by disrupting vesicles that contain them after endocytosis by host cells. For Lm, inflammatory responses of infected macrophages and dendritic cells require LLO (Vazquez et al., 1995; Brzoza et al., 2004). Perhaps inflammatory or immune responses of host cells to invasive microbes are activated by PKCε binding to lipids or proteins which normally reside in the outer (luminal) leaflet of endocytic vesicle membranes, but which are exposed to cytoplasm when the integrity of the bilayer is compromised.

**Experimental procedures**

**Reagents**

The fluorophores Texas Red phallolidin, TRDx (MW = 10 kDa), LY, HPTS, and 4',6-diamindino-2-phenylindole (DAPI) were obtained from Molecular Probes (Eugene, OR). Bafilomycin A₁ was obtained from Calbiochem (La Jolla, CA).

**Bacterial strains**

The Lm wild-type strain DP-L10403, hly deletion strain DP-L2161, plcA- deletion strain DP-L1552, plcB- deletion strain DP-L1935, plcA-plcB- deletion strain DP-L2139 used in this study were gifts from Daniel Portnoy (University of California, Berkeley).

**Bacterial preparation**

*Listeria monocytogenes* were grown overnight at room temperature in Brain Heart Infusion broth. They were subcultured the next day and grown for 1 h to an OD of 0.500 at 37°C. Subcultured bacteria (1 ml) were washed three times in 1 ml Ringer’s buffer (RB): 155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM Hepes and 10 mM glucose, pH 7.2) followed by centrifugation (4500 g). Where indicated, bacteria were pre-labelled with SNARF-1, carboxylic acid, acetate succinimidyl ester (Molecular Probes). Washed bacteria were labelled with 3 μl ml⁻¹ SNARF-1 solution in DMSO for 15 min at 37°C with shaking, then washed four times with 1 ml RB before use.
**Macrophase preparation**

RAW 264.7 macrophages were obtained from ATCC (Manassas, VA) and grown in Advanced Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA), with 2% heat-inactivated FBS (Invitrogen), 100 unit ml⁻¹ of penicillin/streptomycin mixture (Sigma Chemical, St Louis, MO), and L-glutamine, at 37°C with 5% CO₂. Cells were plated the day before the experiment onto 25 mm coverslips, in 6 well plates, at 3 × 10⁵ cells well⁻¹. Macrophages were transfected with plasmids for fluorescent chimera expression using FuGENE 6 transfection reagent, according to the manufacturer’s protocol (Roche Diagnostics, GmbH, Mannheim, Germany).

**Fixed cell assay and immunofluorescence**

Macrophages were infected with Lm (multiplicity of infection -1) for 3 min. Coverslips were washed with RB and incubated with DMEM, 10% FBS, and 25 µg ml⁻¹ gentamicin. Coverslips were fixed with cytoskeletal fix (30 mM Hepes, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO₄, 33 mM potassium acetate, 5% polyethylene glycol 400, and 4% paraformaldehyde) at either 5, 15, 30 or 45 min after infection. Cells were rinsed with phosphate-buffered saline (PBS) and 2% goat serum, permeabilized with 0.3% Triton X-100 in PBS, and incubated for 15 min in PBS and 2% goat serum plus DAPI (2 µg ml⁻¹ from a 100 µg ml⁻¹ stock in water). For immunofluorescence staining, non-transfected macrophages were incubated with 1:50 dilution of mouse monoclonal anti-PKCε antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% goat-serum overnight at 4°C and rinsed three times for 5 min with 2% goat-serum. A 1:1000 dilution of Alexa Fluor 488-labelled secondary antibody (Molecular Probes) in 2% goat serum plus DAPI (2 µg ml⁻¹ from a 100 µg ml⁻¹ stock in water). For immunofluorescence staining, non-transfected macrophages were incubated with 1:50 dilution of mouse monoclonal anti-PKCε antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% goat-serum overnight at 4°C and rinsed three times for 5 min with 2% goat-serum.

**Ratiometric imaging**

Macrophages expressing PKCe-YFP (or untagged YFP when indicated) and CFP were infected with Listeria for 3 min (or 0.5 mg ml⁻¹ of TRDx when indicated), then excess bacteria were washed away with RB (30 times with 1 ml). Where indicated, 500 nM bafilomycin A₁ (from a 100 µM stock in water) was added to the macrophages for 1 h prior to infection and throughout the experiment.

