

cAMP synthesis and degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria

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Summary

We showed recently that actin assembly by phagosomal membranes facilitates fusion with late endocytic organelles in macrophages. Moreover, lipids that induced phagosomal actin also stimulated this fusion process. In macrophages infected with pathogenic mycobacteria actin-stimulatory lipids led to an increase in pathogen destruction, whereas inhibitors facilitated their growth. A model was proposed whereby phagosomal membrane actin assembly provides tracks for lysosomes to move towards phagosomes, thereby facilitating fusion. Here, we investigated how cAMP affected phagosomal actin assembly in vitro, and phagosomal actin, acidification and late fusion events in J774 macrophages. Latex bead phagosomes are shown to possess adenylyl cyclase activity, which synthesizes cAMP, and phosphodiesterase activity, which degrades cAMP. The

system is regulated by protein kinase A (PKA). Increasing cAMP levels inhibited, whereas decreasing cAMP levels stimulated, actin assembly in vitro and within cells. Increasing cAMP levels also inhibited phagosome-lysosome fusion and acidification in cells, whereas reducing cAMP had the opposite effect. High cAMP levels induced an increase in intraphagosomal growth in macrophages of both the non-pathogenic *Mycobacterium smegmatis* and the pathogenic *Mycobacterium tuberculosis*, whereas low cAMP levels or inhibition of PKA correlated with increased bacterial destruction. We argue that the phagosome cAMP-PKA system behaves as a molecular switch that regulates phagosome actin and maturation in macrophages.

Key words: cAMP, Actin, Mycobacteria, Phagosomes

Introduction

When pathogens such as mycobacteria enter macrophages they become enclosed in a specialized membrane compartment, the phagosome (Scott et al., 2003; Vergne et al., 2004; Aderem and Underhill, 1999). This organelle fuses sequentially with early and then late endocytic organelles, including lysosomes. These late events allow hydrolases and the H⁺-ATPase to enter phagosomes and provide a suitable environment for the phagosome to kill and digest pathogens. A number of pathogens, most notably *Mycobacterium tuberculosis* circumvent these late fusion events and thereby grow within the phagosome, causing disease (Russell, 2003; Scott et al., 2003; Vergne et al., 2003; Vieira et al., 2002). The mechanism by which pathogenic mycobacteria, or other pathogens, such as *Salmonella* prevent or alter full phagosome maturation is an important unresolved issue having enormous medical relevance.

The use of latex bead phagosomes (LBPs) allowed a number of functional in vitro assays to be developed to monitor

phagosome fusion with different organelles, and their multiple interactions with microtubules and actin (Desjardins and Griffiths, 2003). Recently, we have focused on the ability of the phagosomal membrane to nucleate the assembly of actin and on the links between this process and the fusion of phagosomes with late-endocytic organelles (Desjardins and Griffiths, 2003; Jahraus et al., 2001; Kjekken, 2004). Isolated LBPs can assemble actin de novo, a process facilitated by ezrin-moesin in conjunction with phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P₂] (Defacque et al., 2002; Defacque et al., 2000a) and gelsolin (Defacque et al., 2000b). More recently we have found that even in vitro this process can be regulated by a large number of signaling proteins, such as PKC, and P38 MAP kinase, as well as lipids such as sphingolipids and fatty acids (Anes et al., 2003; Anes et al., 2006) (M.K., unpublished data). Importantly, many kinases can be activated by adding ATP to LBPs (Emans et al., 1996) and we showed that lipid kinases, such as phosphatidylinositol 4-kinase and sphingosine kinase can synthesize lipids such as phosphoinositides and

sphingosine-1-phosphate in an ATP-dependent fashion (Anes et al., 2003; Defacque et al., 2002).

We have provided extensive evidence that phagosome-membrane-assembled actin filaments facilitate phagosome fusion with late endocytic organelles; a model was proposed in which actin filaments attached to phagosomes and/or late endocytic organelles provide tracks for these organelles to fuse (Jahraus et al., 2001; Kjekken, 2004). Further evidence was presented for a positive role of phagosomal assembled actin by Anes et al. (Anes et al., 2003). Phagosomes containing live, non-pathogenic *Mycobacterium smegmatis* mature normally to the phagosome-lysosome with consequent killing of the bacteria in macrophages. After isolation these phagosomes nucleated actin in vitro in a manner very similar to the LBPs. By contrast, the phagosomes enclosing the pathogenic *M. tuberculosis* or *Mycobacterium avium*, whose maturation is blocked in macrophages, were unable to assemble actin in vitro. Seven, mostly pro-inflammatory lipids were identified that could switch on phagosome actin assembly (in vitro and in macrophages), as well as phagosome maturation and increase the ability of macrophages to kill pathogenic mycobacteria. By contrast, anti-inflammatory lipids inhibit phagosome actin and facilitate pathogen growth (Anes et al., 2003).

Here, we focus on the role of the cAMP-signaling system in phagosome actin assembly and on phagosome maturation. This system mediates a plethora of cellular functions with extensive 'cross-talk' occurs between it and other signaling networks throughout cells (Beavo and Brunton, 2002; Antoni, 2000; Ellerbroek et al., 2003; Frisch, 2000; Houslay and Kolch, 2000; Tasken and Anandahl, 2004; Wong and Scott, 2004; Houslay, 1998; Housley and Milligan, 1997; Howe and Juliano, 2000). Increases in cAMP levels generally compromise the bactericidal activity of the host immune system (Bengis-Garber and Gruener, 1996; Gueirard et al., 1998; Khelef et al., 1993; Reddy et al., 2001). Many pathogens have taken advantage of this phenomenon and evolved mechanisms to elevate intracellular cAMP levels in both macrophages (Gross et al., 2003; Hanski, 1989) and neutrophils (Confer and Eaton, 1982; Gross et al., 2003; Hanski, 1989; Nathan, 2003) – evidently to facilitate their survival within phagosomes. Increased cAMP levels inside phagocytes negatively modulate actin-dependent processes, including chemotactic movement and phagocytosis (Merdrignac et al., 1983; Whelan and Senger, 2003; Wright et al., 1990; Ydrenius et al., 1997). In the case of mycobacterium-infected cells the addition of cAMP inhibits phagosome-lysosome fusion (Lowrie et al., 1979).

