IAI Journals.ASM.org

Coxiella burnetii Alters Cyclic AMP-Dependent Protein Kinase Signaling during Growth in Macrophages

Laura J. MacDonald,[a] Richard C. Kurten,[b,c] and Daniel E. Voth[a]

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA[a]; Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA[b]; and Arkansas Children's Hospital Research Institute, Little Rock, Arkansas, USA[c]

***Coxiella burnetii* is the bacterial agent of human Q fever, an acute, flu-like illness that can present as chronic endocarditis in immunocompromised individuals. Following aerosol-mediated transmission, *C. burnetii* replicates in alveolar macrophages in a unique phagolysosome-like parasitophorous vacuole (PV) required for survival. The mechanisms of *C. burnetii* intracellular survival are poorly defined and a recent Q fever outbreak in the Netherlands emphasizes the need for better understanding this unique host-pathogen interaction. We recently demonstrated that inhibition of host cyclic AMP-dependent protein kinase (PKA) activity negatively impacts PV formation. In the current study, we confirmed PKA involvement in PV biogenesis and probed the role of PKA signaling during *C. burnetii* infection of macrophages. Using PKA-specific inhibitors, we found the kinase was needed for biogenesis of prototypical PV and *C. burnetii* replication. PKA and downstream targets were differentially phosphorylated throughout infection, suggesting prolonged regulation of the pathway. Importantly, the pathogen actively triggered PKA activation, which was also required for PV formation by virulent *C. burnetii* isolates during infection of primary human alveolar macrophages. A subset of PKA-specific substrates were differentially phosphorylated during *C. burnetii* infection, suggesting the pathogen uses PKA signaling to control distinct host cell responses. Collectively, the current results suggest a versatile role for PKA in *C. burnetii* infection and indicate virulent organisms usurp host kinase cascades for efficient intracellular growth.**

Coxiella burnetii is the highly infectious bacterial agent of human Q fever, a zoonotic disease that typically presents as an acute influenza-like illness. By ill-defined mechanisms, the pathogen can also establish a chronic infection resulting in potentially fatal endocarditis. Along with *Bartonella* spp., *C. burnetii* is a leading cause of noncultivatable infectious endocarditis, a condition that is notoriously difficult to treat with current antibiotics (36). *C. burnetii* is naturally spread by contaminated aerosols, and livestock workers are often exposed to the organism while working with infected animals, particularly during parturition. This exposure risk was recently highlighted by a major Q fever outbreak in the Netherlands that resulted in over 4,000 cases and 11 deaths (28). However, due to a reliance on growth inside eukaryotic cells, *C. burnetii* has historically been difficult to study and pathogenic determinants are not well understood.

In vivo, *C. burnetii* targets alveolar phagocytic cells, with macrophages serving as the pathogen's primary growth niche. Following uptake into a host cell, the pathogen is housed for 4 to 6 h in a tight-fitting phagosome that decorates with early endosomal markers including Rab5 and early endosome antigen 1 (7, 24). The early vacuole also interacts with autophagosomes (20, 37) and fluid-phase endosomes before ultimately fusing with lysosomes (43). Although antibacterial acid hydrolases are present in the vacuole, lysosomal fusion triggers acid pH-dependent activation of *C. burnetii* metabolism (21, 32), and the organism replicates in this harsh compartment for many days (2, 14). A prolonged infectious cycle necessitates regulation of host cell survival to ensure a viable replication niche. *C. burnetii* potently inhibits extrinsic and intrinsic apoptosis by triggering a prosurvival transcriptional response and preventing mitochondrial release of cytochrome *c* (30, 45). The pathogen also directs prolonged activation of the prosurvival kinases Akt and Erk1/2 to promote cell survival (44). These events are likely controlled by the organism's Dot/Icm type IV secretion system that translocates effector proteins into the host cytosol, where they regulate intracellular replication and inhibition of apoptosis (4, 10, 29). However, host signaling pathways that regulate parasitophorous vacuole (PV) formation and maintenance have not been defined.

Previous studies uncovered the presence of host vacuolar fusogenic proteins, such as Rab GTPases, on the PV membrane but did not assess the role of kinase-dependent signaling cascades in vacuole biogenesis (7, 9, 37). We recently performed a directed inhibitor screen to identify eukaryotic signaling proteins required for PV generation (25). This study uncovered a role for many kinases in PV formation and suggests signaling cascades may be intimately linked to biogenesis of pathogen replication vacuoles. Eleven kinases were involved in PV formation, including protein kinase C (PKC), myosin light chain kinase, calmodulin-dependent kinase II, and cyclic AMP (cAMP)-dependent protein kinase (PKA). PKA is a versatile host protein that directs many host responses, including cell survival, cytokine production, and cytoskeletal organization (34). PKA target proteins are differentially phosphorylated during *C. burnetii* infection, and the effects of PKA inhibition are reversible (25), indicating the pathogen continually modulates this pathway during intracellular growth.

In the present study, we further probed the importance of PKA

Received 30 January 2012 **Returned for modification** 1 March 2012
Accepted 13 March 2012
Published ahead of print 2 April 2012
Editor: F. C. Fang
Address correspondence to Daniel E. Voth, dvoth@uams.edu.

doi:10.1128/IAI.00101-12

for *C. burnetii* infection and replication. Using PKA-specific pharmacologic inhibitors, we show that PKA activity is critical for proper PV formation and robust bacterial replication. PKA phosphorylation levels indicative of activation increase substantially in *C. burnetii*-infected cells, and a subset of distinct downstream targets are differentially phosphorylated during intracellular growth. Importantly, PKA activity is required for virulent *C. burnetii* infection of primary human alveolar macrophages, which represent the pathogen's *in vivo* target cell. Collectively, these results implicate the PKA signaling cascade as a major determinant of *C. burnetii*-host cell interactions.

