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ATP Stimulates Human Macrophages to Kill Intracellular Virulent *Mycobacterium tuberculosis* Via Calcium-Dependent Phagosome-Lysosome Fusion

David J. Kusner‡± and James A. Barton*†

Advances in therapy for tuberculosis will require greater understanding of the molecular mechanisms of pathogenesis and the human immune response in this disease. Exposure of *Mycobacterium tuberculosis*-infected human macrophages to extracellular ATP (ATP<sub>e</sub>) results in bacterial killing, but the molecular mechanisms remain incompletely characterized. In this study, we demonstrate that ATP<sub>e</sub>-induced bactericidal activity toward virulent *M. tuberculosis* requires an increase in cytosolic Ca<sup>2+</sup> in infected macrophages. Based on our previous work with primary infection of human macrophages, we hypothesized that the Ca<sup>2+</sup> dependence of ATP-induced killing of intracellular *M. tuberculosis* was linked to promotion of phagosome-lysosome fusion. Using confocal laser-scanning microscopy, we demonstrate that ATP<sub>e</sub> induces fusion of the *M. tuberculosis*-containing phagosome with lysosomes, defined by accumulation of three lysosomal proteins and an acidophilic dye. Stimulation of phagosome-lysosome fusion by ATP<sub>e</sub> exhibited distinct requirements for both Ca<sup>2+</sup> and phospholipase D and was highly correlated with killing of intracellular bacilli. Thus, key signal transduction pathways are conserved between two distinct models of human macrophage antituberculous activity: primary infection of naive macrophages and physiologic stimulation of macrophages stably infected with *M. tuberculosis*. *The Journal of Immunology*, 2001, 167:3308–3315.

* tuberculosis is one of the world’s greatest health problems, and its global burden of morbidity and mortality is escalating due to increasing antibiotic resistance and coinfection with HIV (1–3). Despite its importance and widespread distribution, many aspects of human immunity to *Mycobacterium tuberculosis* remain unknown (4, 5). Interactions with macrophages (Mφ) are central to all stages of *tuberculosis* (4, 5), but the molecular mechanisms of antituberculous activity by human Mφ are incompletely understood. We have used an in vitro model in which extracellular ATP (ATP<sub>e</sub>) induces the killing of virulent *M. tuberculosis* within human Mφ to characterize the biochemical pathways that regulate mycobactericidal activity (6). This approach is based on the demonstration by Kaplan and colleagues (7) that ATP<sub>e</sub> stimulates Mφ killing of the attenuated vaccine strain *M. bovis* bacille Calmette-Guérin (BCG) and on studies by Lammas et al. (8) indicating the involvement of P<sub>2</sub>X<sub>7</sub> cell surface receptors for ATP in bactericidal activity toward BCG. We have recently demonstrated that ATP<sub>e</sub> stimulates killing of virulent *M. tuberculosis* in human Mφ and that P<sub>2</sub>X<sub>7</sub>-dependent activation of host phospholipase D (PLD) is tightly coupled to this response (6).

In this study, we present further characterization of the biochemical mechanisms that regulate ATP<sub>e</sub>-induced antimycobacterial activity. We hypothesized that increases in Mφ cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) would be required based on 1) our recent demonstration that pharmacologic elevation of [Ca<sup>2+</sup>]<sub>i</sub> inhibits the intracellular viability of *M. tuberculosis* during initial infection of human Mφ (9); 2) evidence that [Ca<sup>2+</sup>]<sub>i</sub> is a key regulator of PLD activity in phagocytic leukocytes (10–12); and 3) the fact that fission of Mφ P<sub>2</sub>X<sub>7</sub> receptors produces a significant rise in [Ca<sup>2+</sup>]<sub>i</sub> due to influx from the extracellular space via both the rapid opening of a cation-selective channel as well as the subsequent production of a large nonspecific membrane pore permeable to molecules ≤900 Da (13–16). Although Lammas et al. (8) reported that Ca<sup>2+</sup> is not required for ATP<sub>e</sub>-induced killing of intracellular BCG, we reasoned that bactericidal activity toward virulent *M. tuberculosis* might exhibit distinct biochemical requirements.