A central effector of cAMP in cells is cAMP-dependent protein kinase (PKA) (Wong and Scott, 2004; Tasken and Anandahl, 2004; Baillie et al., 2005). Two forms of the PKA holoenzyme are found inside cells, depending on the R subunit: type I is mostly cytoplasmic, and type II is found associated with specific cellular structures and organelles. PKA type II has been found, among other intracellular locations, in endosomal and Golgi membranes (Griffiths et al., 1990; McCahill et al., 2005; Shanks et al., 2002; Tasken et al., 2001; Witczak et al., 1999) and in autophagosomal organelles (Kotoulas et al., 2003). This enzyme is bound to non-enzymatic scaffolding proteins termed A-kinase anchoring proteins (AKAPs). Various AKAPs confer distinct intracellular localization of PKA-RII, which allows for the spatial

separation of cAMP signaling in cells through gradients generated by cAMP hydrolysis by phosphodiesterases (Baillie et al., 2005).

Cyclic AMP is produced in cells through the action of (mostly) plasma membrane localized adenylyl cyclase, which generates cAMP and spatially tethered phosphodiesterases that degrade it (Wong and Scott, 2004; Tasken and Anandahl, 2004; Baillie et al., 2005). Ezrin is an AKAP (Dransfield et al., 1997) that forms part of the cytoskeletal machinery in cells. This prompted us to focus on evaluating the cAMP system in the phagosomal assembly of actin. Towards this goal, we first tested the effects of cAMP on LBP actin assembly in vitro and then extended the analysis to a model mouse macrophage (J774) cell system. There, we monitored phagosome actin, the fusion of phagosomes with late-endocytic organelles, and phagosome acidification. Finally, we describe the effects of either increasing or decreasing intracellular cAMP levels on the ability of J774 cells to kill the non-pathogenic *M. smegmatis* and the pathogenic *M. tuberculosis*. These data provide novel insight into how the regulation of assembly of phagosomal actin is intimately linked to its ability to undergo the full maturation process essential for pathogen destruction.

Results

Effects of modulating cAMP on in vitro assembly of actin by latex bead phagosomes

The in vitro LBP actin assembly assay involves monitoring the growth of Rhodamine-actin filaments from the phagosomal membrane with ATP but in the absence of cytosol and GTP. Throughout this study we used only LBPs following a 2-hour internalization (1 hour pulse and 1 hour chase) of 1 μm latex beads. At this period the phagosomes have maximal actin assembly activity in vitro (Defacque et al., 2000b). Besides thymosin β_4 , which is added to buffer monomeric actin, the key component in the assay is ATP. We recently found that the LBPs are in different signaling states depending on the levels of ATP (Anes et al., 2003). Under the standard conditions of low (0.2 mM) levels of ATP the process of actin assembly is constitutively active (on a sub-population of phagosomes, in vitro and in cells). Different effectors, especially lipids, can variously stimulate or inhibit actin assembly under this condition. However, at physiological levels of ATP (5 mM) the ability of phagosomes to activate actin is repressed, but can be switched on by a number of lipids, such as arachidonic acid and sphingosine-1-phosphate (Anes et al., 2003). For a number of effectors, such as sphingosine and sphingosine-1-phosphate, their effects on phagosomal actin assembly were opposite under high (5 mM) and low (0.2 mM) ATP conditions.

In the in vitro phagosome system, because the cytoplasmic surface of the membrane is exposed to the aqueous medium we could use cAMP itself, rather than the bromo analogue that is usually used because it can cross the plasma membrane of intact cells (see below). As shown in Fig. 1A under the low-ATP regime a significant fraction of the untreated LBP nucleated actin filament growth whereas at high (5 mM) ATP the system was inhibited, in agreement with published results (Anes et al., 2003).

At concentrations ranging from 10 nM to 10 mM, cAMP could strongly inhibit LBP actin assembly at low ATP (Fig. 1A). At high ATP levels (in which the system is already inhibited) there was also a hint of inhibition at all