MATERIALS AND METHODS

***C. burnetii* and mammalian cell culture.** *C. burnetii* phase II (RSA439) organisms (NMII) were purified from infected Vero cells (American Type Culture Collection [ATCC], Manassas, VA) as previously described (14). *C. burnetii* Nine Mile phase I (RSA493) and G (Q212) isolates were cultured in acidified citrate cysteine medium (33) for 7 days at 37°C in 5% CO_2 and 2.5% O_2 and then collected by centrifugation and washed with sucrose phosphate buffer prior to use. Human monocytic THP-1 cells (ATCC) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in 5% CO_2. THP-1 cells were cultured in 24- or 6-well tissue culture plates for infections. Cells were incubated with 200 nM phorbol 12-myristate 13-acetate (PMA; EMD Biosciences, San Diego, CA) overnight to stimulate differentiation into adherent, macrophage-like cells (45). Before infection, medium containing PMA was replaced with PMA-free medium. THP-1 cells were infected with *C. burnetii* in phase I or phase II at a multiplicity of infection (MOI) of 100 or 10, respectively, by addition of organisms to the culture medium. *C. burnetii* in phase II was used at a lower MOI due to increased infectivity of these organisms compared to phase I bacteria (43). All work with virulent *C. burnetii* was performed in the Centers for Disease Control and Prevention-approved biosafety level-3 facility at the University of Arkansas for Medical Sciences. Where indicated, chloramphenicol (10 μg/ml) was added to cell cultures to inhibit bacterial protein synthesis.

Primary human alveolar macrophages were isolated by bronchioalveolar lavage from lung tissue obtained postmortem from the National Disease Research Interchange. Lavage fluid was filtered, subjected to a Ficoll gradient, and cells collected by centrifugation. After processing, cells were placed in 24-well plates in Dulbecco modified Eagle medium/F-12 medium containing 7.5% FBS, penicillin G (50 U/ml), streptomycin sulfate (50 μg/ml), and gentamicin sulfate (50 μg/ml) (Invitrogen) and incubated for 2 h at 37°C in 5% CO_2. Nonadherent cells were then removed, and fresh medium was added to adherent macrophages. The macrophages were infected as described above for THP-1 cells in the absence of penicillin, streptomycin, and gentamicin. Alveolar macrophages were routinely assessed for homogeneity by immunofluorescence and immunoblot analysis (data not shown) with antibodies directed against the macrophage-specific markers CD11/18b, CD14, and CD68 (Abcam, Cambridge, MA).

PKA inhibitor treatments. THP-1 cells or macrophages were cultured in 24-well plates on 12-mm glass coverslips for microscopy analysis. Two hours before infection, PKA was inactivated by pharmacologic inhibition with H-89 (10 μM; Sigma-Aldrich, St. Louis, MO) or Rp-adenosine-3′,5′-cyclic mono-phosphorothioate triethylamine salt (Rp-cAMPS; 100 to 250 μM; Enzo Life Sciences, Plymouth Meeting, PA). Cells were infected with *C. burnetii* isolates as described above and processed for microscopy at 48 h postinfection (hpi) to assess vacuole formation. The cells were considered to contain typical PVs when a large vacuole (>10 μm in diameter) was present and contained replicating organisms.

FFU assays. THP-1 cells were seeded in 24-well plates and differentiated with PMA overnight. Medium was then replaced with medium lacking PMA and cells were pretreated with H-89 (10 μM) or Rp-cAMPS (100 to 250 μM) for 2 h. The cells were then infected with *C. burnetii* as described above. At 48 hpi, the cells were washed and harvested by scraping in inhibitor-free medium supplemented with 2% FBS. The cells were sonicated to release intracellular bacteria, and the samples were applied to Vero cells in 24-well plates. Infected Vero cells were incubated for 120 h and then processed for fluorescence microscopy using an anti-*C. burnetii* primary antibody and an Alexa Fluor-488-conjugated anti-rabbit secondary antibody (Invitrogen) to detect infectious foci. Focus-forming units (FFU) were visualized using a ×40 objective lens and quantified as the FFU/ml as previously described (13, 14).

Fluorescence microscopy. After PKA inhibition and *C. burnetii* infection of THP-1 cells or human alveolar macrophages as described above, the cells were fixed and permeabilized with 100% ice-cold methanol for 3 min, then blocked for 1 h in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Cell Signaling, Danvers, MA) at room temperature. The cells were incubated with mouse anti-CD63 (BD Biosciences, San Jose, CA) and rabbit anti-*C. burnetii* primary antibodies for 1 h at room temperature. The cells were then washed with PBS three times and incubated in PBS containing 0.5% BSA with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 594-conjugated anti-rabbit secondary antibodies (Invitrogen) for 1 h at room temperature. The cells were incubated with DAPI (4′,6′-diamidino-2-phenylindole dilactate; Invitrogen) for 5 min at room temperature to stain eukaryotic and bacterial DNA. Fluorescence microscopy was performed using a Ti-U microscope with a ×60 oil immersion objective (Nikon, Melville, NY). Images were obtained with a D5-QilMc digital camera and analyzed using NIS-Elements software (Nikon).

Immunoblot analysis. *C. burnetii*-infected mammalian cells in six-well plates were directly lysed in buffer containing 1% sodium dodecyl sulfate (SDS) by 10 passages through a 26-gauge needle and boiled for 5 min. Equal amounts of total protein were separated by SDS-PAGE, transferred to a 0.2-μm-pore-size polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA), and blocked in Tris-buffered saline (150 mM NaCl, 100 mM Tris-HCl [pH 7.6]) containing 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature. After blocking, the membranes were probed for equal protein loading using a mouse anti-β-tubulin primary antibody (Sigma-Aldrich) overnight at 4°C. The lysates were probed for PKA activation using rabbit primary antibodies directed against total PKA or phosphorylated PKA (Thr197) (Cell Signaling). The lysates were then probed for total phosphorylated PKA substrates using rabbit primary antibody to phospho-(Ser/Thr) PKA substrates, which detects phosphorylation of S/T residues with arginine at the −3 position (Cell Signaling). Lysates were probed for specific downstream PKA substrates using rabbit primary antibodies directed against total CREB, phospho-CREB (Ser133), total GSK-3β, phospho-GSK-3β (Ser9), total p105, phospho-p105 (Ser933), total Bad, and phospho-Bad (Ser155) (Cell Signaling). Reacting proteins were detected using anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Cell Signaling), which were was used to detect the primary antibody.