Materials and Methods

**Materials and bacteria**

Unless noted, all materials were from previously published sources (6, 9). The H37Rv and Erdman strains of *M. tuberculosis* were obtained from the American Tissue Type Culture Collection (Manassas, VA). The CDC1551 *M. tuberculosis* strain was generously provided by Dr. T. Shinnick (Centers for Disease Control and Prevention, Atlanta, GA). Virulent strains of *M. tuberculosis* (H37Rv, Erdman, and CDC1551) were cultured and prepared for use in experiments as noted previously (17). Briefly, aliquots of frozen *M. tuberculosis* stocks in 7H9 broth were thawed, cultured for 9 days on 7H11 agar at 37°C in 5% CO<sub>2</sub>-95% air, scraped from agar plates, and suspended in RPMI 1640 by vortexing briefly. After settling, the supernatant was transferred to a new tube and allowed to settle once again. An aliquot of this final *M. tuberculosis* suspension was counted in a Petroff-Hauser chamber, and the concentration of bacteria was adjusted for use in experiments. *M. tuberculosis* preparations contained ≥90% single bacteria, with ≥80% viability by determination of CFUs (17). The effects of various experimental manipulations on the viability of *M. tuberculosis* were determined by analysis of CFUs.

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3 Abbreviations used in this paper: Mφ, macrophage; ATP<sub>e</sub>, extracellular ATP; BCG, bacille Calmette-Guérin; PLD, phospholipase D; [Ca<sup>2+</sup>]<sub>i</sub>, cytosolic Ca<sup>2+</sup> concentration; MOI, multiplicity of infection; MAPTAM, Bis-(2-amino-S-methylphenoxy)ethane-N,N,N’,N’-tetraacetic acid tetracetoxymethyl ester; LAMP, lysosome-associated membrane protein; P-L, phagosome-lysosome; 2,3-DPG, 2,3-diphosphoglycerate; PEt, phosphatidylethanol; furA 2, furA 2-acetyl methyl ester.

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Preparation of Mb

PBMC were isolated from healthy, purified protein derivative-negative adult volunteers and were cultured in Teflon wells for 5 days in RPMI 1640 (pH 7.4) with 20% fresh autologous serum as previously described (17). Mb (~2 × 10^6/sample) were purified by adherence to six-well plastic tissue-culture plates or chromic acid-cleaned glass coverslips (25 mm in diameter) for 24 h at 37°C. Monolayers were washed repeatedly and then incubated in RPMI 1640 with 2.5% autologous serum, without antibiotics, for use in experiments. Effects of experimental manipulations on Mb viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with napthol blue-black stain (17, 18).

Infection of Mb with M. tuberculosis and analysis of intracellular survival

Monocyte-derived Mb in RPMI 1640, 20 mM HEPES, and 2.5% human serum were infected with M. tuberculosis at a bacteria/Mb ratio (multiplicity of infection, MOI) of 1:1 and then incubated for 1 h at 37°C. Following infection, monolayers were washed three times with RPMI 1640 at 37°C and incubated with RPMI 1640 and 10% FBS for 24 h, before addition of ATP or buffer control. The MOI of 1:1 was used because it permits long-term cultivation of infected human Mb and the determination of intracellular viability of M. tuberculosis at ~24 h following infection. We have previously used higher levels of MOI, including 10:1, 30:1, and 100:1, but the resultant rapid lysis of the Mb monolayer precludes accurate determination of the bacterium’s intracellular survival (9). Mb cultures were incubated at 37°C for an additional 24 h after the addition of ATP or buffer, followed by quantitation of the growth of M. tuberculosis by determination of CFUs or by the Bactec method, as previously described (6).

Before Mb exhibit a wide variance in the number of M. tuberculosis that grow intracellularly, the results of the CFU assay are generally presented as cumulative data (mean ± range) expressed as a percentage of the paired control samples. The absolute number of M. tuberculosis CFUs in the control samples has been designated in the figure legends. Determinations of mycobacterial viability by the Bectec method were in excellent agreement with the results of the CFU assay (6, 19).