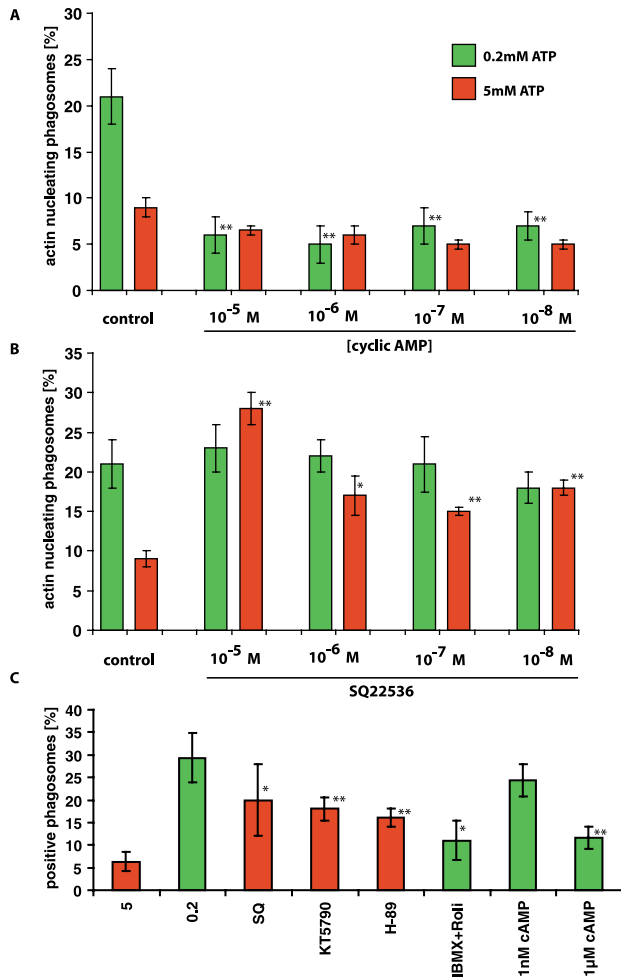


Fig. 1. Cyclic AMP inhibits LBP actin assembly. Effects of cAMP (A) and SQ 22536 (B), on the in vitro de novo actin assembly of latex bead phagosomes isolated by flotation (after 1 hour pulse and 1 hour chase) from J774A.1 mouse macrophages, at low (0.2 mM) and high (5 mM) ATP concentrations. (C) Effect of cAMP-modulating drugs on actin nucleation. Results represent means \pm s.d. from three independent experiments. Asterisks indicate significance as determined by the Student's *t*-test: * P <0.05; ** P <0.01 compared with levels in the control.

concentrations but this was not statistically significant. We then tested the effects of SQ22539, an inhibitor of adenylyl cyclase, the enzyme that synthesizes cAMP. If cAMP could be made locally on the phagosome, this inhibitor would be expected to have the opposite effect to cAMP. Consistent with this hypothesis the addition of this inhibitor at a range of concentrations significantly increased the fraction of actin-positive phagosomes at high ATP levels (Fig. 1B). However, at low ATP levels the inhibitor had no effect on the (already high) signal seen under this condition. These data argue that a local synthesis of cAMP is required to inhibit the phagosomal actin assembly machinery at high ATP, and when cAMP synthesis is blocked, the system is activated. By contrast, at low ATP levels, the levels of cAMP are seemingly too low to effect inhibition, although under this condition the system can still be inhibited by exogenous cAMP (Fig. 1A).

If the cAMP that is generated at 5 mM ATP conditions is

responsible for the inhibition of phagosomal actin then blocking cAMP synthesis should stimulate actin assembly. In agreement with this, the addition of SQ 22539 at high ATP levels elevated the assembly of actin (Fig. 1C). Conversely, the addition of 1 μ M cAMP (but not 1 nM) could induce an inhibition of the (normally constitutively active) process at low ATP levels (Fig. 1C). The same effect was seen with a combination of two inhibitors of cAMP phosphodiesterase (10 μ M IBMX and 1 μ M rolipram) at low ATP levels the low turnover of cAMP (see below) could be shifted towards accumulation of cAMP, which then inhibited the actin assembly. When these inhibitors were used by themselves, a partial inhibitory effect was observed (not shown).

As PKA provides a key target for cAMP action in cells, we evaluated its role by testing the effects of H89, an inhibitor of PKA catalytic activity on phagosomal actin. As shown in Fig. 1C, the addition of H89 significantly stimulated the assembly of actin at high ATP concentration (i.e. the normally blocked state), whereas it had no effect at the normally permissive low ATP condition. Similar results were seen with another PKA inhibitor (KT5720) (data not shown). These results argue that the ability of cAMP to inhibit LBP actin assembly in vitro is mediated via PKA activity.

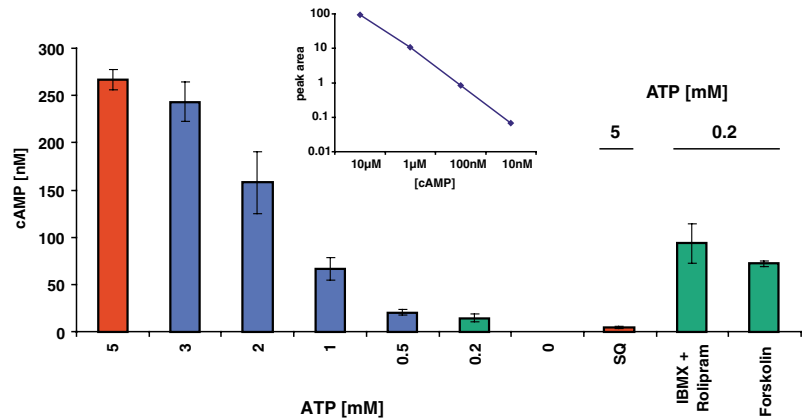
Synthesis of cAMP by phagosomes in vitro

The above results led us to test directly whether LBPs have the capacity to synthesize cAMP. For this, we established a HPLC system that can detect cAMP down to nanomolar levels (Fig. 2 inset). As shown in Fig. 2B when phagosomes were incubated at low ATP levels (0.2 mM) there was a hardly detectable release of cAMP. However, as the ATP was increased towards the 'high-ATP' condition (5 mM) an increasing amount of cAMP could be detected. At 5 mM ATP, the synthesis of cAMP was totally blocked by the adenylyl cyclase inhibitor SQ 22539 (Fig. 2 inset). At 0.2 mM (low) ATP the low level of cAMP could be significantly elevated by stimulation of adenylyl cyclase with the direct activator, forskolin. Moreover, a similar increase in cAMP was seen when phosphodiesterase activity was inhibited using IBMX and rolipram (Fig. 2). Thus, even at low ATP concentrations, there is a significant synthesis of cAMP that is balanced by degradation through phosphodiesterases. These results are consistent with the actin assembly data and fit the model that phagosomes can synthesize and degrade cAMP, whose levels act as a switch: high cAMP levels inhibit phagosome actin assembly whereas low cAMP levels around phagosomes allow actin to be nucleated.