RESULTS

PKA activity is required for *C. burnetii* PV formation and bacterial replication. Our previous studies showed that the PKA inhibitor H-89 prevents prototypical PV formation (25). H-89 is commonly used as a specific inhibitor of PKA (3) but can less efficiently impact the activity of other kinases, including mitogen- and stress-activated kinase 1 (MSK1) and p70 ribosomal protein S6 kinase (S6K), in some systems. Thus, we sought to confirm that PKA is involved in PV formation by using a second specific inhibitor. Rp-cAMPS is a PKA-specific inhibitor that competes with endogenous cAMP for binding sites on PKA, efficiently preventing activation (6). It is important to note that the effective dose of Rp-cAMPS is higher than that of H-89 due to lower cell permeability (6, 8), and the inhibitor was therefore tested at 100 to 500

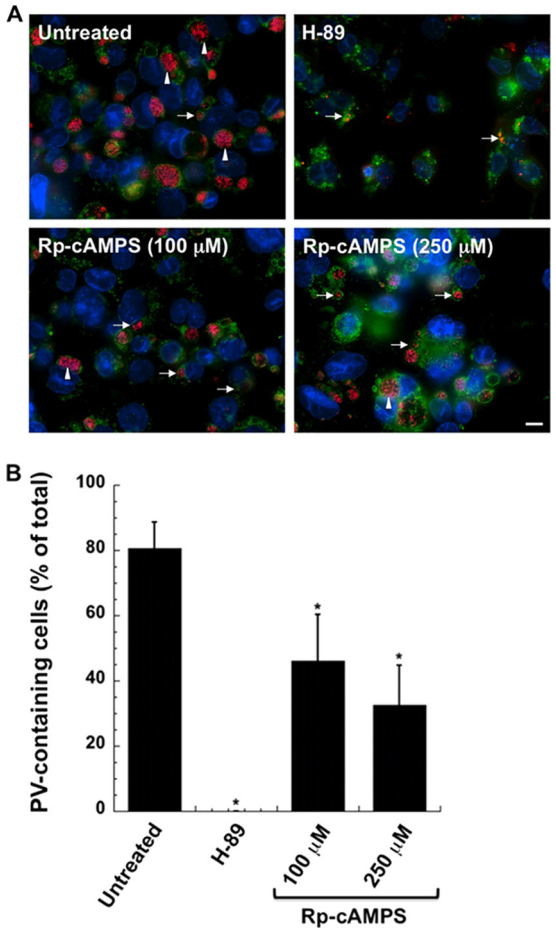

FIG 1 PKA activity is required for *C. burnetii* PV formation. THP-1 cells were infected with phase II *C. burnetii* (NMII) after treatment with H-89 (10 μM) or Rp-cAMPS (100 or 250 μM) 2 h prior to infection. Inhibitors were present through 48 hpi, and then the cells were prepared for microscopy. (A) The lysosomal marker CD63 (green) was used to confirm PV formation, DNA was stained with DAPI (blue), and bacteria were detected using a *C. burnetii*-specific antibody (red). Arrowheads indicate large, typical PV (>10 μm) at 48 hpi, and arrows denote small, atypical PV. Bar, 10 μm. (B) PV in at least five fields of 20 cells/field were counted for each treatment condition, and the percentages of cells forming large vacuoles (>10 μm) containing replicating organisms were quantified. Error bars indicate the standard deviation from the mean, and the results are representative of three independent experiments. An asterisk indicates a *P* value of <0.005 compared to untreated cells as determined using the Student *t* test. These results show that H-89 and Rp-cAMPS antagonize PV formation.

μM. For these studies, we used PMA-differentiated human macrophage-like THP-1 cells, which are a reliable *in vitro* model of *C. burnetii*-macrophage interactions (45). The cells were pretreated with H-89 or Rp-cAMPS for 2 h and then infected with avirulent *C. burnetii* NMII for 48 h in the presence of inhibitors. The cells were processed for fluorescence microscopy using an antibody directed against the late phagosome/lysosome marker CD63 to label PV (Fig. 1A). Single, large PVs were present in fewer than 50% of cells treated with Rp-cAMPS versus 80% in untreated cells (Fig. 1B), indicating PKA is required for optimal vacuole formation. Rp-cAMPS-treated cultures contained significantly more cells harboring multiple, small PVs with fewer organisms in each vacuole. In addition, typical PV were present in <5% of H-89-treated cells (Fig. 1B), confirming our previous results.

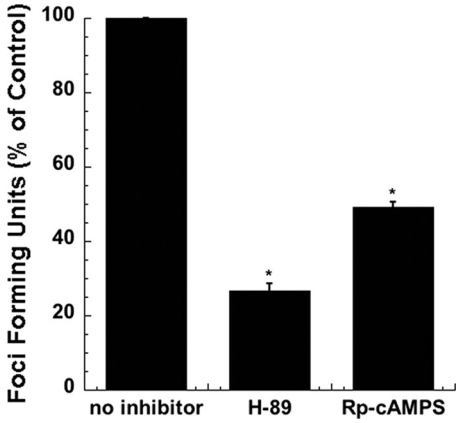

FIG 2 PKA inhibitors antagonize *C. burnetii* replication. THP-1 cells were infected with NMII *C. burnetii* after treatment with H-89 (10 μM) or Rp-cAMPS (100 μM) 2 h prior to infection. Inhibitors were present throughout the infection time course and, at 48 hpi, the cells were sonicated to release intracellular organisms. Harvested organisms were incubated with Vero cells for 120 h and then processed for immunofluorescence microscopy. The vacuoles representative of infectious foci were enumerated as FFU/ml. Error bars indicate the standard deviations from the mean. An asterisk indicates a *P* value of <0.05 compared to untreated cells as determined using the Student *t* test. Samples were analyzed in triplicate and are representative of at least two independent experiments. Treatment with either PKA inhibitor prevents *C. burnetii* replication.

