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In Fig. 7C, the 1 μM and 5 μM bars were incorrectly labelled. The corrected figure and legend are shown below.

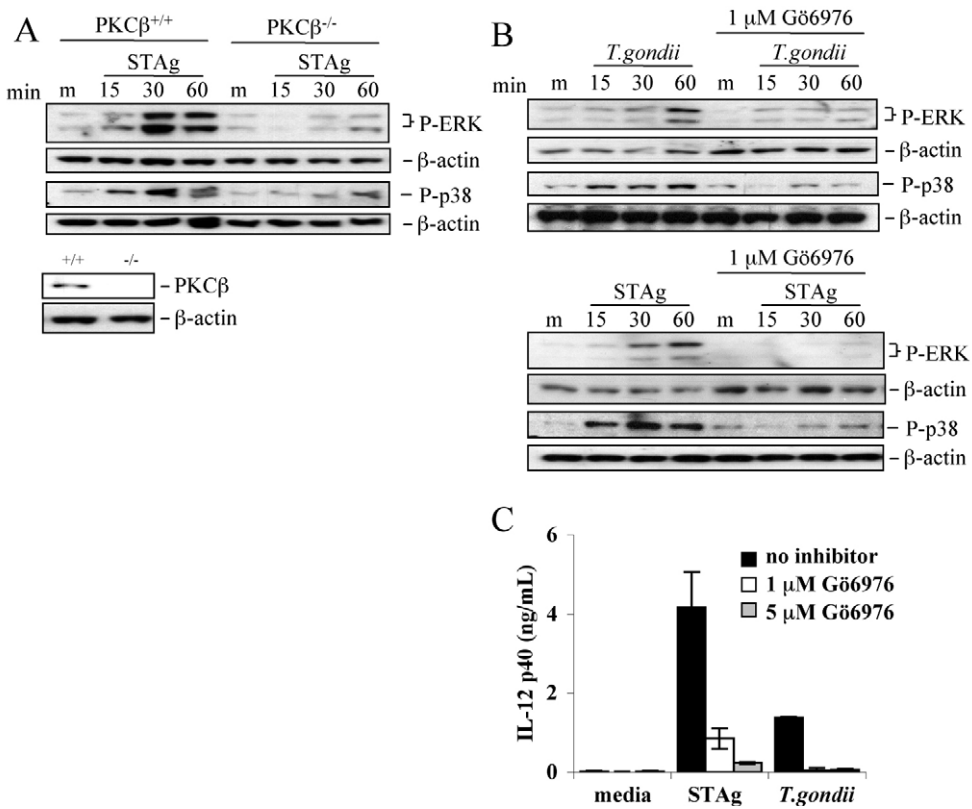

Fig. 7. Conventional PKC regulate *T. gondii*-induced MAPK activation and production of IL-12. (A) PKCβ$^{-/-}$ and WT macrophages were stimulated with medium (m) or STAg (50 μg/ml) for the times indicated and whole cell lysates were used for immunoblotting for phospho-ERK1/2 and phospho-p38. Blots were then stripped and reprobed for β-actin (top panels) and PKCβ (bottom panel). (B) WT macrophages were pre-treated with medium (m) or the conventional PKC inhibitor Gö6976 (1 μM), then treated with medium (m), infected with *T. gondii* (5:1) or stimulated with STAg (50 μg/ml). Whole cell lysates collected at the times indicated were immunoblotted for phospho-ERK1/2 and phospho-p38, then stripped and reprobed for β-actin. (C) Macrophages treated with media (black bars), 5 μM (grey bars), or 1 μM (white bars) Gö6976 were infected (1:1) or stimulated with STAg (50 μg/ml) overnight, and the supernatants collected at 20 hours post-infection were assayed for IL-12p40 production by ELISA (error bars indicate s.e.m.). In each panel, results are representative of four to five experiments.

The authors apologise for this error.

Host cell Ca^{2+} and protein kinase C regulate innate recognition of *Toxoplasma gondii*

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Summary

In healthy hosts, acute infection with the opportunistic pathogen *Toxoplasma gondii* is controlled by innate production of IL-12, a key cytokine crucial for the development of protective immunity. Previous work has established that the mitogen-activated protein kinases (MAPK), particularly p38 and ERK1/2, are important regulators of *T. gondii*-induced IL-12 synthesis. Here we report that host cell Ca^{2+} is required for activation of MAPK by *T. gondii*, as well as LPS and CpG, and for parasite-induced synthesis of IL-12. In addition, pharmacological mobilization of Ca^{2+} stores in macrophages treated with parasites or LPS enhanced MAPK phosphorylation initiated by these stimuli. Investigation of the upstream mechanism by which Ca^{2+} regulates MAPK activation revealed that *T. gondii* induced acute activation of conventional, Ca^{2+}-dependent PKCα and PKCβ, which are required for infection-induced MAPK activation and production of IL-12. Despite these findings, neither acute parasite infection nor LPS initiated a measurable Ca^{2+} response in macrophages, suggesting that low levels of Ca^{2+} are permissive for initiation of pro-inflammatory signaling. Together these data identify host cell Ca^{2+} and PKC as crucial regulators of the innate immune response to microbial stimuli, including *T. gondii*.

Key words: *Toxoplasma gondii*, Interleukin 12, Mitogen-activated protein kinase, Ca^{2+}, Macrophage/monocyte, Protein kinase C

Introduction

Toxoplasma gondii is a protozoan pathogen that chronically infects a significant percentage of the world's population. In healthy hosts, infection stimulates strong cell-mediated immunity dominated by T-cell production of interferon-γ (IFN-γ), which is required for long-term parasite control (Gazzinelli et al., 1993; Scharton-Kersten et al., 1996; Suzuki et al., 1988). In patients with primary or acquired defects in T-cell function, the inability to control parasite replication can result in overt disease (Denkers and Gazzinelli, 1998; Gazzinelli et al., 1992). Many of the factors that are required for the development of protective T-cell responses have been identified, and the cytokine IL-12 has a critical role in promoting synthesis of IFN-γ (Gazzinelli et al., 1993; Gazzinelli et al., 1994). Studies from several laboratories have determined that macrophages (Scharton-Kersten et al., 1996), dendritic cells (Reis e Sousa et al., 1997) and neutrophils (Bliss et al., 1999; Del Rio et al., 2004) can respond directly to *Toxoplasma* or parasite-derived antigens to produce IL-12. In the past decade, significant progress has been made toward defining the parasite-derived factors and host signaling pathways that are involved in the innate recognition of *Toxoplasma* by these cell populations.