Effects of modulating cAMP on phagosomal-associated actin in cells

The synthesis and degradation of cAMP by phagosomes and its consequences for the actin assembly process in vitro led to the obvious question whether the regulation of the system would be similar in macrophages. We first tested the effects of altering the levels of intracellular cAMP on LBP-associated actin. For this, beads were internalized for 15 minutes followed by a 1-hour and 15-minute chase in medium without beads in J774 cells. Effectors that modulate cAMP levels were always added at the beginning of the chase period to avoid the complications of effects of the cAMP on phagocytic uptake (Oropeza-Rendon et al., 1980; Zalavary et al., 1994). Intracellular F-actin was detected using Rhodamine-phalloidin

Fig. 2. LBPs can synthesize and degrade cAMP. HPLC analysis of cAMP on phagosomes treated as indicated. Red color indicates high (5 mM) ATP, blue, intermediate ATP conditions and green low ATP conditions (200 μ M ATP). The inset shows a calibration of the system with pure cAMP. The absorbance values represent the peak areas of cAMP. SQ, SQ22356.



and visualized by confocal microscopy. The fraction of phagosomes having actin dots or patches was quantified (Fig. 3A,B). We have shown previously that this parameter correlates well with the results obtained from the in vitro assay under different conditions (Anes et al., 2003; Defacque et al., 2000a; Defacque et al., 2000b).

In these experiments we used the cell-permeable 8-bromo(Br)-cAMP rather than cAMP itself. As shown in Fig. 3B, 1 μ M 8-Br-cAMP decreased modestly, but significantly, the fraction of phalloidin-labeled phagosomes. SQ22536, the inhibitor of adenylyl cyclase, had no effect on this parameter. The addition of the PKA inhibitor H89 significantly enhanced the fraction of actin-positive phagosomes. This is consistent with the notion that cAMP-dependent activation of PKA is one mechanism through which cAMP effects its inhibition of the phagosome actin assembly process in macrophages. Except for the effects of the inhibitor of adenylyl cyclase, which had no effect in these experiments, these data are consistent with our in vitro data.

Localization of the RII subunit on phagosomes

We next addressed the question whether PKA could be detected on phagosomes and took advantage of an antibody that we had previously used to localize the RII regulatory subunit of this enzyme to endosomes and the TGN (Griffiths et al., 1990). We therefore tested this antibody by immunofluorescence microscopy in J774 cells with internalized latex beads. As shown in Fig. 3A a distinct spot or patches of signal for RII were seen on a significant fraction of phagosomes. In untreated cells 65% of LBPs scored positive for this protein (Fig. 3B). In cells treated with the PKA inhibitor H89 or the adenylyl cyclase inhibitor SQ22536 there was no significant increase or decrease in labeled phagosomes; by contrast, with 8-Br-cAMP this parameter was significantly inhibited (Fig. 3B). These data show that PKA-RII is localized on phagosomes in a manner that can be modulated by cAMP. It is also noteworthy that the fraction of phagosomes that labeled for PKA was very similar to the fraction that labeled with phalloidin (cf. Fig. 3A,B). The presence of the PKA RII was also confirmed by western blot analysis. PKA appeared as a band of 57 kDa (Fig. 3B).

Effects of the cAMP system on phagosomal fusion with lysosomes

From our previous results (summarized in the Introduction) we

would postulate that effectors that stimulated phagosome actin assembly in vitro and phagosome-associated actin in cells would also stimulate phagosome-lysosome fusion. To address this issue we used a recently developed assay for monitoring this process for mycobacterial phagosome fusion with late-endocytic organelles (Anes et al., 2006). This involves pre-internalizing 10 nm colloidal gold for 2 hours before adding the latex beads labeled with rhodamine. We have found that the colloidal gold label is more reliable than other methods we have used. The co-localization of the label with the green fluorescent beads was then scored as a positive fusion event.

As shown in Fig. 3C at the end of the 75-minute bead internalization, just under 60% of phagosomes were found to have fused with late-endocytic organelles. When 8-Br-cAMP was added there was a small, but significant, inhibition of phagosomal-lysosomal fusion. The same level of inhibition was seen when we treated the cells with the IBMX and rolipram cAMP phosphodiesterase inhibitor combination, whose effect should therefore be similar to the addition of cAMP. By contrast, both SQ22536, the inhibitor of adenylyl cyclase and H89, the inhibitor of PKA, had the opposite effect: they significantly stimulated phagosomal-lysosomal fusion. Thus cAMP and PKA are not only negative effectors of phagosomal actin, but also of phagosomal-lysosomal fusion, providing further evidence of intimate links between them.

Effects of the cAMP system on phagosome acidification

The fusion of late-endocytic organelles with phagosomes delivers the H⁺-ATPase that allows the phagosomal pH to be lowered to levels around pH 5.5 or below. The early phagosomes, before these fusion events, like the early endosomes, maintain a pH that is mildly acidic (6-6.2). Whereas the fully matured late phagosomes can accumulate acidotropic dyes such as LysoTracker red, the early phagosomes are not sufficiently acidic to label with these dyes. We therefore monitored the effects of the different cAMP effectors on the fraction of LysoTracker-positive phagosomes. As shown in Fig. 3D, in untreated macrophages after internalization of beads for 75 minutes, about 55% of the phagosomes were positive for LysoTracker (see also Anes et al., 2006), a value which is very close to the 58% that had fused with the gold compartments. More striking was the fact that the effects of increasing cAMP (8-Br-cAMP, IBMX and rolipram) and of decreasing cAMP (SQ22536 and H-89) were essentially quantitatively superimposable on the fusion data

(cf. Fig. 3C,D). As in the fusion process, increases in the cAMP level led to a reduction in the fraction of acidified phagosomes, whereas decreasing cAMP concentration, or inhibiting PKA increased this parameter.