We next assessed the requirement of PKA activity for *C. burnetii* replication using a standard FFU assay (13, 14). THP-1 cells were infected for 48 h in the presence of H-89 or Rp-cAMPS and then sonicated to release intracellular organisms. Sonicated samples were applied to a monolayer of Vero cells for 120 h, and PVs indicative of infectious foci were enumerated. As shown in Fig. 2, FFU significantly decreased in inhibitor-treated, infected cells compared to untreated, infected cells. Rp-cAMPS-treated cells resulted in ca. 50% fewer FFU and H-89-treated cells resulted in an ca. 70% decrease in FFU. Together, these results indicate PKA activity is critical for *C. burnetii* replication that relies on formation and expansion of the phagolysosome-like PV.

PKA is activated during *C. burnetii* infection. Because PKA inhibition impairs PV formation and bacterial replication, we predicted that *C. burnetii* activates PKA and downstream signaling during intracellular growth. To assess PKA activity, we examined phosphorylation of the kinase during infection by immunoblot. For complete activation, PKA must be bound by cAMP and phosphorylated on Thr197 by either the upstream kinase phosphoinositide-dependent kinase 1 or autophosphorylation (34). As shown in Fig. 3, PKA phosphorylation levels in *C. burnetii*-infected cells were substantially greater than those in uninfected cells at 24 hpi and continued to increase through 96 hpi (Fig. 3A). PKA phosphorylation returned to uninfected cell levels when cells were infected in the presence of chloramphenicol to inhibit bacterial protein synthesis (Fig. 3B), indicating *C. burnetii* actively triggers PKA activation. In addition, PKA phosphorylation levels did not increase during infection of cells treated with H-89 (data not shown), further confirming the PKA-specific effects of this widely used inhibitor.

PKA is activated during virulent *C. burnetii* infection of primary human alveolar macrophages. Most studies of *C. burnetii*-host cell interactions are performed with avirulent phase II organ-

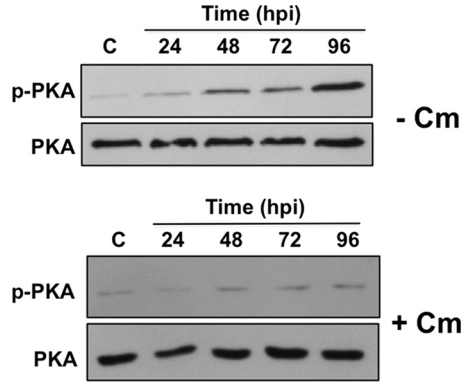

FIG 3 PKA is activated during *C. burnetii* infection. THP-1 cells were infected with NMII *C. burnetii* in the absence (− Cm) or presence (+ Cm) of chloramphenicol to inhibit bacterial protein synthesis. Lysates were harvested at the indicated times postinfection and subjected to immunoblot analysis with antibodies directed against total or phosphorylated PKA. C, control, uninfected cells; p-, phosphorylated. PKA phosphorylation levels continually increase from 24 to 96 hpi, suggesting the kinase is activated throughout intracellular growth. Treatment with chloramphenicol abrogates increased phosphorylation of PKA, indicating *C. burnetii* actively triggers activation of the kinase.

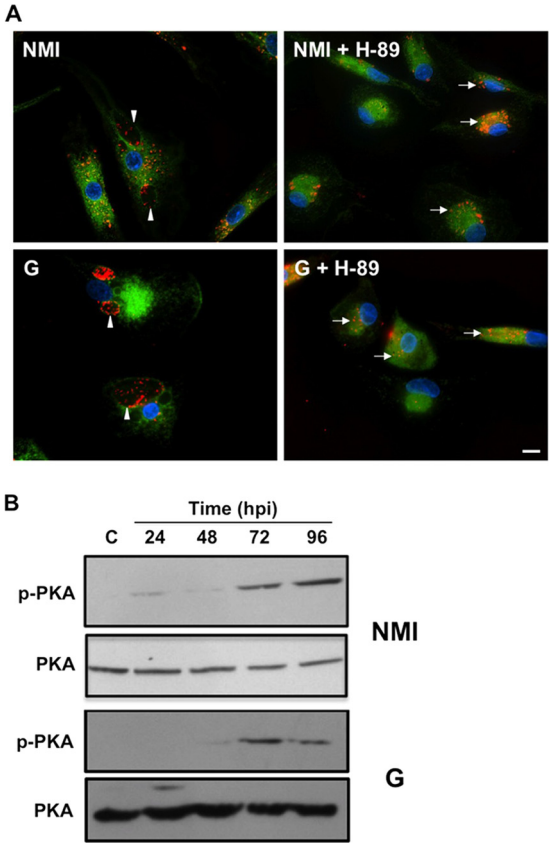

FIG 4 PKA activity is required for PV formation by virulent *C. burnetii* during infection of primary human alveolar macrophages. (A) Alveolar macrophages were pretreated for 2 h with H-89 and then infected with *C. burnetii* Nine Mile I (NMI) or G. Infections proceeded for 48 h, and then the cells were prepared for microscopy. The lysosomal marker CD63 (green) was used to confirm PV formation, DNA was stained with DAPI (blue), and bacteria were detected using a *C. burnetii*-specific antibody (red). Arrowheads indicate typical PV (>10 μm) at 48 hpi, and arrows denote small, atypical PV. Bar, 10 μm. PKA inhibition by H-89 antagonizes virulent *C. burnetii* PV formation. (B) Alveolar macrophages were infected with *C. burnetii* NMI or G for the indicated times, and then cell lysates were harvested and subjected to immunoblot analysis using primary antibodies to detect total or phosphorylated PKA. C, control, uninfected cells; p-, phosphorylated. Similar to the *in vitro* THP-1 model, virulent *C. burnetii* isolates trigger increasing levels of phosphorylated PKA indicative of activation at 72 to 96 hpi.