Macrophages are a prominent source of IL-12 during early *Toxoplasma* infection and historically have served as a robust model to study cellular responses to *T. gondii*, as well as other intracellular pathogens. Work from several laboratories has shown that *Toxoplasma* interacts with macrophage signaling pathways in multiple ways to evade the immune response. For example, the parasite inhibits transcription factors associated with host defense such as NF-κB (Butcher et al., 2001; Shapira et al., 2002) and STAT1α (Luder et al., 2001), while promoting activation of STAT3, which suppresses synthesis of IL-12 (Butcher et al., 2005). Nevertheless, live parasites and parasite-derived products induce pro-inflammatory signaling in macrophages, including activation of the IKK complex (Shapira et al., 2005), as well as phosphorylation of the mitogen-activated protein kinases (MAPK) p38 and ERK1/2, which have been shown in several studies to be important regulators of the macrophage IL-12 response (Kim et al., 2005; Mason et al., 2004). Previous work has demonstrated that induction of IL-12 synthesis by *T. gondii* requires the Toll-like receptor (TLR) adaptor molecules MyD88 (Scanga et al., 2002) and TRAF6 (Mason et al., 2004), and there is evidence that parasite recognition is mediated by one or more TLRs (Hitziger et al., 2005; Mun et al., 2003; Scanga et al., 2002; Yarovinsky et al., 2005). Additionally, there is a report of a MyD88-independent pathway to IL-12 production in dendritic cells, involving a secreted parasite cyclophilin binding to the chemokine receptor CCR5 (Aliberti et al., 2000; Aliberti et al., 2003). Recent efforts have begun to define some of the links between these upstream events and the initiation of pro-inflammatory

signaling following *T. gondii* infection. ERK1/2 and p38 activation are both activated in a TRAF6-dependent manner (Mason et al., 2004), whereas only p38 phosphorylation requires the presence of MyD88 (Kim and Denkers, 2006). Moreover, in macrophages, CCR5 has been shown to be dispensable for activation of both of these MAP kinases (Kim and Denkers, 2006). Nevertheless, the upstream signaling factors connecting parasite recognition with the subsequent pro-inflammatory response are still unclear.

In addition to those described above, *Toxoplasma* has developed several strategies to bypass the anti-microbial activities of its host, including the rapid establishment of a non-fusogenic compartment, the parasitophorous vacuole (PV), within which the parasite is able to replicate, protected from host cell hydrolases (Joiner et al., 1994; Schwab et al., 1994). Through the cytoplasmic surface of the PV, *T. gondii* associates specifically with host cell endoplasmic reticulum and mitochondria soon after invasion (Sinai et al., 1997). The biological significance of this interaction remains unclear; however, both the ER and mitochondria regulate intracellular Ca^{2+}, which is implicated in host cell responses to microbial products in several pathogen systems (Gewirtz et al., 2000; Goldfine and Wadsworth, 2002; Goodridge et al., 2003; Yadav et al., 2004). Moreover, *T. gondii* lysate is reported to mobilize Ca^{2+} in CCR5-transfected cells (Aliberti et al., 2003). These observations, coupled with previous reports that Ca^{2+} levels influence pro-inflammatory signaling pathways in lymphocytes (Dolmetsch et al., 1997; Dolmetsch et al., 1998), led to the hypothesis that host cell Ca^{2+} might influence the innate recognition of *T. gondii*. Consistent with this concept, the studies presented here show that Ca^{2+} is required for parasite-mediated MAPK activation and IL-12 synthesis, and its pharmacological mobilization enhances MAPK signaling. Moreover, Ca^{2+} also modulates MAPK signaling induced by other microbial stimuli, including LPS and CpG. In addition, *T. gondii* induces acute activation of Ca^{2+}-dependent PKC isoforms α and β, and their inhibition reduces MAPK activation and IL-12. Despite these findings, none of these stimuli induces acute Ca^{2+} elevations, suggesting that low cytoplasmic levels of host cell Ca^{2+} are permissive for pro-inflammatory signaling in macrophages. Together these data indicate that host cell Ca^{2+} and PKC play crucial roles in the macrophage innate immune response to diverse microbial stimuli.

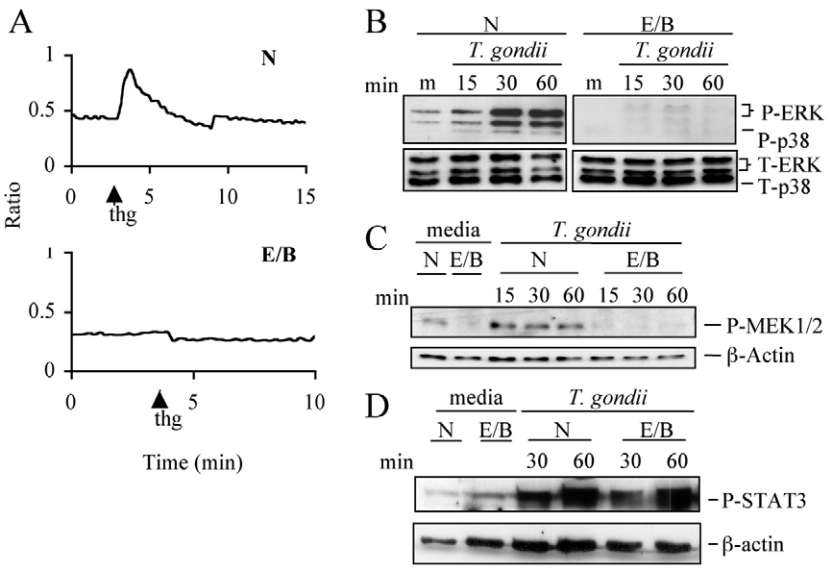

Fig. 1. MAPK signaling induced by *T. gondii* requires host Ca^{2+}. (A) Fura-2-loaded macrophages were incubated in normal buffer (N) or BAPTA-AM (15 μM) in Ca^{2+}-free EGTA medium (E/B) as described in Materials and Methods, then transferred to the Ca^{2+} imaging chamber, and stimulated with thapsigargin (thg; 1 μM, arrowhead) in normal buffer or Ca^{2+}-free EGTA buffer, respectively, to release available intracellular Ca^{2+} stores. Images were captured at 5 frames per minute and 340/380 ratios were calculated and averaged to create the line tracings depicted. Averages are based on ratios collected from approximately 60 cells in each imaging field. (B-D) Macrophages were pre-treated with normal medium (N) or BAPTA-AM (15 μM) in Ca^{2+}-free EGTA medium (E/B) for 45 minutes, washed, then treated with medium (m) as a control or infected with live *T. gondii* at a ratio of 5:1. Whole cell lysates were collected at the times indicated, and subjected to immunoblotting for phospho-ERK1/2 and phospho-p38 using individual antibodies specific to each MAPK (B, top panels). Membranes were then stripped and reprobed for total levels of ERK1/2 and p38 (bottom panels). Separate lysates were collected and used to immunoblot for phospho-MEK1/2 (C) or phospho-STAT3 (D). The membranes were stripped and reprobed for β-actin to determine loading. Each experiment was repeated two to four times, and representative results are presented.