Calcium measurements were performed as previously described (9). Briefly, Mb were adhered to collagen-coated glass coverslips and incubated in 10 µM fura 2-aceethylmethyl ester (fura 2) in HBSS for 30 min at 37°C. Levels of [Ca^{2+}]i in single Mb, or the mean [Ca^{2+}]i, of groups of 10–20 cells, was determined using a Photoscan II spectrofluorometer (Photon Technology International, Lawrenceville, NJ). [Ca^{2+}]i was determined from the ratio of fluorescence emission intensities at 510 nm following excitation at 340 and 380 nm, respectively, as previously described (20). To analyze the contribution of [Ca^{2+}]i-mediated signaling pathways to ATP-dependent mycobactericidal activity, both extracellular and intracellular [Ca^{2+}]i were experimentally modified. Extracellular [Ca^{2+}]i was depleted by washing Mb twice in Ca^{2+}-free buffer containing 5 mM EGTA, followed by resuspension in the same buffer and incubation for 5 min at 37°C before addition of ATP. Inhibition of changes in [Ca^{2+}]i, were also produced by preincubation of Mb with the intracellular Ca^{2+} chelator, bis-(2-amino-S-methylphenoxy)ethane-N,N,N’,N’-tetraacetic acid tetraacetoxymethyl ester (MAPTAM) (25 µM), in EGTA-containing buffer for 20 min at 37°C. The effects of EGTA- and MAPTAM-induced alterations in [Ca^{2+}]i were verified in parallel experiments by direct determination of [Ca^{2+}]i in control and treated Mb via fluorescence of fura 2 (20). Levels of [Ca^{2+}]i were <20 nM in MAPTAM-treated cells following addition of ATP or control Ca^{2+}-mobilizing agonists, platelet-activating factor (100 nM) or complement-opsinized zymosan, demonstrating the efficacy of MAPTAM-induced [Ca^{2+}]i, buffering (9, 21). The effects of these modulators of extracellular and intracellular [Ca^{2+}]i on Mb viability and monolayer density were determined in parallel experiments, and no significant differences from control cells were observed. EGTA and MAPTAM did not alter the viability of M. tuberculosis, either when incubated directly with mycobacteria in TH9 medium for 24 h at 37°C or when added to infected Mb in the absence of ATP.

Confluent microscopy

The degree of maturation of phagosomes containing live or killed M. tuberculosis was assessed by colocalization of the bacilli with the acidophilic dye LysoTracker Red (Molecular Probes, Eugene, OR) and the lysosomal protein markers LAMP-1, cathepsin D, or CD63, and lysosome-associated membrane protein (LAMP)-1, as described previously (9, 22). LysoTracker Red was incubated at a 1/10,000 dilution with Mb monolayers in RPMI 1640, 20 mM HEPES, and 2.5% autologous serum for 2 h at 37°C. Unincorporated dye was removed by washing, followed by infection with M. tuberculosis. After removal of nonadherent bacilli, LysoTracker Red was added to each well at the same concentration used for initial labeling. At 30 min postfection, Mb were fixed in 3.75% paraformaldehyde for 15 min and permeabilized with ice-cold methanol-acetone (1:1). Detection of the lysosomal protein markers LAMP-1, cathepsin D, or CD63 was accomplished by incubating coverslips with blocking buffer (PBS, 5% BSA, and 10% goat serum) for 1 h, followed by the appropriate 1st Abs (diluted in blocking buffer) for 1 h, repeated washings, and incubation with Texas Red-conjugated secondary anti-IgG Ab for 1 h, all at 25°C. The localization of M. tuberculosis was determined by incubating monolayers with auramine for 20 min at 25°C, followed by a 3-min incubation in 0.5% acid alcohol. Following repeated washings, coverings were mounted with buffered glycerol solution and nail polish.

Confocal microscopy was performed on a Zeiss Laser Scan inverted 510 microscope (Zeiss, Oberkochen, Germany). An argon-krypton laser (excitation, 488 nm; emission band pass, 505–530 nm) was used for detection of auramine fluorescence, and a helium-neon laser (excitation, 543 nm; emission limit of pass, 585 nm) was used for detection of Texas Red and LysoTracker Red. The percentage of M. tuberculosis phagosomes colocalizing with the marker of interest was determined by counting 25 phagosomes from each sample. The effects of modulation of Mb [Ca^{2+}]i on the response to ATP, was performed by preincubation with EGTA or MAPTAM, respectively. Neither EGTA nor MAPTAM directly affected the fluorescence of auramine, LysoTracker Red, or Texas Red (9). At a MOI of 1:1, <10% of Mb contained two or more bacteria. Therefore, confocal images of Mb containing single bacilli are shown as most representative.