isms (43). Although phase II bacteria provide a reliable model of *in vitro* host cell interactions, it is important to examine infection-altering events in the context of virulent bacteria to assess whether these events occur during natural infection. We recently developed a new *in vitro* model for *C. burnetii*-host cell interactions using primary human alveolar macrophages obtained from bronchioalveolar lavage samples. During natural infection, *C. burnetii* targets alveolar macrophages for replication. The pathogen replicates efficiently in a large PV in these cells *in vitro* (J. G. Graham and D. E. Voth, unpublished results), representing the most appropriate model of *in vivo* cellular interactions. Here, alveolar macrophages were treated with H-89 and then infected with virulent Nine Mile I (acute disease isolate) or G (endocarditis isolate) organisms for 48 h and processed for fluorescence microscopy. As shown in Fig. 4A, virulent *C. burnetii* isolates formed large CD63-positive PV in primary alveolar macrophages. However, macrophages treated with H-89 do not support PV expansion, suggesting that PKA activity is required for natural *C. burnetii* infection. Inhibitor treatment results correlated well with increased PKA phosphorylation levels in infected macrophages at 72 to 96 hpi (Fig. 4B). Together, these results implicate a major role for PKA during *in vivo* infection.

PKA substrates are differentially phosphorylated during *C. burnetii* infection. PKA regulates numerous downstream targets to efficiently control the host response to infection (34). We previously showed that PKA substrates are differentially phosphorylated during infection using a pan-phospho-PKA substrate antibody (25). Here, we used the same antibody to determine whether *C. burnetii* actively regulates downstream PKA-dependent events. Lysates were harvested from THP-1 cells infected in the presence or absence of chloramphenicol. As shown in Fig. 5, *C. burnetii* induced an overall response distinct from that seen after antibiotic treatment, indicating the pathogen actively modulates PKA signaling during intracellular growth. Similar to our previous results (25), infection in the absence of chloramphenicol resulted in differential phosphorylation of 8 PKA substrates. This response was also negated when infected cells were treated with H-89 (data not shown), indicating *C. burnetii* activates a specific PKA signaling regimen.

Individual PKA targets altered during infection and the consequences of this differential regulation are not known. To address this gap in our understanding of *C. burnetii*-regulated PKA activity, we assessed phosphorylation of four potential PKA target proteins during infection. Increased phosphorylation of cAMP response element binding protein (CREB) indicates activation of the protein ((5, 40), while phosphorylation of Bad, p105 (an NF-κB family member), and glycogen synthase kinase-3β (GSK-3β) correlates with inactivation (5, 12, 41). We first monitored phosphorylation of CREB, which is a common readout of PKA activity and is generally phosphorylated when PKA activity increases (5, 19, 40). Interestingly, phosphorylated levels of CREB did not differ substantially from uninfected cells at any time point postinfection (Fig. 6A). Similar to CREB, GSK-3β phosphoryla-

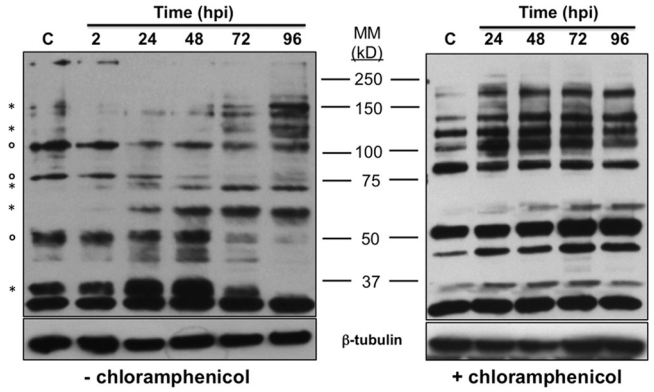

FIG 5 *C. burnetii* actively regulates PKA substrate phosphorylation. THP-1 cells were infected with NMII *C. burnetii* in the presence or absence of chloramphenicol. Lysates were harvested and probed for phosphorylated PKA substrates and tubulin as a loading control. C, control, uninfected cells. The molecular mass (MM) is shown in kilodaltons. Asterisks indicate proteins with increased phosphorylation levels at 24 to 96 hpi and circles denote proteins with decreased phosphorylation levels after 48 hpi that are distinct from profiles observed in chloramphenicol-treated cells. PKA substrates are differentially phosphorylated during infection and chloramphenicol treatment abrogates these changes, further indicating *C. burnetii* actively modulates PKA signaling.

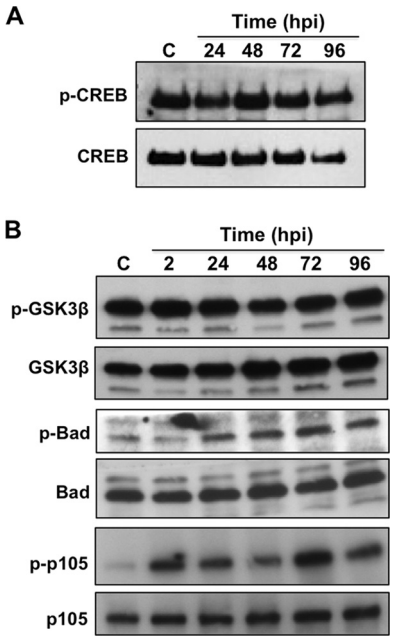

FIG 6 Distinct downstream PKA targets are phosphorylated during *C. burnetii* infection. THP-1 cells were infected with NMII *C. burnetii* and lysates harvested at the indicated points of time. Lysates were probed for total or phosphorylated forms of CREB (A) or Bad, GSK-3β, or p105 (B). C, control, uninfected cells; p-, phosphorylated. *C. burnetii* infection does not induce substantial changes in CREB or GSK-3β phosphorylation. In contrast, the phosphorylated levels of Bad and p105 increase at 24 and 2 hpi, respectively, and the levels remained elevated above those of control cells throughout infection, indicating continual altered regulation of these PKA targets.

tion levels did not change compared to uninfected cells (Fig. 6B). In contrast, increased phosphorylation was observed for Bad between 24 and 96 hpi and for p105 between 2 and 96 hpi (Fig. 6B). Taken together, these results suggest *C. burnetii* regulates PKA signaling for distinct purposes during infection and does not alter all PKA-dependent responses. Furthermore, levels of phosphorylated Bad and p105 also increased during virulent *C. burnetii* infection of primary human alveolar macrophages (data not shown), further supporting a role for PKA-dependent responses in natural infection.