Results

MAPK activation by *T. gondii* requires host cell Ca^{2+}

To determine the role of Ca^{2+} in *T. gondii*-induced activation of MAPK, bone-marrow macrophages (BMMϕ) were infected under culture conditions that depleted extracellular and intracellular Ca^{2+} sources. The extent of Ca^{2+} depletion was assessed using Fura-2-loaded macrophages pre-treated in normal-Ca^{2+} or chelating conditions as described in Materials and Methods. Macrophages pretreated in normal, Ca^{2+}-containing media (N) exhibited an acute Ca^{2+} response following stimulation with thapsigargin that was absent in EGTA/BAPTA-AM (E/B)-pre-treated cells (Fig. 1A), indicating that the depletion protocol effectively eliminates releasable Ca^{2+} stores. Under normal-Ca^{2+} conditions, macrophages exhibited low level phosphorylation of both ERK1/2 and p38, which was acutely upregulated by *T. gondii* (Fig. 1B) as previously reported (Kim, L. et al., 2004). However, Ca^{2+} depletion reduced basal levels of ERK1/2 and p38 phosphorylation in untreated cells, and prevented *T. gondii*-induced activation of both MAPK. Activation of ERK1/2 occurs as a result of phosphorylation by the upstream MAPKK, MEK1/2 (Dong et al., 2002), which is activated in response to *T. gondii* (Kim and Denkers, 2006). To determine whether Ca^{2+} acts proximally to ERK1/2, the effect of Ca^{2+} depletion on MEK1/2 activation by *T. gondii* was evaluated. Under normal Ca^{2+} conditions, MEK1/2 showed low-level activation in resting cells, and was rapidly phosphorylated after infection (Fig. 1C). When Ca^{2+} was depleted, basal levels of MEK1/2 phosphorylation were

eliminated in resting cells and kinase activation was prevented following *T. gondii* challenge, suggesting that Ca^{2+} mediates its effects upstream of the MAPK. Previously it was reported that chelation of parasite Ca^{2+} stores adversely affects parasite invasion of host cells (Lovett and Sibley, 2003). To control for the possibility that invasion might be compromised under Ca^{2+}-depleted conditions, macrophages were plated on coverslips and infected in parallel in each experiment and the percentage of cells containing intracellular parasites was identical under normal and Ca^{2+}-depleted conditions (data not shown). Thus, the absence of MEK1/2 and MAPK activation under Ca^{2+}-depleted conditions is not the result of delayed or compromised invasion, but rather appears to be due to the specific lack of host cell Ca^{2+}.

The observation that Ca^{2+} is required for infection-induced activation of MAPK raised the question of whether other host cell responses to *T. gondii* were similarly Ca^{2+} dependent. Recent studies have reported that invasion with live *Toxoplasma* results in the rapid phosphorylation of STAT3, which negatively regulates infection-induced IL-12 synthesis (Butcher et al., 2005). In agreement with these previous studies, STAT3 was rapidly phosphorylated following *T. gondii* infection in normal Ca^{2+}, and its activation was not prevented by depletion of Ca^{2+} stores (Fig. 1D). This observation was confirmed by densitometry analysis where the immunoblot signal from phospho-STAT3 was normalized to its corresponding β-actin band, and the fold change of STAT3 phosphorylation was determined. By 60 minutes post-infection, phospho-STAT3 expression was induced 3.9-fold in normal Ca^{2+}, and 3.0-fold under Ca^{2+}-depleted conditions (data not shown), suggesting that Ca^{2+} is dispensable for parasite-induced activation of STAT3. Together these findings indicate that parasite invasion is not compromised in Ca^{2+}-depleted host cells, and that Ca^{2+} is not required for all infection-induced signaling.

We next examined whether Ca^{2+}-dependent MAPK activation is restricted to live infection. Soluble *Toxoplasma* antigen (STAg), a soluble parasite lysate, has been used to study IL-12 production in macrophages (Li et al., 1994; Scanga et al., 2002), and has been shown to activate ERK1/2 and p38 in these cells (Li et al., 1994; Mason et al., 2004). Macrophages were stimulated with STAg in normal media, or under Ca^{2+}-depleted conditions. Similarly to the findings with live infection, MAPK activation by STAg was abrogated under Ca^{2+}-chelated conditions (Fig. 2A). To confirm the effect of Ca^{2+} depletion on phosphorylation of p38, we conducted a densitometry analysis in which the signal from phospho-p38 was normalized to its corresponding Total-p38 band. STAg stimulated a more than twofold increase in phospho-p38 of under normal Ca^{2+} conditions, and this was completely abolished by BAPTA treatment (data not shown). These findings indicate that both live parasites and STAg induce MAPK activation in a Ca^{2+}-dependent manner. Moreover, this result argues against the possibility that Ca^{2+} depletion prevents MAPK activation by affecting parasite viability. We also examined whether other microbial stimuli use a Ca^{2+}-dependent pathway to activate MAPK. Macrophages were stimulated with the TLR4 agonist LPS (Fig. 2B) or the TLR9 agonist CpG (Fig. 2C) under normal or Ca^{2+}-depleted conditions. Similarly to *T. gondii*, MAPK activation by these preparations was abrogated by BAPTA treatment. Thus, these

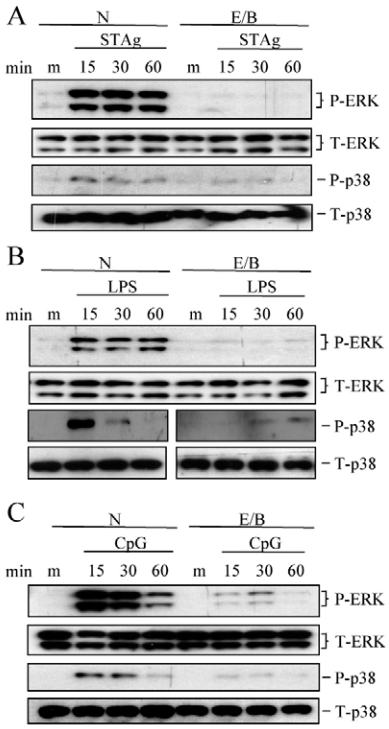

Fig. 2. MAPK activation by parasite lysate, LPS, and CpG requires host cell Ca^{2+}. Macrophages were depleted of Ca^{2+} by pre-treating with normal media (N) or BAPTA-AM (15 μM) in Ca^{2+} free EGTA medium (E/B) then treated with medium (m), STAg (A; 50 μg/ml), LPS (B; 100 ng/ml) or CpG (C; 1 μg/ml) for the times indicated. Whole cell lysates collected at the times indicated were used for immunoblotting with phospho-ERK1/2 and phospho-p38 antibodies. Blots were then stripped and reprobed for total MAPK. Results are representative of three experiments each.

results indicate that Ca^{2+} is a necessary factor required for pro-inflammatory signaling in response to multiple pathogen-derived stimuli.