For quantitative analysis of phagosomal maturation, the range of fluorescence intensity of the Texas Red 2° Ab or LysoTracker Red was recorded along the major axis of the bacillus (defined by auramine staining). Samples in which the mean fluorescent intensity of the given lysosomal marker was greater than the mean of control, uninfected Mb were scored as positive. For a given phagosome to be scored as a mature phagolysosome, each of the four lysosomal markers (LAMP-1, cathepsin D, CD63, and LysoTracker Red) must have all been positive. Because the confocal measurements were acquired in a blinded fashion, inherent limitations of the technique (e.g., the lack of a single, fully specific marker of Mb lysosomes) should be distributed equally among the experimental groups. The use of this technique by our laboratory (9, 22) and others (23–25) has resulted in strong interobserver correlations, as well as excellent agreement with the results of immunoelectron microscopy (25–27).

Data analysis

Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of six or more data sets were analyzed for significance using Student’s t test. Nonparametric evaluation of other data sets was performed with the Mann-Whitney rank sum test. Analysis of correlation was performed with the Spearman rank order test (28).

Results

ATP-induced killing of virulent M. tuberculosis within human Mb requires increases in [Ca^{2+}]i

To characterize the biochemical determinants of ATP-stimulated killing of intracellular M. tuberculosis, we used an in vitro model system in which blood monocytes are differentiated to a Mb phenotype by cultivation for 5 days, followed by purification via adherence to tissue culture plates (6, 17, 22). Mb were infected with the virulent H37Rv strain of M. tuberculosis, at a MOI of 1:1 for 24 h, followed by incubation with 3 mM ATP. Treatment with ATP reduced the viability of intracellular M. tuberculosis by 83 ± 7% at 24 h and 3.62 ± 0.41 logs at day 7 compared with control samples treated with buffer alone (p < 0.001 for each time point, n = 9) (6).

To begin to evaluate the Ca^{2+}-dependence of this ATP-induced bactericidal activity, we determined the effect of ATP on levels of [Ca^{2+}]i in M. tuberculosis-infected Mb labeled with the fluorescent Ca^{2+} indicator fura 2. ATP stimulated a rapid and sustained increase in [Ca^{2+}]i in infected Mb that was indistinguishable from the [Ca^{2+}]i response of uninfected cells (Fig. 1A) (29–31). This ATP-induced Ca^{2+} response was due to stimulation of two classes of Mb cell surface purinergic receptors, P2Y1, G protein-coupled...
The inhibitory effect of Ca\(^{2+}\) chelation on ATP-induced killing of *M. tuberculosis* was reversed by increasing the concentration of extracellular Ca\(^{2+}\) to exceed that buffered by EGTA and MAPTAM (data not shown). Reversal of microbicidal activity by excess CaCl\(_2\) supports the specificity of the Ca\(^{2+}\) chelators and strengthens the conclusion that Ca\(^{2+}\) is required for ATP-induced mycobacterial killing. Finally, the killing of two other well-characterized, virulent strains of *M. tuberculosis*, Erdman (26, 27) and CDC1551 (32), by ATP\(_e\) exhibited a similar Ca\(^{2+}\) dependence (Fig. 2B, *p* < 0.001 for each strain and *n* = 5). Taken together, these data demonstrate that ATP\(_e\)-induced killing of virulent *M. tuberculosis* within human Møs requires an increase in [Ca\(^{2+}\)]. Because specific stimulation of Mø P\(_2\)X\(_2\) receptors via UTP does not result in killing of intracellular *M. tuberculosis* (6), these data are consistent with a requirement for P\(_2\)X\(_2\)-mediated influx of extracellular Ca\(^{2+}\) for tuberculocidal activity.