DISCUSSION

Eukaryotic PKA is required for PV formation and bacterial replication during virulent and avirulent *C. burnetii* infection of macrophages. An increase in PKA phosphorylation indicative of activation occurs throughout infection, suggesting prolonged stimulation of the signaling cascade. *C. burnetii*-directed alteration of PKA activity triggers downstream phosphorylation of distinct targets. Interestingly, *C. burnetii* does not activate all PKA-dependent events, indicating the pathogen cleverly usurps a kinase cascade to elicit specific downstream cellular responses. These results open a new avenue to understanding how *C. burnetii* alters host cell physiology via signaling cascades to survive in a degradative phagolysosomal compartment.

To confirm previous studies using the common PKA inhibitor H-89, we used a more specific antagonist, Rp-cAMPS. Although Rp-cAMPS negatively impacts the formation of large, prototypical PVs and *C. burnetii* replication, it is less efficient than H-89 treatment. One explanation for this discrepancy may be differences in cell permeability of the two inhibitors, with H-89 more efficiently crossing cellular membranes to access PKA. A second, more intriguing possibility is the involvement of other kinases reportedly inhibited by H-89 in certain systems. For example, H-89 can influence activity of the kinases MSK and S6K (15). Although it is not readily apparent how these kinases would influence PV formation, we are currently probing the potential role of these proteins in *C. burnetii* infection. We do not predict S6K involvement in PV formation, since the kinase is not activated during *C. burnetii* infection (44). Furthermore, inhibitor specificity is often analyzed in cell-free biochemical assays or in specific cell lines. Thus, non-PKA effects of H-89 must be determined in macrophages to understand their role in *C. burnetii* infection.

cAMP-dependent signaling, including the PKA cascade, is manipulated by other intracellular pathogens. *Brucella suis* causes increased cAMP accumulation and CREB phosphorylation in macrophages and H-89 treatment prevents bacterial replication (19). Similarly, treating J774 macrophages with H-89 prevents intracellular replication of *Mycobacterium smegmatis* and *M. tuberculosis* (26). These results are similar to our data showing that H-89 or Rp-cAMPS treatment prevent typical PV formation and bacterial replication. cAMP and PKA control many events that alter eukaryotic cellular physiology, including phagocytosis and phagosome biogenesis (35). Indeed, cAMP signaling controls endosome acidification in some systems (42) and PKA regulates autophagosome formation through interactions with LC3 (11, 31). Overall, levels of cAMP do not change significantly during *C. burnetii* infection (L. J. MacDonald and D. E. Voth, unpublished results). However, cAMP compartmentalization is critical for controlling distinct downstream signaling events (35). PKA activity also depends on subcellular localization regulated by A-kinase anchor proteins (AKAPs) that tether the kinase to specific organelles (23). Therefore, proper cAMP and PKA localization may be important for *C. burnetii* infection and this possibility is under investigation.

PKA potentially controls PV expansion by regulating cytoskeleton-related proteins. F-actin is recruited to the PV early during infection and actin depolymerizing agents disrupt vacuole maturation (1). The small GTPases RhoA and Cdc42 are also recruited to the PV and are predicted to control actin assembly on the maturing vacuole. PKA is known to regulate activity of RhoA and Cdc42. PKA inactivates RhoA by phosphorylation, promoting RhoA interaction with Rho guanine dissociation inhibitor proteins that maintain the protein in an inactive cytosolic form (16, 17). Conversely, PKA promotes the activation of Cdc42, allowing the GTPase to associate with membranes and alter actin polymerization (18). AKAPs can tether PKA to cytoskeletal components (23), further suggesting the importance of PKA localization during infection. Thus, PKA may contribute to PV expansion through regulating actin polymerization around the maturing vacuole.

We predict that PKA has a dual role in *C. burnetii* infection. First, PKA is involved in PV expansion and thus must act during the early stages of infection. Second, phosphorylation of PKA and target proteins increases throughout infection at times when the PV has fully matured and expanded. Specific downstream targets altered during infection, including Bad and p105, do not have a predicted role in phagosome maturation but may be critical for other aspects of infection following PV establishment. Bad is a mitochondrial proapoptotic protein that regulates cytochrome *c* release (27). *C. burnetii* potently inhibits host cell apoptosis in a cytochrome *c*- and effector-dependent manner (29, 30, 45); however, the host components involved have not been fully defined. Bad phosphorylation by PKA on Ser155, which inactivates Bad, likely contributes to preventing apoptosis. Bad can also be phosphorylated on Ser136 by Akt, a kinase activated during infection (44), to regulate mitochondrial-dependent apoptosis, suggesting that *C. burnetii* uses both PKA and Akt to target Bad and prevent apoptosis.

p105 phosphorylation levels also remain elevated above basal level throughout *C. burnetii* intracellular growth. In eukaryotic cells, p105, also known as NF-κB1, is bound to p50 in the cytoplasm and is phosphorylated by PKA to regulate tumor necrosis factor alpha (TNF-α) production (5). After phosphorylation and proteolysis of p105, the p50 dimer is released and translocates to the nucleus to control transcription of host response genes. PKA signaling is required for TNF-α production by macrophages in response to *M. smegmatis* (47), supporting a role for PKA in regulating the cytokine response to infection. *C. burnetii*-infected dendritic cells produce high levels of TNF-α (38), suggesting the p105 pathway may regulate this cytokine response to the pathogen.