We recently showed that the gold-enriched late-endocytic organelles are distinct from those enriched in the H^+ -ATPase and that these different compartments fuse with *M. smegmatis* phagosomes with quite distinct kinetics (Anes et al., 2006). By contrast, our data here argue that regulation of the fusion of these compartments with latex bead phagosomes by the cAMP system is quite similar. Presumably, the difference seen with *M. smegmatis* phagosomes reflects regulation of the system by the live bacteria.

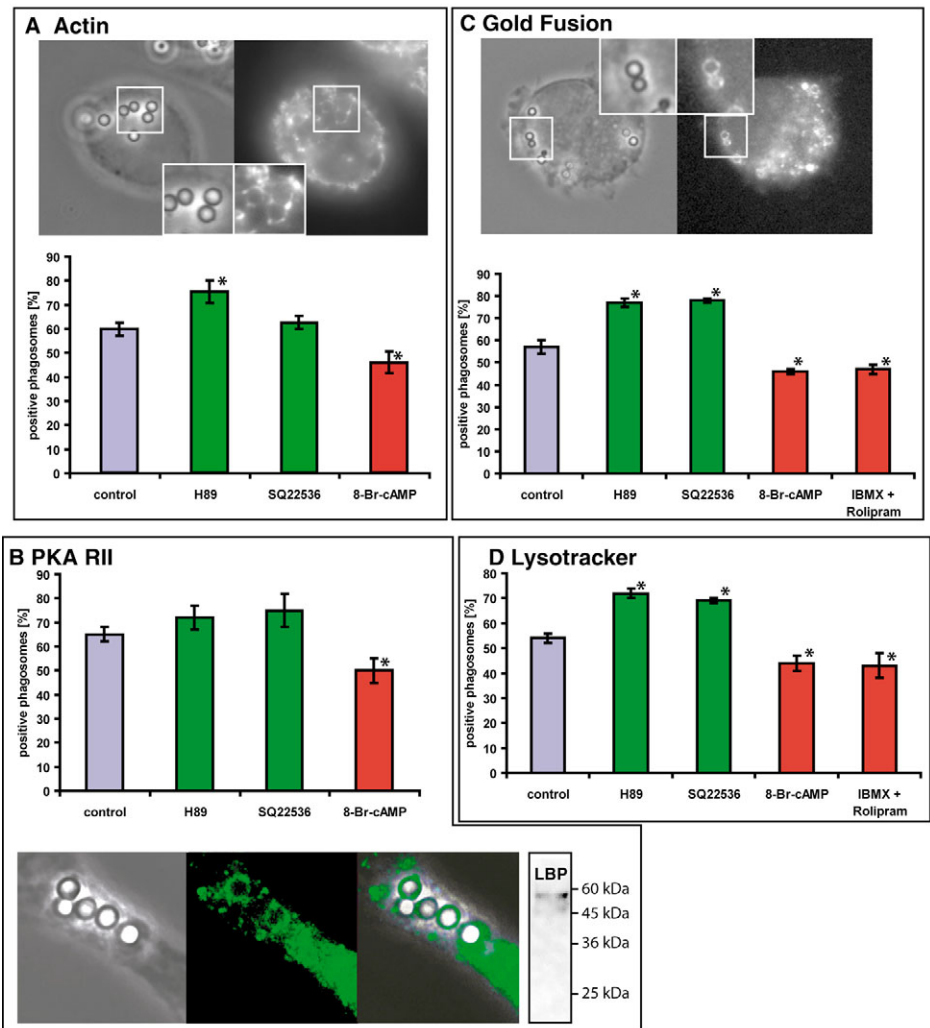
Effects of the cAMP system on mycobacterial growth in macrophages

Based on our recent work (see Introduction) we hypothesized that conditions inducing an increase in intracellular cAMP levels, that reduce phagosomal actin, should facilitate the growth of intraphagosomal mycobacteria, whereas conditions that reduce its concentration should facilitate bacterial

destruction. In order to test this hypothesis we tested the effects of modulating cAMP levels on the intracellular growth of a fast-growing, non-pathogenic species of mycobacteria, *M. smegmatis* (cell doubling time 3 hours) and a slow-growing pathogenic one, *M. tuberculosis* (M.tb-H37 Rv; doubling time ~24 hours). *M. smegmatis* can be completely killed within 48 hours by J774 cells whereas *M. tuberculosis* grows steadily in J774 cells over a 15-day period, after which time the macrophages die (Kuehnel et al., 2001; Anes et al., 2003; Anes et al., 2006).

The effects of the modulation of the cAMP system in mycobacterium-infected cells supported the above hypothesis, albeit at higher concentrations than used for the other macrophage experiments (above). For cells infected with *M. smegmatis* and *M. tuberculosis*, the addition of 50 mM (but not 1 or 10 mM) 8-Br-cAMP itself led to a modest, but statistically significant increase in multiplication of these mycobacteria at early, but not late times of infection; this is shown as CFU of bacteria isolated from macrophages (Fig. 4A,B). For *M. tuberculosis* these results, and the need for higher concentrations of 8-Br-cAMP, are in agreement with