**ATP induces Ce\(^{2+}\)-dependent maturation of *M. tuberculosis* phagosomes**

The specific antimicrobial mechanism(s) by which ATP\(_e\) induces intracellular mycobacterial killing are unknown (6, 8). Because Ca\(^{2+}\) regulates several phagocyte antimicrobial responses including generation of reactive oxygen species (33) and reactive nitrogen intermediates (34, 35), granule secretion (36), synthesis of cytokines (37), and, in certain cases, phagosome-lysosome (P-L) fusion (9), the Ca\(^{2+}\)-dependence of ATP\(_e\)-induced killing of *M. tuberculosis* may be due to multiple effects. We tested the hypothesis that increases in [Ca\(^{2+}\)] promote ATP\(_e\)-induced killing of *M. tuberculosis* via enhancement of P-L fusion based on the following...
The extent of maturation of *M. tuberculosis*-containing phagosomes was quantitated by laser scanning confocal microscopy using three protein markers of lysosomes/late endosomes (CD63, cathepsin D, and LAMP-1) and the acidophilic dye LysoTracker Red (9). Although there is no single, unambiguous marker for lysosomes, the relative accumulation of several distinct proteins (both soluble and membrane-associated) and pH-sensitive dyes provides accurate determination of the identity of vesicular compartments (22, 25–27, 29, 38). As previously demonstrated, phagosomes containing live H37Rv *M. tuberculosis* exhibited low levels of the lysosomal markers, consistent with an immature matutational state, i.e., inhibition of P-L fusion (Fig. 3). Incubation with ATP for 30 min resulted in marked increases in each of the lysosomal markers, consistent with promotion of P-L fusion. Inhibition of ATP<sub>p</sub>-induced increases in [Ca<sup>2+</sup>]<sub>c</sub>, with MAPTAM/EGTA resulted in a complete reversal of ATP-stimulated phagosomal maturation (Fig. 3). In fact, the level of lysosomal markers on *M. tuberculosis* phagosomes in MΦ incubated with MAPTAM/EGTA followed by ATP was lower than that present in control MΦ. These data support the novel hypotheses that ATP<sub>p</sub>-induced antituberculous activity involves the promotion of P-L fusion, and the obligatory role of Ca<sup>2+</sup> in ATP<sub>p</sub>-stimulated mycobacterial killing is due, at least in part, to the Ca<sup>2+</sup><sub>c</sub>-dependence of P-L fusion.

A quantitative assessment of the strength of the associations between three variables, 1) ATP<sub>p</sub>-induced elevation of [Ca<sup>2+</sup>]<sub>c</sub>, 2) P-L fusion, and 3) reductions in intracellular viability of *M. tuberculosis*, was made via an analysis of correlation using the Spearman rank order correlation test. The strength of the proposed association was determined via an analysis of correlation rather than regression, because the former makes no assumptions about the directionality between variables and is therefore more rigorous in complex, multivariate situations in which unambiguous designations of “independent” and “dependent” variables cannot be assigned (28). The level of [Ca<sup>2+</sup>]<sub>c</sub> in ATP-stimulated MΦ was highly correlated with P-L fusion (defined as presence of all four lysosomal markers; $r = 0.916$, $p < 0.001$, and $n = 9$). Similarly, the degree of P-L fusion correlated highly with reductions in *M. tuberculosis* viability ($r = 0.926$ and $p < 0.001$). Finally, $ATP_{p}$-induced levels of [Ca<sup>2+</sup>]<sub>c</sub> correlated highly with reductions in mycobacterial viability ($r = 0.942$ and $p < 0.001$). Although these high degrees of correlation between each of the variables do not prove a causal connection, they strongly support our hypothesis.

### ATP<sub>p</sub>-induced killing of intracellular *M. tuberculosis* exhibits distinct requirements for both Ca<sup>2+</sup> and PLD activity

We have recently demonstrated that ATP<sub>p</sub>-stimulated killing of *M. tuberculosis* within human MΦ is tightly coupled to activation of the PLD signal transduction pathway (6). Therefore, we sought to determine whether the requirements for increases in [Ca<sup>2+</sup>]<sub>c</sub>, and PLD activity were independent functions of distinct signaling pathways or whether they exhibited some degree of interdependence. The importance of this distinction arises from the fact that both Ca<sup>2+</sup><sub>c</sub>-dependent PLD activities as well as PLD-dependent mycobacterial killing have been previously demonstrated (10, 39).

To determine whether the ATP<sub>p</sub>-induced increases in MΦ [Ca<sup>2+</sup>]<sub>c</sub> require stimulation of PLD activity, we used two well-characterized, mechanistically distinct inhibitors of PLD activity, 2,3-diphosphoglycerate (2,3-DPG) and ethanol. Although no specific pharmacologic inhibitor of PLD has been identified, the combined use of these complementary inhibitors has yielded substantial insights into the role of PLD in diverse physiologic functions (6, 17). 2,3-DPG is a competitive inhibitor of PLD that exhibits low toxicity to intact cells (17, 40). Preincubation of MΦ monolayers with 2,3-DPG (0.1–3 mM) for 15 min at 37°C produced...
dose-dependent inhibition of ATP-stimulated PLD activity (6) with a maximal reduction of 91% (range 87–93% reduction, \( p < 0.001 \) and \( n = 5 \)) at 5 mM 2,3-DPG. Despite this marked inhibition of ATP-stimulated PLD activity, 2,3-DPG had no detectable effect on the accompanying increase in \( [\text{Ca}^{2+}]_c \) (Fig. 4A).