PKA activity is also required for optimal PV formation in primary macrophages. Alveolar macrophages are central to *C. burnetii* infection, representing the pathogen's initial target cell upon aerosol-mediated uptake by a host. Virulent *C. burnetii* efficiently infects and replicates in primary human alveolar macrophages *in vitro* (Graham and Voth, unpublished) and increased PKA phosphorylation is apparent at 3 to 4 days postinfection. PKA alters alveolar macrophage responses to microbial pathogens by regulating antimicrobial molecule production. Specifically, PKA regulates production of TNF-α and H_2O_2 (46), defenses used by eukaryotic cells to degrade intracellular organisms. Avirulent *C. burnetii* disrupts assembly of the NADPH oxidase complex in neutrophils and mouse macrophages, lowering reactive oxygen species (ROS) levels (22, 39). Thus, it is tempting to predict the pathogen stimulates PKA activity to alter ROS-directed signaling.

In conclusion, we have uncovered a role for host PKA in *C. burnetii* PV formation. Similar to previous findings on Akt and Erk1/2 (44), PKA activity is stimulated throughout infection, further indicating that *C. burnetii* continually manipulates host signaling long after uptake by macrophages. Modulation of PKA activity is predicted to be important during *in vivo* infection, since the kinase is activated and involved in PV generation in primary human alveolar macrophages. Our results present yet another example of how intracellular pathogens adeptly subvert host cell functions using complex kinase signaling pathways. Future studies on PKA control of cytoskeletal organization, inhibition of apoptosis, and cytokine production will provide a better understanding of the scope of host signaling required for *C. burnetii* infection.

ACKNOWLEDGMENTS

We thank Joseph Graham for critical reading of the manuscript.

This research was supported by funding to D.E.V. from the American Heart Association (BGIA3080001) and NIH/NIAID (R01AI087669) and to R.C.K. from the UAMS Translational Research Institute (1UL1RR029884).

REFERENCES

1. **Aguilera M, et al.** 2009. Actin dynamics and Rho GTPases regulate the size and formation of parasitophorous vacuoles containing *Coxiella burnetii*. Infect. Immun. **77**:4609–4620.
2. **Akporiaye ET, Rowatt JD, Aragon AA, Baca OG.** 1983. Lysosomal response of a murine macrophage-like cell line persistently infected with *Coxiella burnetii*. Infect. Immun. **40**:1155–1162.
3. **Aronoff DM, Canetti C, Serezani CH, Luo M, Peters-Golden M.** 2005. Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1. J. Immunol. **174**:595–599.
4. **Beare PA, et al.** 2011. Dot/Icm type IVB secretion system requirements for *Coxiella burnetii* growth in human macrophages. mBio **2**:e00175–11. doi:10.1128/mBio.00175-11.
5. **Beinke S, Ley SC.** 2004. Functions of NF-κB1 and NF-κB2 in immune cell biology. Biochem. J. **382**:393–409.
6. **Bell D, McDermott BJ.** 1994. Use of the cyclic AMP antagonist, Rp-cAMPS, to distinguish between cyclic AMP-dependent and cyclic AMP-independent contractile responses in rat ventricular cardiomyocytes. J. Mol. Cell. Cardiol. **26**:1439–1448.
7. **Beron W, Gutierrez MG, Rabinovitch M, Colombo MI.** 2002. *Coxiella burnetii* localizes in a Rab7-labeled compartment with autophagic characteristics. Infect. Immun. **70**:5816–5821.
8. **Bouschet T, et al.** 2003. Stimulation of the ERK pathway by GTP-loaded Rap1 requires the concomitant activation of Ras, protein kinase C, and protein kinase A in neuronal cells. J. Biol. Chem. **278**:4778–4785.
9. **Campoy EM, Zoppino FC, Colombo MI.** 2011. The early secretory pathway contributes to the growth of the *Coxiella*-replicative niche. Infect. Immun. **79**:402–413.
10. **Carey KL, Newton HJ, Luhrmann A, Roy CR.** 2011. The *Coxiella burnetii* Dot/Icm system delivers a unique repertoire of type IV effectors into host cells and is required for intracellular replication. PLoS Pathog. **7**:e1002056.
11. **Cherra SJ, III, et al.** 2010. Regulation of the autophagy protein LC3 by phosphorylation. J. Cell Biol. **190**:533–539.
12. **Cieslak D, Lazou A.** 2007. Regulation of BAD protein by PKA, PKCδ, and phosphatases in adult rat cardiac myocytes subjected to oxidative stress. Mol. Cells **24**:224–231.
13. **Cockrell DC, Beare PA, Fischer ER, Howe D, Heinzen RA.** 2008. A method for purifying obligate intracellular *Coxiella burnetii* that employs digitonin lysis of host cells. J. Microbiol. Methods **72**:321–325.
14. **Coleman SA, Fischer ER, Howe D, Mead DJ, Heinzen RA.** 2004. Temporal analysis of *Coxiella burnetii* morphological differentiation. J. Bacteriol. **186**:7344–7352.
15. **Davies SP, Reddy H, Caivano M, Cohen P.** 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**:95–105.