Experiments used an inverted fluorescence microscope (Nikon TE300, Japan) equipped with transmitted light and a mercury arc lamp with epifluorescence illumination. To measure YFP and CFP fluorescence, two filter wheels (Lambda 10–2, Sutter Instruments, Novato, CA) held excitation filters (S500/20x and S436/10x, for YFP and CFP respectively, Chroma Technology Corporation, Rockingham, VT) and emission filters (S535/30m and S470/30m) with the dichroic mirror set (86002v1bs, Chroma Technology Corporation). A cooled CCD camera (Quantix Photometrics, Tucson, AZ) collected images and Metamorph software version 6.3 (Universal Imaging, West Chester, PA) controlled the equipment and image processing.

A ratio image was obtained by dividing each YFP image by the corresponding CFP image and multiplying by 1000. A binary mask was produced from the addition of the YFP and CFP images followed by application of a manual threshold. The binary and divided images were combined in a logical AND to produce ratio images that excluded non-cellular signals. A region was drawn around the phagosome in the ratio image and the average ratio of YFP/CFP in the phagosome was calculated. A second region drawn around the entire cell was used to measure the average fluorescence intensities of YFP and CFP over the entire cell. Relative ratios of YFP/CFP in the phagosome (Rₐ) were then divided by the YFP/CFP for the entire cell (Rₑ) to obtain a cell-normalized phagosome ratio (Rᵦ/Rₑ).

**Measurement of vacuole perforation by Lm**

In a method adapted from previous work (Shaughnessy et al., 2006), macrophages expressing PKCe-YFP were infected with a 100 µl mixture of wild-type Lm, TRDx (0.5 mg ml⁻¹) and LY (0.5 mg ml⁻¹) for 3 min. After infection, cells were washed thoroughly with RB. Lm-infected macrophages were located by phase-contrast optics. Using the 86006 dichroic filter set (Chroma Technology Corporation), four images were taken every minute for 30 min: phase-contrast, LY (exc. 436 nm/em. 535 nm), TRDx (exc. 580 nm/em. 630 nm) and YFP (exc. 492 nm/em. 535). The sequential loss of fluorescence from LY and TR was recorded relative to the timing of PKCe-YFP localization.

**Purification of LLO**

Recombinant LLO was purified from E. coli strain BL21(DE3) transformed with the pET29b vector expressing LLO with a C-terminal six-histidine tag as previously described (Mandal and Lee, 2002). The protein yield was measured using the BCA assay ( Pierce, Rockford, IL), and protein purity was analysed using SDS-PAGE. For some experiments, LLO was heat-inactivated at 70°C for 10 min. Haemolytic activity was measured using the sheep red blood cell-based haemolysis assay, as previously described (Mandal and Lee, 2002).

**Preparation of LLO liposomes**

LLO/HPTS, hiLLO/HPTS, or HPTS liposomes were prepared with phosphatidylethanolamine (Avanti, Alabaster, AL) and cholesterol/hemisuccinate (Sigma) in a 2:1 molar ratio using the thin film method (Lee et al., 1996; Mandal and Lee, 2002). LLO and HPTS were encapsulated inside liposomes at 0.25 mg ml⁻¹ and 35 mM HPTS, respectively, in 30 mM Tris buffer, 100 mM NaCl, at pH 8.5, under non-reducing conditions. Liposomes underwent repeated sonication and freeze-thaw cycles. Unencapsulated protein and HPTS were removed by purification on a Sepharose CL-4B column (Amersham Pharmacia, Uppsala, Sweden).

**Osmotic lysis of pinosomes**

Macrophage pinosomes were osmotically lysed according to previously published methods and adapted for this work (Okada and Rechsteiner, 1982; Moore et al., 1988). RAW macrophages expressing PKCe-YFP were exposed to hypertonic medium (0.5 M sucrose, 10% polyethylene glycol 1000, in RB) for 10 min (when stated, polyethylene glycol 1000 was not added). Cover-
slips were then washed five times and medium was replaced with hypotonic medium (60% RB and 40% water) for 3 min, then washed again and replaced with isotonic medium (RB). Phase-contrast and YFP images were taken after osmotic lysis.

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