Ethanol (and other short-chain primary alcohols) inhibit PLD-mediated generation of phosphatidic acid by substituting for water as the attacking nucleophile, yielding the metabolically inert alternate product, phosphatidylethanol (PEt) (17, 41). Thus, ethanol inhibits PLD-dependent responses without blocking enzyme turnover, providing a complementary method of PLD inhibition that is mechanistically distinct from that of 2,3-DPG. Incubation of M\( \Phi \) with ethanol (0.03–1.0%, v/v) for 2 min at 37°C before addition of ATP resulted in concentration-dependent inhibition of phosphatidic acid generation (6) with a 93% reduction produced by addition of 1.0% ethanol (between 89 and 95% reduction, \( p < 0.001 \) and \( n = 5 \)). Similar to the results with 2,3-DPG, ethanol had no significant effect on ATP\(_e\)-stimulated increases in M\( \Phi \) [Ca\(^{2+}\)]\(_c\) (Fig. 4A). At the concentrations used, 2,3-DPG and ethanol had no effect on M\( \Phi \) viability or monolayer density (data not shown). Taken together, the results with 2,3-DPG and ethanol demonstrate that ATP\(_e\)-induced increases in M\( \Phi \) [Ca\(^{2+}\)]\(_c\) are independent of the accompanying stimulation of PLD activity.

To evaluate whether ATP-stimulated increases in PLD activity were dependent on [Ca\(^{2+}\)]\(_c\), M\( \Phi \) were incubated in various concentrations of extracellular Ca\(^{2+}\) (buffered with EGTA) before addition of 3 mM ATP. PLD activity was determined by accumulation of the PLD-specific product PEt in the presence of 0.5% ethanol. In M\( \Phi \) incubated in 25 \( \mu \)M MAPTAM and 3 mM EGTA for 20 min before addition of ATP, Ca\(^{2+}\) was not required for activation of PLD but, rather, augmented the level of PLD activity stimulated by ATP\(_e\). These data on the relationship between Ca\(^{2+}\) and PLD activity in M. tuberculosis-infected human M\( \Phi \) are in close agreement with previous work by el-Moatassim and Dubyak in uninfected murine M\( \Phi \) (30, 42).

Our previous report (6) and the current data strongly suggest that ATP\(_e\)-induced killing of intracellular M. tuberculosis requires both elevation of [Ca\(^{2+}\)]\(_c\) and activation of PLD. Furthermore,
ATP₆-stimulated bactericidal activity is tightly coupled to [Ca²⁺],-dependent promotion of P-L fusion. Thus, we hypothesize that activation of PLD may also be required for maturation of the M. tuberculosis-containing phagosomes to phagolysosomes. To test this hypothesis, levels of P-L fusion were determined in Mφ in which PLD activity was inhibited by 2,3-DPG, ethanol, or MAPTAM/EGTA. In parallel experiments, the viability of intracellular M. tuberculosis was determined to assess its correlation with the extent of P-L fusion. We have previously reported the dose-dependent inhibition of ATP₆-stimulated PLD activity by 2,3-DPG and ethanol (6). In the current experiments, specific concentrations of these inhibitors were selected that either maximally inhibited ATP₆-induced PLD activity (5 mM 2,3-DPG, 1% ethanol) or inhibited PLD activity to the same extent as 25 μM MAPTAM/3 mM EGTA (2 mM 2,3-DPG, 0.5% ethanol), i.e., ~40% of the maximum (Fig. 4B).