16. **Dong JM, Leung T, Manser E, Lim L.** 1998. cAMP-induced morphological changes are counteracted by the activated RhoA small GTPase and the Rho kinase ROKα. J. Biol. Chem. **273**:22554–22562.
17. **Ellerbroek SM, Wennerberg K, Burridge K.** 2003. Serine phosphorylation negatively regulates RhoA *in vivo*. J. Biol. Chem. **278**:19023–19031.
18. **Feoktistov I, Goldstein AE, Biaggioni I.** 2000. Cyclic AMP and protein kinase A stimulate Cdc42: role of A(2) adenosine receptors in human mast cells. Mol. Pharmacol. **58**:903–910.
19. **Gross A, Bouaboula M, Casellas P, Liautard JP, Dornand J.** 2003. Subversion and utilization of the host cell cyclic adenosine 5′-monophosphate/protein kinase A pathway by *Brucella* during macrophage infection. J. Immunol. **170**:5607–5614.
20. **Gutierrez MG, et al.** 2005. Autophagy induction favors the generation and maturation of the *Coxiella*-replicative vacuoles. Cell. Microbiol. **7**:981–993.
21. **Hackstadt T, Williams JC.** 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. U. S. A. **78**:3240–3244.
22. **Hill J, Samuel JE.** 2011. *Coxiella burnetii* acid phosphatase inhibits the release of reactive oxygen intermediates in polymorphonuclear leukocytes. Infect. Immun. **79**:414–420.
23. **Howe AK.** 2004. Regulation of actin-based cell migration by cAMP/PKA. Biochim. Biophys. Acta **1692**:159–174.
24. **Howe D, Mallavia LP.** 2000. *Coxiella burnetii* exhibits morphological change and delays phagolysosomal fusion after internalization by J774A. 1 cells. Infect. Immun. **68**:3815–3821.
25. **Hussain SK, Broederdorf LJ, Sharma UM, Voth DE.** 2010. Host kinase activity is required for *Coxiella burnetii* parasitophorous vacuole formation. Front. Microbiol. **1**:137.
26. **Kalamidas SA, et al.** 2006. cAMP synthesis and degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria. J. Cell Sci. **119**:3686–3694.
27. **Levine B, Sinha S, Kroemer G.** 2008. Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy **4**:600–606.
28. **Limonard GJ, et al.** 2010. One-year follow-up of patients of the ongoing Dutch Q. fever outbreak: clinical, serological and echocardiographic findings. Infection **38**:471–477.
29. **Luhrmann A, Nogueira CV, Carey KL, Roy CR.** 2010. Inhibition of pathogen-induced apoptosis by a *Coxiella burnetii* type IV effector protein. Proc. Natl. Acad. Sci. U. S. A. **107**:18997–19001.
30. **Luhrmann A, Roy CR.** 2007. *Coxiella burnetii* inhibits activation of host cell apoptosis through a mechanism that involves preventing cytochrome *c* release from mitochondria. Infect. Immun. **75**:5282–5289.
31. **Mavrakis M, Lippincott-Schwartz J, Stratakis CA, Bossis I.** 2007. mTOR kinase and the regulatory subunit of protein kinase A (PRKAR1A) spatially and functionally interact during autophagosome maturation. Autophagy **3**:151–153.
32. **Omsland A, Cockrell DC, Fischer ER, Heinzen RA.** 2008. Sustained axenic metabolic activity by the obligate intracellular bacterium *Coxiella burnetii*. J. Bacteriol. **190**:3203–3212.
33. **Omsland A, et al.** 2009. Host cell-free growth of the Q. fever bacterium *Coxiella burnetii*. Proc. Natl. Acad. Sci. U. S. A. **106**:4430–4434.
34. **Pearce LR, Komander D, Alessi DR.** The nuts and bolts of AGC protein kinases. Nat. Rev. Mol. Cell. Biol. **11**:9–22.
35. **Pryzwansky KB, Kidao S, Merricks EP.** 1998. Compartmentalization of PDE-4 and cAMP-dependent protein kinase in neutrophils and macrophages during phagocytosis. Cell. Biochem. Biophys. **28**:251–275.
36. **Raoult D, Marrie T, Mege J.** 2005. Natural history and pathophysiology of Q. fever. Lancet Infect. Dis. **5**:219–226.
37. **Romano PS, Gutierrez MG, Beron W, Rabinovitch M, Colombo MI.** 2007. The autophagic pathway is actively modulated by phase II *Coxiella burnetii* to efficiently replicate in the host cell. Cell. Microbiol. **9**:891–909.
38. **Shannon JG, Howe D, Heinzen RA.** 2005. Virulent *Coxiella burnetii* does not activate human dendritic cells: role of lipopolysaccharide as a shielding molecule. Proc. Natl. Acad. Sci. U. S. A. **102**:8722–8727.
39. **Siemsen DW, Kirpotina LN, Jutila MA, Quinn MT.** 2009. Inhibition of the human neutrophil NADPH oxidase by *Coxiella burnetii*. Microbes Infect. **11**:671–679.
40. **Siu YT, Jin DY.** 2007. CREB: a real culprit in oncogenesis. FEBS J. **274**: 3224–3232.
41. **Tsujio I, et al.** 2000. Inactivation of glycogen synthase kinase-3 by protein kinase C delta: implications for regulation of tau phosphorylation. FEBS Lett. **469**:111–117.
42. **Van Dyke RW, Root KV, Hsi RA.** 1996. cAMP and protein kinase A stimulate acidification of rat liver endosomes in the absence of chloride. Biochem. Biophys. Res. Commun. **222**:312–316.
43. **Voth DE, Heinzen RA.** 2007. Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. Cell. Microbiol. **9**:829–840.
44. **Voth DE, Heinzen RA.** 2009. Sustained activation of Akt and Erk1/2 is required for *Coxiella burnetii* antiapoptotic activity. Infect. Immun. **77**: 205–213.
45. **Voth DE, Howe D, Heinzen RA.** 2007. *Coxiella burnetii* inhibits apoptosis in human THP-1 cells and monkey primary alveolar macrophages. Infect. Immun. **75**:4263–4271.
46. **Wall EA, et al.** 2009. Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105. Sci. Signal. **2**:ra28.
47. **Yadav M, Roach SK, Schorey JS.** 2004. Increased mitogen-activated protein kinase activity and TNF-alpha production associated with *Mycobacterium smegmatis*- but not *Mycobacterium avium*-infected macrophages requires prolonged stimulation of the calmodulin/calmodulin kinase and cyclic AMP/protein kinase A pathways. J. Immunol. **172**:5588–5597.