Ca^{2+} is required for *T. gondii*-induced IL-12

Work from several laboratories has previously established that p38 and ERK1/2 are important regulators of IL-12 production in macrophages stimulated with live infection or STAg (Kim et al., 2005; Mason et al., 2004). Given that both MAPK are Ca^{2+} dependent, we next determined whether Ca^{2+} is also required for IL-12 production in response to STAg. Concerns about cell viability after long-term Ca^{2+} depletion led us to use a real-time PCR approach, which could be performed following acute stimulation. Under normal conditions, STAg induced transcription of IL-12p40 and IL-12p35 mRNA (Fig. 3), both of which were significantly reduced in the absence of Ca^{2+} sources. Cell viability, assessed by Alamar Blue reduction assay, was minimally affected by this short-term depletion [reduced by 11±1.8% (± s.e.m.) relative to untreated controls; data not shown]. Host cell Ca^{2+} is therefore required for activation of MAPK, as well as downstream synthesis of IL-12.

Ca^{2+} entry enhances *T. gondii*-induced MAPK

The findings presented above establish that host cell Ca^{2+} is

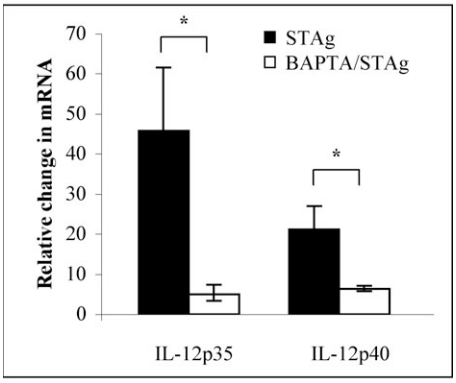

Fig. 3. Ca^{2+} is required for STAg-induced production of IL-12. Macrophages pre-treated with normal buffer (black bars) or BAPTA-AM (15 μM) in Ca^{2+} free/EGTA (white bars) were stimulated with STAg (50 μg/ml) for 5 hours in normal media or Ca^{2+} free EGTA medium, respectively. mRNA was isolated and reverse transcribed to cDNA, which was used in real-time PCR reactions for IL-12p35 and IL-12p40. All amplifications were conducted in triplicate and results are normalized to endogenous 18S and expressed as fold change in mRNA relative to untreated controls for each condition, according to the ΔΔCt method. Experiments were repeated at least three times with similar results. The data presented are from a representative experiment. *$P \leq 0.01$ by Student's *t*-test.

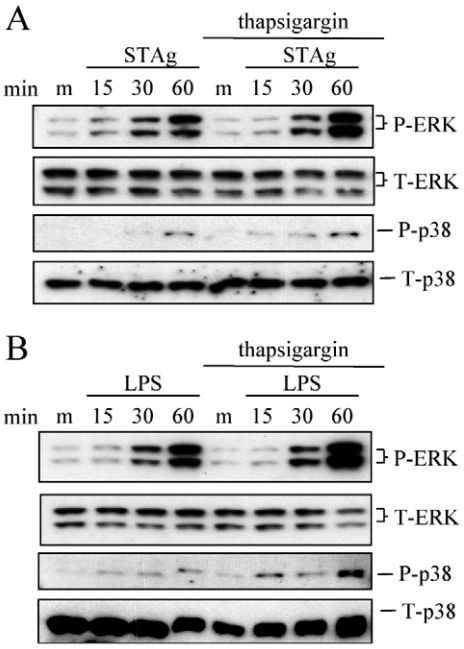

Fig. 4. Ca^{2+} elevation enhances MAPK activation by parasite lysate or LPS. Macrophages were treated with medium (m) or stimulated with STAg (A; 50 μg/ml) or LPS (B; 100 ng/ml) either in the presence or absence of the Ca^{2+}-mobilizing stimulus thapsigargin (1 μM) in normal Ca^{2+} media. Whole cell lysates were collected at the times indicated and immunoblotted for phospho-ERK1/2 and phospho-p38 then stripped and re-probed for total MAPK. Each experiment was repeated at least three times, and representative results are presented.

necessary for MAPK activation by several microbial treatments. We next determined whether elevations in Ca^{2+} levels would influence phosphorylation of MAPK. Assessing the effects of Ca^{2+} mobilization during live infection is complicated by the fact that abrupt elevations in host cytoplasmic Ca^{2+} induce egress of intracellular parasites (reviewed by Arrizabalaga and Boothroyd, 2004). Therefore, macrophages were treated with STAg or LPS in normal Ca^{2+} media and simultaneously treated with thapsigargin to induce an elevation in cytoplasmic Ca^{2+} (Fig. 1A, top panel). As previously noted in studies using B lymphocytes (Dolmetsch et al., 1997), Ca^{2+} elevation alone did not stimulate MAPK phosphorylation (Fig. 4A,B). However, when combined with signals generated by STAg (Fig. 4A) or LPS (Fig. 4B), elevated Ca^{2+} induced stronger MAPK activation than either stimulus alone. Together, these data indicate that increases in cytoplasmic Ca^{2+} can influence the strength of the signals induced by microbial stimuli.

Microbial stimuli fail to induce a detectable Ca^{2+} flux

The studies described above demonstrate that Ca^{2+} is required for MAPK activation by *T. gondii* as well as several other microbial preparations, and that Ca^{2+} mobilization enhances the MAPK response. These results suggested that these stimuli might induce changes in cytoplasmic Ca^{2+} levels in treated cells. To test this, BMMϕ were loaded with Fura-2 to assess real-time changes in intracellular Ca^{2+} following stimulation. Unexpectedly, no changes in Ca^{2+} levels were recorded following addition of *T. gondii* (Fig. 5A right), STAg (Fig. 5B) or LPS (Fig. 5C) within the timeframe of detectable MAPK activation (Fig. 1). However, the macrophages showed a typical pattern of Ca^{2+} release following exposure to thapsigargin (Fig. 5A left, B and C), indicating that they were viable and responsive. These findings suggest that these microbial stimuli may induce small changes in Ca^{2+} that are local or below the level of detection in this system. Alternatively, basal Ca^{2+} levels in activated macrophages may be sufficient for MAPK activation. This latter possibility is supported by the observation that Ca^{2+} depletion reduces basal levels of phosphorylated MEK1/2 and MAPK in resting, unstimulated cells (Fig. 1).