Fig. 3. Effects of cAMP on actin (A), PKA RII (B), fusion (C) and acidification (D) in J774 macrophages. (A) Effects of indicated treatments on LBP-associated actin. Micrograph shows the effect of actin on intracellular LBPs visualized by Rhodamine-phalloidin labeling with a magnification of the LBP area in untreated cells. (Left) DIC-image; (right) Rhodamine-phalloidin image. The histogram shows percentage of LBPs positive for actin under indicated conditions. (B) Analysis of the co-localization of PKA RII. Micrograph shows DIC image of LBPs in macrophages (left), immunofluorescent labeling of PKA RII on LBPs (middle) and the overlay (right). The histogram shows the percentage of LBPs co-localizing with PKA RII under indicated conditions. The blot shows identification of PKA RII on LBPs. (C) Analysis of fusion of LBPs with Rhodamine-gold-filled late endosomes and lysosomes. Micrograph shows an example of lysosomal Rhodamine-gold particles co-localizing with LBPs with a magnification of the LBP area in untreated cells: DIC image (left), Rhodamine-gold image (right). The histogram shows the percentage of LBPs co-localizing with lysosomal Rhodamine-gold under indicated conditions. (D) Analysis of phagosomal acidification. The histogram shows the percentage of LBPs that co-localize with LysoTracker Red DND-99 under indicated conditions. All histograms represent mean \pm s.d. of three individual experiments. Asterisks indicate significance as determined by the Student's *t*-test: * P <0.01 compared with levels in the control.



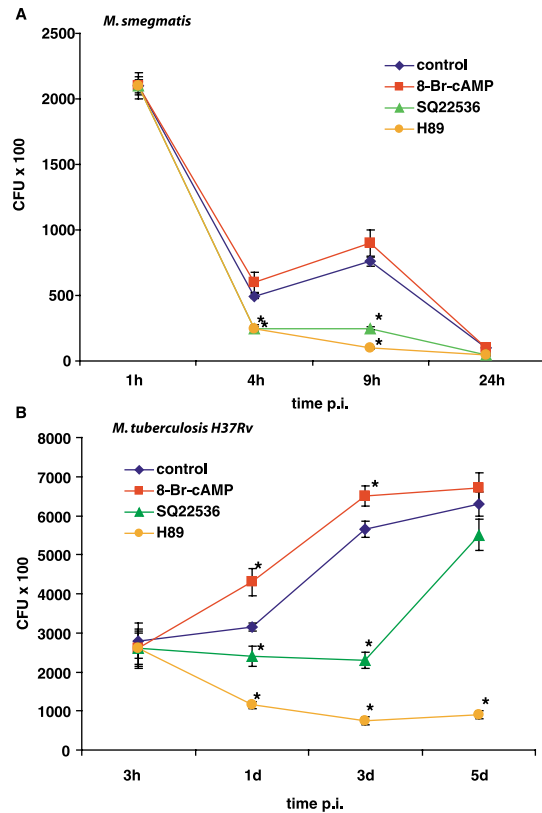


Fig. 4. Effects of cAMP on the destruction of mycobacteria as viewed by CFU plating assay. (A) *M. smegmatis* survival. Results represent mean \pm s.d. from three independent experiments. (B) *M. tuberculosis* H37Rv survival. Reagents were added as follows: 8-Br-cAMP (50 μ M) added at 3 hours and 2 days post infection. SQ22536 (50 μ M) added at 3 hours and 2 day post infection. H-89 (10 μ M) added at 3 hours post infection. Asterisks indicate significance as determined by the Student's *t*-test: * P <0.01 compared with levels in the control.

Majumder and Gupta (Majumder and Gupta, 1994). By contrast, the adenylyl cyclase inhibitor SQ22536, also at 50 μ M (but not 1 or 10 μ M) led to a significant drop in the number of bacterial colonies in both cases. Treatment with 10 μ M H89, to block PKA also led to an increase in destruction of both bacteria. Treatment of free bacteria in culture with 8-Br-cAMP, SQ22536 or H89 had no effect on bacterial viability at any concentration up to 50 μ M (results not shown). These results fit the notion that with increasing macrophage cAMP levels, active PKA is beneficial to the bacteria, whereas decreasing cAMP or inhibiting PKA facilitates their destruction by macrophages.

Discussion

Here, we investigated the role of cAMP and PKA in phagosome cell biology: first by using isolated latex bead phagosomes and subsequently by analyzing these model particles in J774 macrophages. The ensuing results then provided the basis for predicting how a change in cAMP would affect the intracellular survival of mycobacteria. Given the extensive data describing cAMP as a negative regulator of actin in cells we expected that increasing the cAMP concentration

would inhibit assembly of phagosomal actin and would be associated with more pathogen growth, and vice versa. Our data here are fully consistent with this hypothesis and extend our understanding of how the cAMP/PKA system regulates phagosome biology.

We show that after isolation, the phagosomal membrane has adenylyl cyclase activity that synthesizes cAMP, which is able to activate LBP-bound PKA, as well as phosphodiesterase activity that degrades cAMP. The basal LBP system is constitutively active at low (0.2 mM) ATP levels and inhibited by high (5 mM) ATP levels (Anes et al., 2003). Here, we reveal that this process is also strongly regulated by the synthesis and degradation of cAMP by machinery on the LBP membrane. With low ATP levels the adenylyl cyclase system is kept at a low level, whereas above 2 mM ATP (approaching physiological levels) it becomes activated. Increasing cAMP levels then switch off the phagosomal actin-assembly process, at least in part, via PKA activity. Below 1-2 mM ATP, net levels of cAMP synthesis are kept low by a balance between synthesis and degradation, and the actin process is constitutively 'on'; it can however be switched off by exogenous cAMP. It should be noted that the enzymes that make and degrade cAMP are functionally active in an in vitro system free of cytosol or GTP. The absence of cytosol rules out any recruitment of cytoplasmic proteins to the phagosomal membrane under conditions in which it polymerizes actin.