Inhibition of ATP₆-stimulated PLD activity (Fig. 5A) was accompanied by concordant reductions in P-L fusion (Fig. 5B) and inversely proportional increases in M. tuberculosis viability (Fig. 5C). These data support the hypothesis that ATP₆-induced stimulation of PLD activity is coupled to promotion of P-L fusion and reductions in intracellular viability of M. tuberculosis. Of note, incomplete inhibition of PLD activity by 2 mM 2,3-DPG or 0.5% ethanol (to the level of PLD activity present in MAPTAM-treated Mφ) resulted in partial inhibition of P-L fusion (Fig. 5B) and partial restoration of mycobacterial viability (Fig. 5C). These results contrast with the complete inhibition of ATP₆-stimulated P-L fusion and mycobacterial killing in MAPTAM-treated Mφ, in which [Ca²⁺]ₕ was completely inhibited. Taken together, these data support the hypothesis that increases in PLD activity and [Ca²⁺]ₕ make distinct and experimentally separable contributions to ATP-induced P-L fusion and mycobacterial killing.

**Discussion**

The dynamic interactions between M. tuberculosis and human Mφ are central to each of the complex stages of tuberculosis, from initial infection through the development of active disease. A crucial feature of pathogenesis is the ability of tubercle bacilli to...
induced increases in [Ca$^{2+}$]/H9278 host PLD activity (6, 8). However, the M$\delta$ stimulated PLD activity). Little information is available concerning the intracellular compartment in which the bacilli reside as the duration of infection increases. Knowledge of the biochemical composition and regulatory determinants of these intracellular havens is essential to the development of rational, targeted interventions to modify the survival of M. tuberculosis within M$\delta$. Therapeutic augmentation of host immunity could contribute both to the treatment of patients with active disease, as well to the prevention of tuberculosis in the third of the world’s population (~2 billion persons) latently infected with M. tuberculosis (1, 2).

We have recently demonstrated that ATP$\gamma$ stimulates human M$\delta$ to kill intracellular virulent M. tuberculosis (6). ATP$\gamma$-induced restriction of mycobacterial viability requires stimulation of M$\delta$ cell surface P$\gamma$X$\gamma$ receptors and is tightly coupled to stimulation of host PLD activity (6, 8). However, the M$\delta$ bactericidal mechanism(s) that directly kills intracellular M. tuberculosis is unknown, as is its relation to occupancy of P$\gamma$X$\gamma$ receptors and activation of PLD. In this paper, we present several novel features of ATP$\gamma$-induced killing of virulent M. tuberculosis in human M$\delta$. First, the mycobactericidal effect of ATP$\gamma$ required elevation of [Ca$^{2+}$]$_i$ based on the significant inhibition by the extracellular Ca$^{2+}$ chelator, EGTA, and complete inhibition by the intracellular Ca$^{2+}$ buffer, MAPTAM. Second, incubation of infected M$\delta$ with ATP resulted in the maturation of M. tuberculosis phagosomes to phagolysosomes, reversing the characteristic inhibition of P-L fusion that is a hallmark of tuberculous pathogenesis. Third, ATP$\gamma$-induced increases in [Ca$^{2+}$]$_i$ and PLD activity exert distinct and complementary roles in the induction of P-L fusion and killing of intracellular M. tuberculosis. Specifically, ATP$\gamma$-stimulated increases in [Ca$^{2+}$]$_i$ are independent of PLD, and Ca$^{2+}$ is not required for ATP$\gamma$-induced activation of PLD (although micromolar concentrations of Ca$^{2+}$ maximally augment the level of ATP$\gamma$-stimulated PLD activity).

The requirement for increases in [Ca$^{2+}$]$_i$ and PLD activity for both P-L fusion and restriction of the intracellular viability of M. tuberculosis in ATP$\gamma$-stimulated M$\delta$ supports the hypothesis that phagosomal maturation directly contributes to mycobactericidal activity. These data are also consistent with the corollary hypothesis that the requirements for Ca$^{2+}$ and PLD activity in ATP$\gamma$-induced antymycobacterial activity are due to the Ca$^{2+}$ and PLD dependence of P-L fusion. Further testing of these hypotheses is currently hampered by our limited understanding of the molecular mechanisms of tuberculous pathogenesis. For example, lack of information regarding the biochemical signals that regulate the maturation of M. tuberculosis phagosomes hinders the identification of compounds that might inhibit P-L fusion in a Ca$^{2+}$- and PLD-independent manner. If available, such inhibitory compounds could be used to directly test whether P-L fusion is required for ATP$\gamma$-induced reductions in mycobacterial viability. Similarly, because the specific mechanism(s) by which P-L fusion leads to killing of M. tuberculosis in human M$\delta$ is unknown, it is not yet possible to evaluate the Ca$^{2+}$ and PLD dependence of specific mycobactericidal reactions.