Conventional PKC regulate MAPK activation and IL-12 production by *T. gondii*

In a search to identify Ca^{2+}-sensitive pathways that could lie upstream of MAPK activation in *T. gondii*-infected macrophages, we evaluated the role of the protein kinase C (PKC) family of enzymes. The PKC family includes the conventional members (cPKC) PKCα and PKCβI/II, which are regulated by intracellular Ca^{2+} (Parker and Murray-Rust, 2004). The various family members are involved in diverse cellular functions, including cell survival and inflammation, and have been shown to direct MAPK phosphorylation in multiple cell types (Yang and Kazanietz, 2003). The initiation of cPKC activity can be determined by translocation of isoforms from the cytosol to the plasma membrane. In macrophages, LPS stimulation has been shown to induce a one- to twofold increase in membrane-associated PKCα and PKCβ (Fronhofer et al., 2006; Kontny et al., 2000; Shinji et al., 1994). To determine whether *T. gondii* also activates cPKC, whole cell lysates from macrophages challenged with *T. gondii* and STAg,

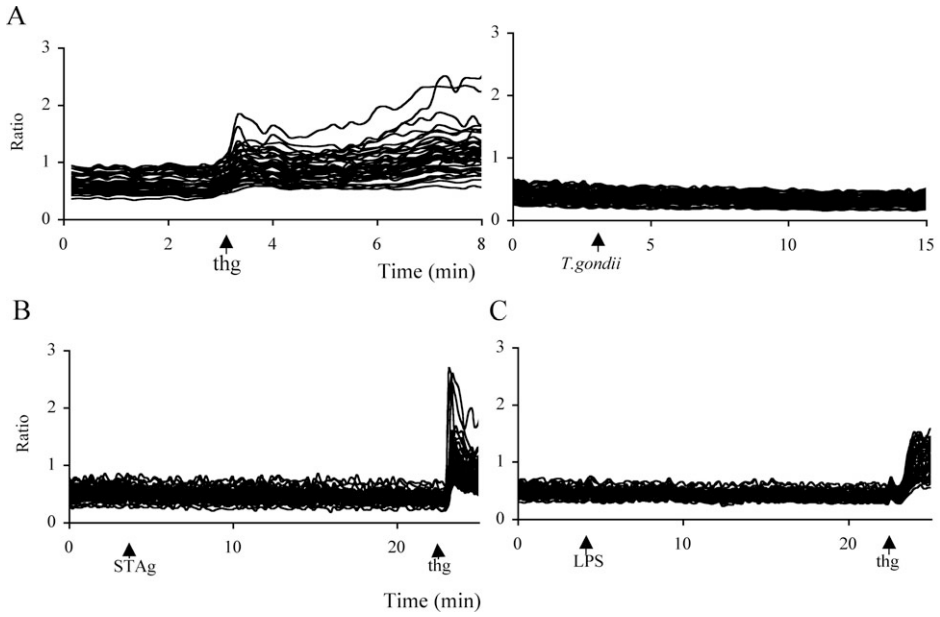

Fig. 5. Microbial stimuli fail to induce an acute Ca^{2+} flux. Macrophages were loaded with Fura-2 and used for live cell Ca^{2+} imaging. (A, left panel) Fura-2-loaded macrophages were treated with thapsigargin (thg; 1 μM) after 3 minutes of baseline recording to demonstrate a typical Ca^{2+} response in these cells. Live *T. gondii* (A, right panel; 5:1), STAg (B; 50 μg/ml) or LPS (C; 100 ng/ml) was added to Fura-2-loaded macrophages following 3 minutes of baseline recording (arrows) and intracellular Ca^{2+} was recorded over the time period indicated. At the end of experiments in B and C, thapsigargin (thg; 1 μM) was added to demonstrate a positive response. Individual lines in each panel represent the intracellular Ca^{2+} level within each individual cell in the imaging field expressed as a 340/380 ratio. Experiments typically included 55-65 cells per imaging field. Recordings are representative of five to seven experiments for each condition.

as well as LPS for comparison, were separated into cytosolic and membrane fractions by high-speed ultracentrifugation. Consistent with previous reports, LPS activated both PKC isoforms within minutes of stimulation (Fig. 6A) (Fronhofer et al., 2006; Kontny et al., 2000; Shinji et al., 1994). STAg and *T. gondii* also induced translocation of PKCα and PKCβ to the membrane fraction (Fig. 6A), and the fold increase in membrane-associated PKCα was higher following parasite treatment compared with LPS treatment. Peak activation by parasites occurred between 5 and 15 minutes, and was consistently greater in magnitude for PKCα compared with PKCβ (Fig. 6B). In addition, live infection induced higher levels of PKCβ activation than seen with STAg.

The kinetics of infection-induced PKC activation suggested that it could lie upstream of the MAPK (Fig. 1). To test this possibility, we assessed the ability of macrophages derived from $PKC\beta^{-/-}$ mice to activate MAPK in response to STAg. Treatment-induced phosphorylation of ERK1/2 and p38 was prevented in knockout cells relative to the WT, and this effect was more pronounced for ERK than for p38 (Fig. 7A). To further assess the contributions of both cPKC isoforms, WT macrophages were stimulated in the presence of Gö6976, an

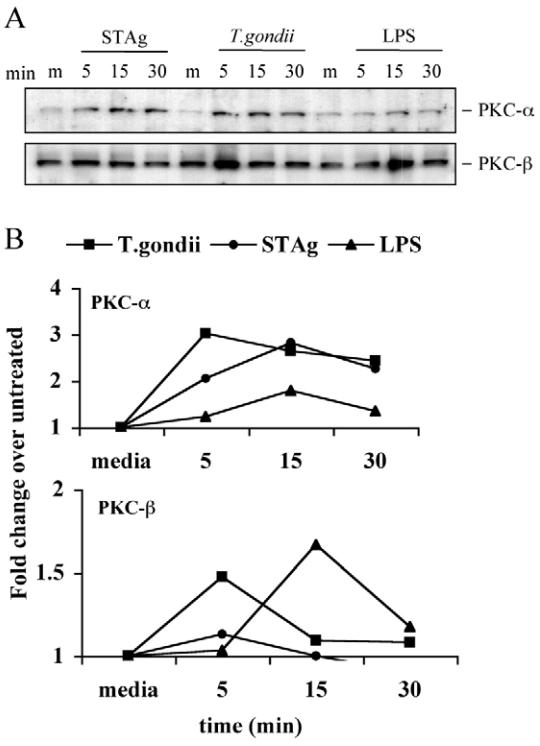

Fig. 6. *T. gondii* infection induces activation of conventional PKC. (A) Macrophages were infected with live *T. gondii* (5:1) or stimulated with media (m), STAg (50 μg/ml) or LPS (100 ng/ml) for the times indicated, then whole cell lysates were collected and fractionated by high-speed ultracentrifugation. Membrane fractions were used to immunoblot for PKCα and PKCβ as noted. Activation of PKC isoforms is determined by translocation to the membrane contained in the pellet fraction. (B) Densitometry of immunoblots in A, where data are represented as fold change over media-treated controls. Results are representative of seven experiments in which *T. gondii* induced a significant ($P \leqslant 0.03$) accumulation of both isoforms of PKC in the pellet fraction.