Together with ATP the cAMP system provides a regulatory switch for phagosomal actin assembly. At high ATP levels, cAMP is actively synthesized and PKA is activated, with the overall effect of inhibiting the phagosome actin system. In the presence of the inhibitor of adenylyl cyclase, however, the synthesis of cAMP is inhibited and the LBP actin levels are now elevated. When the ATP level drops below 1-2 mM the cAMP synthesis is kept low, allowing the actin system to be turned on.

The LBP system now offers a powerful in vitro system to decipher the connections between the cAMP system and the other signaling networks we have found to operate on the phagosomal membrane during the process of actin assembly – both by LBPs and mycobacterial phagosomes (Anes et al., 2003) (our unpublished data).

Although most of our recent analysis has focused on the LBP lipids, one of the key proteins identified as being essential for the LBP actin assembly process is ezrin (and/or moesin) (Defacque et al., 2000b); moreover ezrin needs to be bound to phagosomally synthesized PIP2 for optimal activity (Defacque et al., 2002). Since the N-terminal domain of ezrin can bind directly to PKA (Zhou et al., 2003), it is tempting to speculate that PKA bound to ezrin on isolated phagosomes and in macrophages becomes activated by cAMP to phosphorylate key target molecules in the machinery for assembling actin. The effect of this proposed phosphorylation would be to inhibit the actin system. Recent evidence in parietal cells shows that phosphorylation of ezrin on Ser66 by PKA is important for acid secretion by these cells (Zhou et al., 2003; Wang et al., 2005). Whether this, or another site (Ser518) identified in the ERM-related protein merlin (Alfthan et al., 2004) is important in the regulation of actin assembly on phagosomes remains to be determined.

The cellular-signaling state that allows phagosomes to assemble actin also facilitates phagosomal fusion with late

endosomes and lysosomes, as well as the acquisition (from a distinct late-endocytic organelle) of the H⁺-ATPase that acidifies the lumen (Kjeken, 2004; Anes et al., 2003; Anes et al., 2006). Conditions that activate these processes also lead to increased destruction of mycobacteria, whereas negative regulation of phagosomal actin and fusion facilitate pathogen growth (Anes et al., 2003). These observations led to the actin track model (Kjeken, 2004). A similar pattern of correlation is again seen here with the cAMP system: lowering cAMP levels elevated phagosomal actin, acidification and fusion with gold-filled late-endocytic organelles while increasing mycobacterial death in macrophages. By contrast, increasing cAMP levels lowered the actin, acidification and fusion processes and increased the destruction of both the non-pathogenic *M. smegmatis* and the pathogenic *M. tuberculosis* H37 Rv.

Although the magnitude of the effects we observed on bacterial survival was modest, it was both statistically significant, and in agreement with the modest, but significant differences seen with the different cAMP modulators on phagosomal actin, phagosome-lysosome fusion and acidification. One caveat here is that although the main effects are likely to be due to activity of macrophage enzymes we cannot rule out the fact that the effectors and/or inhibitors we used may also target the equivalent enzymes known to be expressed by the mycobacteria themselves (Shenoy et al., 2005; Lowrie et al., 1975; Castro et al., 2005; Cann et al., 2003; Reddy et al., 2001). Complex effects of modulating cAMP levels in mycobacterium-infected macrophages have been described in many studies making it difficult to provide a simple model (e.g. Gazdik and McDonough, 2005; Yadav et al., 2004; Roach et al., 2005; Schorey and Cooper, 2003). It is even conceivable that mycobacteria can make and secrete cAMP into the phagosomal lumen, as suggested from earlier studies (Lowrie et al., 1975). If so, it remains an open question how this pool might communicate with the macrophage-made pool on the other side of this membrane.

The cAMP system is clearly a key factor in determining whether cells such as macrophages kill intraphagosomal pathogens or whether the pathogens survive and grow. A key finding of this study is that we could pinpoint many of the effects of cAMP to the phagosomal membrane itself. With respect to the macrophage goal of achieving full phagosome maturation, the cell presumably needs to keep local cAMP levels low around the phagosome. Under this condition, provided other signals are present, phagosomal actin can be dynamically activated, a process that has recently been observed in living macrophages. These studies show that the growth of actin from late phagosomes is a very transient phenomenon, occurring in the second-to-minute time scale; the actin is subsequently just as rapidly depolymerised (Yam and Theriot, 2004) (M.K. and D. Holzer, unpublished data). Our data in the present study lead us to speculate that the continuous synthesis of cAMP by phagosomes would provide a way to keep the phagosomal-plasma membrane actin system switched off. Only by lowering cAMP transiently can the membrane-catalyzed assembly of actin be transiently activated.

From the perspective of pathogens that multiply within phagosomes, the optimal outcome would appear to be one in which the cellular cAMP levels are sustained at high levels, in order to reduce actin assembly, fusion and other events. As pointed out in the introduction, this is precisely the fascinating

route that many pathogens have followed. By contrast, from the macrophage perspective it would seem beneficial to maintain a low-cAMP zone, at least around the phagosomes. The competition between host and pathogen for the control of this key regulatory system highlights one mechanism that helps to determine which of the protagonists wins the war for survival.

Materials and Methods

Cells and bacteria

The J774 A.1 mouse macrophage cell line and the strains *M. smegmatis* mc2 155, and *M. tuberculosis* H37Rv, were obtained and maintained as previously described. Cells used in this study were between passages 5-25 (Anes et al., 2003; Defacque et al., 2002).