Our previous work (9, 22) and the results presented herein demonstrate that [Ca$^{2+}$]$_i$ regulates the intracellular viability of M. tuberculosis in two different experimental models designed to represent distinct stages in the host-pathogen interaction. In the first case, primary infection of naive human M$\delta$, the intracellular survival of M. tuberculosis requires inhibition of M$\delta$ Ca$^{2+}$ signaling, which closely correlates with inhibition of P-L fusion (9, 22). Transient (20 min) pharmacologic elevation of [Ca$^{2+}$]$_i$ at the time of infection results in P-L fusion and decreased viability of intracellular bacilli. In the second case (this paper), in M$\delta$ stably infected with M. tuberculosis for 24 h (Figs. 3 and 5) to 7 days (data not shown), elevation of [Ca$^{2+}$]$_i$ is required for ATP$\gamma$-induced P-L fusion and reduction in the intracellular survival of tubercle bacilli.

Characterization of the Ca$^{2+}$ dependence of mycobacteria-M$\delta$ interactions in these two distinct experimental models will require more detailed kinetic analysis and further identification of the specific target proteins (22) that directly mediate killing of intracellular M. tuberculosis during both primary and established infection of human M$\delta$. We have recently demonstrated that Ca$^{2+}$-dependent killing of M. tuberculosis during the initial infection of M$\delta$ requires specific activation of calmodulin and calmodulin-dependent protein kinase II on the phagosomal membrane (22). Studies to determine whether this signal transduction pathway functions in ATP$\gamma$-induced killing of M. tuberculosis following stable infection of human M$\delta$ are in progress. The current data, demonstrating that mycobacterial phagosomes exhibit Ca$^{2+}$-regulated maturation as late as 7 days following infection, has novel and important implications from the perspectives of both pathogenesis and therapeutics. Regarding the former, these findings establish Ca$^{2+}$ as the first known molecular regulator of phagosomal physiology at this late time point. Furthermore, the data suggest a certain degree of stability of the phagosomal phenotype over, at least, the first week of infection. Regarding the later point, this study furthers our understanding of the molecular mechanisms that regulate ATP-dependent killing and supports the general therapeutic model that physiologic host immune defenses can be mobilized to treat tuberculosis. Definition of the mechanisms by which physiologic compounds such as ATP (6–8), cytokines (23), and T cell components (44, 45) exert mycobactericidal activity may provide important therapeutic advances, which are particularly critical due to increasing antimicrobial resistance in M. tuberculosis.

Because M. tuberculosis is a virulent pathogen specific to humans, it is difficult to estimate the degree to which results with murine M$\delta$ or attenuated strains (e.g., BCG) correlate with human immunity in tuberculosis. For example, the ability of ATP$\gamma$, to stimulate killing of M. tuberculosis in murine M$\delta$ that lack P$\gamma$X$\gamma$ receptors may indicate the presence of additional bactericidal mechanisms in normal mice. Alternatively, it may be due to the acquisition of compensatory mechanisms in mice that develop in the absence of the P$\gamma$X$\gamma$ receptor, that normally regulates several critical M$\delta$ functions, including transcription, secretion, giant cell formation, and apoptosis (8, 14–16, 25). A second example of a result obtained from a different experimental system that conflicts with our data is the demonstration that ATP$\gamma$-induced killing of the attenuated BCG strain is Ca$^{2+}$ independent (8). This difference in the requirement for Ca$^{2+}$ between our studies may be due to the operation of distinct regulatory mechanisms involved in M$\delta$ that encounter a virulent pathogen (M. tuberculosis, this study) vs an attenuated vaccine strain (8). Additionally, differences in experimental methods may have contributed to the conflicting results, because intracellular Ca$^{2+}$ chelators, which can completely inhibit agonist-dependent increases in [Ca$^{2+}$]$_i$, were not used in the BCG study (8). Molecular definition of the Ca$^{2+}$-dependent steps in the killing of intracellular M. tuberculosis will be required to clarify these points and to establish the specific similarities and differences in the interactions of human M$\delta$ with M. tuberculosis vs BCG.

Note added in proof. During revision of this manuscript, Lammas and colleagues (8) reported, in contrast to their earlier findings,
that ATP-induced killing of BCG is Ca\(^{2+}\) dependent and is associated with phagosomal acidification (46).

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