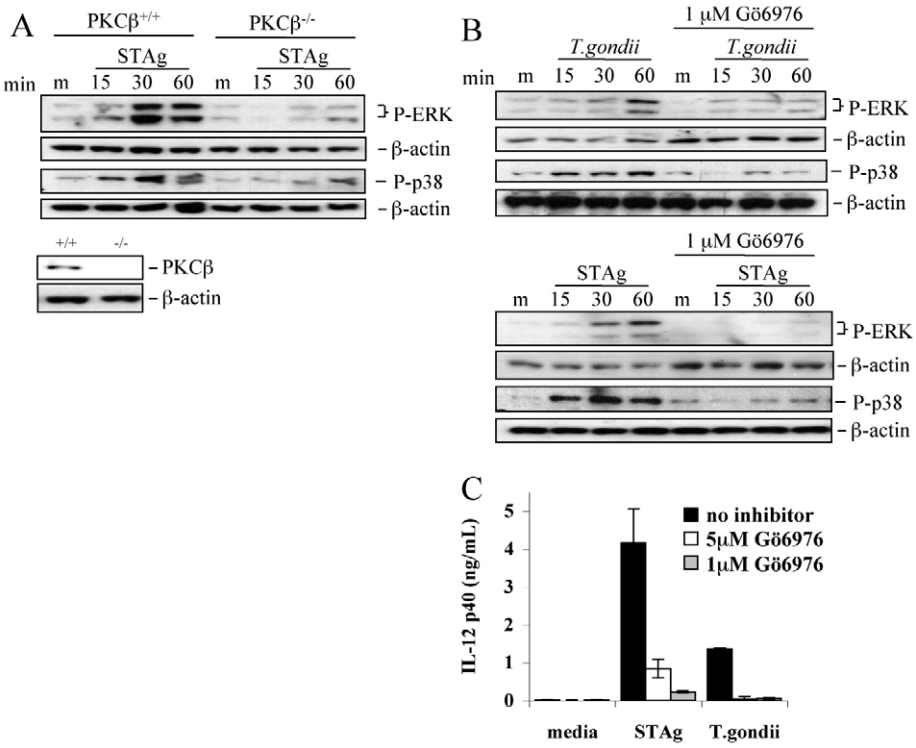

Fig. 7. Conventional PKC regulate *T. gondii*-induced MAPK activation and production of IL-12. (A) PKCβ$^{-/-}$ and WT macrophages were stimulated with medium (m) or STAg (50 μg/ml) for the times indicated and whole cell lysates were used for immunoblotting for phospho-ERK1/2 and phospho-p38. Blots were then stripped and reprobed for β-actin (top panels) and PKCβ (bottom panel). (B) WT macrophages were pre-treated with medium (m) or the conventional PKC inhibitor Gö6976 (1 μM), then treated with medium (m), infected with *T. gondii* (5:1) or stimulated with STAg (50 μg/ml). Whole cell lysates collected at the times indicated were immunoblotted for phospho-ERK1/2 and phospho-p38, then stripped and reprobed for β-actin. (C) Macrophages treated with media (black bars), 5 μM (white bars), or 1 μM (grey bars) Gö6976 were infected (1:1) or stimulated with STAg (50 μg/ml) overnight, and the supernatants collected at 20 hours post-infection were assayed for IL-12p40 production by ELISA (error bars indicate s.e.m.). In each panel, results are representative of four to five experiments.

inhibitor that targets PKCα and PKCβ. Phosphorylation of ERK1/2 and p38 by *T. gondii* was diminished in a dose-dependent manner, despite normal levels of infection (Fig. 7B top, and data not shown). Moreover, this compound also blocked MAPK activation by STAg (Fig. 7B, bottom), arguing against a non-specific effect on the parasite. Consistent with these findings, Gö6976 dose-dependently limited production of IL-12 from infected macrophages (Fig. 7C), without affecting cell viability during the overnight treatment, or LPS-induced IL-12 synthesis (data not shown). Together, these results implicate PKC in directing MAPK activation and production of IL-12 by *T. gondii*.

Discussion

Ca^{2+} is an important regulator of many fundamental cellular processes that include cell growth, proliferation, and death (reviewed by Berridge et al., 2000). In recent years there has been a growing appreciation of this second messenger in immune function. In T and B lymphocytes, detailed studies of the mechanisms by which Ca^{2+} signals are 'decoded' have shown that incremental increases in cytoplasmic Ca^{2+} can activate various transcription factors, including members of the MAPK, NF-κB, and NFAT families (Dolmetsch et al., 1997; Dolmetsch et al., 1998). These pathways are variably sensitive to the amplitude and duration of the Ca^{2+} flux, providing a means by which Ca^{2+} signals can be translated into a phenotypic effect. Nevertheless, little is known about the role of this ion in the regulation of signaling during an innate immune response. The studies reported here establish that the ability of macrophages to activate MAPK in response to *T. gondii*, parasite lysate, LPS or CpG, is dependent on host cell Ca^{2+} concentration. Moreover, when induced in combination with a primary microbial stimulus, Ca^{2+} mobilization enhances phosphorylation of MAPK. Furthermore, live parasites, STAg and LPS induce activation of Ca^{2+}-dependent PKC isoforms, which directs MAPK activation in *T. gondii*-infected cells. Despite these findings, neither live parasites, STAg nor LPS initiate a detectable Ca^{2+} response in macrophages. LPS has previously been demonstrated to use a Ca^{2+}-dependent pathway for activation of ERK (Goodridge et al., 2003), and a large body of literature reports that it activates cPKC as well as other Ca^{2+}-sensitive signaling mediators in macrophages (Chano and Descoteaux, 2002; Chen et al., 2001; Chen et al., 1998; Fujihara et al., 1994; Giroux and Descoteaux, 2000; Goodridge et al., 2003; Kontny et al., 2000; Mishra et al., 2005; Novotney et al., 1991; Salonen et al., 2006; Shinji et al., 1997; St-Denis et al., 1998; Sweet and Hume, 1996). These observations represent indirect evidence that LPS treatment induces changes in intracellular Ca^{2+}, however direct demonstration of a Ca^{2+} response is controversial (Drysdale et al., 1987; Kim, Y. et al., 2004; Letari et al., 1991a; Letari et al., 1991b; Lowry et al., 1999; Maudsley and Morris, 1987; McLeish et al., 1989; Prpic et al., 1987; Waga et al., 1993). It may be the case that LPS, as well as the other microbial stimuli examined here, induce small or localized Ca^{2+} changes that are undetectable with our Fura-2-based, whole cell imaging system, and this signaling is sufficient to facilitate phosphorylation of MAPK. Alternatively, we have observed that treatment with BAPTA in Ca^{2+} free/EGTA medium not only depletes Ca^{2+} stores, but also lowers the basal, cytoplasmic Ca^{2+} levels in resting macrophages (Fig. 1A; compare baselines before stimulation with thapsigargin in top and bottom panels). Thus, another possibility is that the existing cytoplasmic Ca^{2+} levels in activated macrophages are sufficient to permit pro-inflammatory signaling upon microbial contact. This possibility is supported by the observation that

basal levels of phosphorylated MEK1/2 and MAPK in resting, untreated cells are eliminated when Ca^{2+} sources are depleted (Fig. 1A-C and Fig. 2A, m lanes).