Agents and antibodies

Carboxylated polystyrene beads (uniform microspheres; mean diameter 0.93 μ m) were from Bangs Laboratories. LysoTracker Red DND-99 as 1 mM solution in DMSO and Avidin (NeutrAvidin) were from Molecular Probes (Molecular Probes, Leiden, The Netherlands). Mowiol 4-88 was from Calbiochem. The anti-R11 polyclonal antibody was described (Griffiths et al., 1990a). FITC donkey anti-rabbit antibody was from Dianova. Cyclic AMP concentration affecting reagents H-89, SQ 22536, 8-Br-cAMP, rolipram, IBMX, as well as the rest of the agents (analytical grade) used in this study, were from Sigma.

Solutions of cAMP-modulating agents

For the macrophage experiments, reagents used for the modulation of intracellular cAMP concentrations were prepared as described below and used at following concentrations: H-89 (1 μ M) (Davies et al., 2000), SQ 22536 (10 μ M), IBMX (10 μ M) plus rolipram (1 μ M) or 8-Br-cAMP (1 μ M) (Blocker et al., 1997). The PKA inhibitor H-89 was dissolved in methanol at a concentration of 10 mM. For experiments, the stock solution was diluted 1:10 in double-distilled water and used after an additional 1:1000 dilution in complete medium.

The adenylyl cyclase inhibitor SQ 22536 was dissolved in water as a 10 mM stock solution (Sigma S-153) and used at a final concentration of 10 μ M. 8-Br-cAMP, the membrane permeable analogue of cAMP was stored as a 1 mM stock solution (Sigma B7880) in water and used at 1 μ M concentration in complete medium.

Inhibition of phosphodiesterase was done by a combination of IBMX and rolipram; a 10 mM stock solution of Rolipram in double-distilled water and a 10 mM stock solution of IBMX in DMSO were prepared. Rolipram was used at 1 μ M concentration and IBMX at a 10 μ M concentration in whole medium. IBMX is a non-selective phosphodiesterase inhibitor and rolipram is a PDE4 selective inhibitor.

Mycobacteria CFU after growth in J774 macrophages

CFU counts were performed as described (Anes et al., 2003).

Avidin coating of latex beads

The conjugation of 1 μ m latex beads with avidin was described earlier (Anes et al., 2003; Defacque et al., 2000b; Desjardins et al., 1994).

Preparation of LBPs

Phagosomes containing 1 μ m latex beads, were prepared and kept in liquid nitrogen until the day of use, as previously described (Kuehn et al., 2006; Defacque et al., 2000a; Defacque et al., 2000b; Desjardins et al., 1994).

In vitro actin assembly assay

In vitro actin assembly assay at high (5 mM) and low (0.2 mM) ATP concentrations, was performed as described before (Kuehn et al., 2006; Anes et al., 2003; Defacque et al., 2000b).

Western blot of PKA RII

The protein content of phagosome preparations was measured using the Bio-Rad protein quantification kit and 20 mg of protein were resolved on a 10% polyacrylamide gel and then blotted onto a nitrocellulose membrane. The membrane was washed with 0.1% PBS-Tween 20 and incubated overnight in PBS containing 5% skimmed milk powder and washed in 0.1% PBS-Tween. Protein kinase A regulatory subunit II was detected using an anti-PKA RII antibody (Griffiths et al., 1990) in 0.1% PBS-Tween 20 containing 0.1% BSA for 1 hour. After three washes in 0.1% PBS-Tween 20, bound antibody was detected using an anti-rabbit HRP antibody in 0.1% PBS-Tween 20 containing 0.1% BSA. After intensive washing with 0.1% PBS-Tween 20, the HRP localization was revealed by using the ECL kit from Amersham.

Measurement of cAMP by HPLC

HPLC analysis of cAMP was done using a Beckman Gold HPLC System equipped

with a Phenomenex Spherclone 5 m SAX. Samples for cAMP quantification were generated as follows: $\sim 5 \times 10^8$ phagosomes were incubated in 10 mM Tris-HCl buffer pH 7.4 containing 2 mM $MgCl_2$, 200 μM $CaCl_2$ and 50 mM KCl with 5 mM Mg-ATP for 30 minutes with or without cAMP-modifying drugs as described above. Phagosomes were removed by centrifugation and supernatants containing cAMP were analyzed.

Macrophage assays

J774 macrophages grown on coverslips were used. Cells were pulsed with latex beads for 15 minutes, rinsed in PBS to wash away the remaining beads, followed by a 1 hour chase in complete medium alone, or in medium containing cAMP-modulating agents at the indicated concentrations. Microscopy sample preparation was done as described (Anes et al., 2003; Anes et al., 2006).

Phalloidin staining of J774 cells

Phalloidin staining of macrophages pulsed with beads for 15 minutes and then chased with J774 for 1 hour, was performed as before (Anes et al., 2003; Defacque et al., 2000a). Phagosomes were considered as positive for actin, when exhibiting a signal in the form of spot(s) or patch(es). Only beads that were clearly distinct from the cortical actin were evaluated in this analysis.

Assay of phagosomal acidification with LysoTracker Red DND-99

For phagosomal acidification assays, the acidotropic, fixation-viable, fluorescent agent LysoTracker Red DND-99, was used as described by Anes et al. (Anes et al., 2003).

In vivo fusion assay

Preparation, and labeling of lysosomes was done as described in Anes et al. (Anes et al., 2006). The essence of this method is to internalize colloidal gold (10 nm) coated with bovine serum albumin conjugated to Rhodamine for 1 hour followed by a 1-hour chase. Then, beads were added to the cells for 15 minutes. Non-internalized beads were removed by washing and phagosomes were allowed to mature for 1 hour. Phagosomes were considered positive for fusion with endocytic organelles, when the ~ 1 mM latex beads were surrounded by a homogenous or patchy Rhodamine fluorescence signal.

Immunofluorescence microscopy

This was done as described by Anes et al. (Anes et al., 2003).

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