In addition to the MAPK, we have found that degradation of IκBα following *T. gondii* infection is also prevented by Ca^{2+} depletion (data not shown), which is consistent with previously published reports implicating Ca^{2+} in NF-κB activation by pathogens such as *Salmonella typhimurium* (Gewirtz et al., 2000). However, not all acute signaling events induced by *T. gondii* are Ca^{2+} dependent, because phosphorylation of STAT3 occurs in BAPTA-treated cells (Fig. 1). Recent work has started to define the events that lead to the activation of MAPK in macrophages infected with *T. gondii*. Activation of p38 is reported to occur by autophosphorylation (Kim et al., 2005) and requires MyD88, whereas ERK1/2 phosphorylation can occur independently of MyD88, and may proceed through a G_i protein-linked PI3K signaling pathway (Kim and Denkers, 2006). Despite these differences, there is a common requirement for the adapter molecule TRAF6 (Mason et al., 2004) and for Ca^{2+} in both pathways. The precise mechanism by which Ca^{2+} affects ERK1/2 and p38 activation in our system is unknown, however the finding that it is required for activation of upstream MEK1/2 suggests that it acts proximally, perhaps through one or more Ca^{2+}-sensitive kinases. A previous study implicated a role for PKC in production of TNF-α and to a lesser extent IL-12 in STAg-treated macrophages (Grunvald et al., 1996). Here we establish that infection induces activation and translocation of conventional PKC isoforms α and β to the cell membrane, and that MAPK activation is compromised in $PKC\beta^{-/-}$ macrophages. Chemical inhibition of both isoforms decreases both MAPK activation and IL-12 synthesis following parasite challenge, indicating that host cell Ca^{2+} is required for *T. gondii*-induced MAPK activation and subsequent IL-12 production through conventional PKC. In addition, preliminary data indicate that *Toxoplasma* induces rapid activation of the Ca^{2+}-sensitive enzyme calmodulin-dependent protein kinase II (CaMKII), although its chemical inhibition has no effect on MAPK activation (data not shown). These results reveal that, in addition to PKC, at least one other Ca^{2+}-sensitive pathway is activated by infection, however its function is unclear.

Recent progress has begun to identify some of the immunoregulatory products produced by parasites and the host receptors that recognize them. *T. gondii* is reported to produce a prophilin molecule recognized by the newly described TLR11 (Yarovinsky et al., 2005; Zhang et al., 2004), as well as a cyclophilin, C-18, which induces IL-12 synthesis by DC through the endogenous host chemokine receptor CCR5 (Aliberti et al., 2003). The signaling events initiated in host cells contacted by these parasite products have not been defined, however it has been reported that purified C-18, as well as unpurified STAg, induces a transient Ca^{2+} response in cells transfected with the CCR5 receptor (Aliberti et al., 2003). We do not detect changes in intracellular Ca^{2+} in primary bone-marrow-derived macrophages stimulated with STAg, however, this apparent discrepancy may arise as a result of imaging technique (flow cytometry versus live cell imaging), or may be attributed to cell type, or differences in receptor expression between primary and transfected cells. Nevertheless, it will be important to characterize the signaling events induced by these parasite products, and particularly, to determine whether they initiate Ca^{2+}-dependent signaling downstream.

This work has focused primarily on the macrophage response to *T. gondii*, however, we have found that MAPK activation by classic TLR agonists, LPS and CpG, is also Ca^{2+} dependent, implicating an important role for this ion in the innate immune response. These studies suggest that microbial stimulation and intracellular Ca^{2+} levels can converge to augment and fine-tune innate signaling pathways. Thus, at low levels, Ca^{2+} acts permissively to facilitate pro-inflammatory signaling, whereas at higher concentrations it enhances the signaling response. These findings imply that the responses of macrophages contacted by microbial products could be amplified by simultaneous exposure to stimuli that induce changes in intracellular Ca^{2+} levels such as chemokines or Fcγ-receptor-mediated phagocytosis of microbes and dead cells (Myers and Swanson, 2002). Indeed this concept is supported by the recent observation that PKCα activation is enhanced when LPS-treated macrophages are simultaneously stimulated through the Fcγ receptor (Fronhofer et al., 2006). In this regard, Ca^{2+}-dependent signals appear to facilitate cross-talk between these pathways, helping to integrate and fine-tune the overall innate response. In addition to *T. gondii*, studies on other pathogens, including *Salmonella* (Gewirtz et al., 2000) and mycobacteria (Yadav et al., 2004), have identified a role for Ca^{2+} in facilitating pro-inflammatory signaling. Thus, there is growing evidence that Ca^{2+} not only mediates many of the basic processes that govern cell survival, proliferation, and growth (reviewed by Berridge et al., 2000), but also acts as an important regulator of the innate immune response to infection. As such, elucidation of the upstream components that effect these Ca^{2+}-dependent events may present attractive targets for anti-inflammatory therapeutics.

Materials and Methods

Mice and cell culture

Bone-marrow-derived macrophages were cultured from female C57BL/6 (age 4-6 weeks; Jackson Laboratories, Bar Harbor, ME), or $PKC\beta^{-/-}$ mice, as previously described (Caamano et al., 1999). For all experiments, macrophages were harvested on day 7, rested overnight, then primed for 16 hours with 100 U/ml rmIFN-γ (BD Pharmingen, San Diego, CA) in macrophage medium containing the following: DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (HIFCS; US Bio-Technologies, Parkerford, PA), 1% penicillin and streptomycin (Invitrogen), 25 mM HEPES, and 1 mM sodium pyruvate. Macrophages were not primed with IFN-γ in experiments examining phosphorylation of STAT3.

Parasites and STAg

RH strain *T. gondii* tachyzoites were maintained in vitro by twice-weekly passage through human foreskin fibroblast monolayers in medium containing DMEM supplemented with 10% HIFCS, 1% penicillin, streptomycin and gentamicin (Invitrogen). For in vitro infections, intracellular tachyzoites were harvested by passage through a 22-gauge needle and washed with Ca^{2+}-free, Mg^{2+}-free dPBS (Cellgro, Mediatech, Herndon, VA). For Ca^{2+}-depletion experiments, tachyzoites were then resuspended in either normal buffer or Ca^{2+}-free medium/EGTA (see below) immediately before infection. Soluble *Toxoplasma* antigen (STAg) was prepared from RH tachyzoites as previously described (Sharma et al., 1983).

Manipulation of host cell Ca^{2+} and microbial reagents

For Ca^{2+}-depletion experiments, 8×10^5 macrophages were washed three times in warm dPBS to remove Ca^{2+}-containing media, then pre-treated for 45 minutes with either normal buffer (N condition; 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM NaOH-HEPES, 10 mM glucose, pH 7.3, Osm 290-300), or 15 μM BAPTA-AM (Invitrogen Molecular Probes) in Ca^{2+}-free EGTA medium (E/B condition; 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM Glucose, 10 mM NaOH-HEPES, 0.25 mM EGTA, pH 7.3, Osm 290-300). They were then washed twice with normal buffer (N condition), or Ca^{2+}-free EGTA medium (E/B condition) to remove extracellular BAPTA. Macrophages pre-treated as described above were

stimulated with STAg (50 μg/ml), LPS (*S. typhimurium*; 100 ng/ml, Sigma), rmTNF-α (10 ng/ml; EMD Biosciences, San Diego, CA), CpG (1 μg/ml; Invitrogen), or infected at a ratio of five parasites to one macrophage (5:1) in either normal buffer (N) or Ca^{2+}-free EGTA medium (E/B). The effect of Ca^{2+} mobilization on MAPK activation was assessed by stimulating 8×10^5 macrophages with medium, STAg, or LPS and simultaneously inducing a Ca^{2+} flux by co-treatment with thapsigargin (1 μM; Sigma) in normal buffer.

Inhibitors

Macrophages were pre-treated with Gö6976 (1-5 μM; Calbiochem, EMD Biosciences) for 60 minutes before addition of stimuli then whole cell lysates were collected at the times indicated. For all live infection experiments, macrophages were plated on coverslips in parallel to lysate samples, stained, and the percentage of infected cells determined. Infection rates were identical under media-treated and inhibitor-treated conditions (data not shown).

PKC translocation

Activation of PKC was assessed by translocation of the various isoforms from the cytosol to the membrane in macrophages following stimulation with *T. gondii* or STAg. Live parasites (8:1) or STAg (50 μg/ml) were applied to 4×10^6 macrophages and whole cell lysates were collected 5-30 minutes later in 20 mM Tris-HCl, pH 7.4, containing 5 mM EGTA and 10 μl/ml Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) and Phosphatase inhibitor cocktail 2 (Sigma). Lysates were sonicated and separated by high-speed centrifugation for 30 minutes at 40,000 rpm (86,500 ***g***) on a Beckman Coulter (Fullerton, CA) TL-100 ultracentrifuge. Supernatants were reserved as the cytosolic fraction, and the pellet was washed three times in cold dPBS then resuspended in Tris-EGTA buffer.

Preparation of cell lysates, immunoblotting and antibodies

Whole cell lysates were prepared at the times indicated by washing cells with ice-cold dPBS, and lysing for 10 minutes on ice in HNTG buffer (0.1% Triton X-100, 20 mM HEPES, 10% glycerol, 150 mM NaCl) supplemented with Complete protease inhibitor and phosphatase inhibitor cocktails as above. Lysates were subjected to immunoblotting as described previously (Mason et al., 2004). Total ERK1/2, phospho-ERK1/2, phospho-p38, phospho-STAT3, phospho-MEK1/2 and β-actin antibodies were from Cell Signaling Technology (Beverly, MA). Total p38 was from Santa Cruz Biotechnology (Santa Cruz, CA). PKCα (clone M4) and PKCβ antibodies were from Upstate (Lake Placid, NY) and BD Transduction Laboratories (San Diego, CA) respectively. All primary antibodies were detected by incubation with appropriate HRP-linked secondary antibodies from Pierce Biotechnology (Rockford, IL). Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). In some experiments, membranes were stripped by incubation in stripping buffer (2 M Tris, 10% SDS, 14.4 M 2-ME) for 30 minutes at 65°C. In most experiments, blots were then reprobed with β-actin, or in some instances total ERK or p38, to determine loading.

Measurement of IL-12 and cell viability

For real-time PCR, RNA was isolated with TRIzol Reagent (Invitrogen) from 4×10^6 macrophages that were pre-treated for 45 minutes with normal buffer or EGTA/BAPTA-AM as described above, and treated with STAg (50 μg/ml) for 5 hours in normal buffer or Ca^{2+}-free-EGTA medium. RNA was isolated and reverse transcribed to cDNA, and 100 ng was used in real-time PCR using pre-mixed Taqman probe and primer sets for IL-12p40, IL-12p35 and 18S (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Real-time PCR was performed using the Applied Biosystems 7500 Real-time PCR system and data were analyzed using the manufacturer's software. Each amplification was performed in triplicate, data were normalized to the endogenous 18S gene, and the relative change in mRNA levels was determined using the ΔΔCt method (reviewed by Livak and Schmittgen, 2001). In parallel, live macrophages treated in each condition were assessed for viability at the 5-hour time point with the Alamar Blue reduction assay (Biosource International, Camarillo, CA) according to manufacturer's instructions. IL-12p40 levels in culture supernatants were measured by ELISA using mAb C15.6 and biotinylated C17.8 (eBioscience, San Diego, CA) as previously described (Caamano et al., 1999).

Intracellular Ca^{2+} imaging

Macrophages (1×10^6) plated on sterilized 22 mm coverslips (Fisher Scientific, Pittsburgh, PA) and primed overnight with rm-IFN-γ were loaded with the Ca^{2+}-sensitive indicator Fura-2 AM (5 μM; Molecular probes) in macrophage media containing 0.2% Pluronic-F127 (Sigma-Aldrich) for 60 minutes at 37°C. Following loading, coverslips were transferred to a microscope recording chamber and perfused with normal buffer to remove extracellular Fura-2. Discrete bandwidth excitation light (340±10 nm, 380±10 nm) from a xenon source coupled to a computer-controlled monochromator (TILL, Applied Scientific Imaging, Eugene, OR) was delivered to the epifluorescence attachment of the Nikon microscope through a quartz fiber optic guide. Excitation light was directed through the fluorescence objective (40×, Nikon, USA) via a dichroic mirror. The emitted fluorescence from Fura-2-loaded cells was passed through a 470 nm long-pass filter and images were obtained with an intensified charge coupled video camera (Hammamatsu Model C2400-68, Japan) connected to the side port of the inverted microscope. Four fluorescent video images were averaged and digitized using Image Pro Plus 5.1 software (Media Cybergenics, Silver Spring, MD). Within cursor-defined areas of interest, paired 340/380 images were background subtracted and the ratio was calculated. Images were captured at 5 frames per minute for the durations indicated and data is expressed as the 340/380 ratio. In all experiments, tachyzoites (5:1), STAg (50 μg/ml), or LPS (100 ng/ml) were added in normal buffer after 3 minutes of baseline recording. Some experiments were ended by addition of 1 μM thapsigargin in normal buffer to demonstrate responsiveness.

Statistics

IL-12 ELISA data were analyzed by unpaired two-tailed Student's *t*-tests, and a P value of $\leqslant0.05$ was considered significant. For analysis of PKC translocation experiments, densitometry values were converted to fold change over media controls and analyzed by Wilcoxon Signed Rank Test where $P\leqslant0.05$ was significant. All analysis was with GraphPad Prism software (GraphPad Software, San Diego, CA).

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