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pH and Calcium Dependence of Hemolysis Due to *Rickettsia prowazekii*: Comparison with Phospholipase Activity

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Rickettsia prowazekii **invades nucleated cells through phagocytosis and subsequently proliferates in the cytoplasm of the host cell. Hemolysis and a phospholipase A_2 (PLA_2) activity at neutral pHs have previously been reported, even though the phagosomal environment is most likely acidic. We here show that *R. prowazekii* and *R. typhi* also lyse erythrocytes at mildly acidic pHs, compatible with an early phagosomal compartment. For *R. prowazekii*, hemolysis at an acidic pH but not a neutral pH is enhanced by Ca^{2+}, raising the possibility that more than one membranolytic factor may be produced by the rickettsiae. The rickettsiae alone display PLA_2 activity, implying that the enzyme is of bacterial rather than erythrocyte or host cell origin. Moreover, the PLA_2 activity requires divalent cations (Ca^{2+} or Mg^{2+}), and, as with many extracellular PLA_2s from other species, it has a preference for acidic over neutral phospholipids. The pH dependence of PLA_2 is similar to that of the hemolysis without Ca^{2+}, but in the presence of the hemolysis buffers (which contain Mg^{2+}), there is no calcium-induced enhancement at acidic pHs. Thus, these rickettsiae are endowed with a membranolytic activity that could contribute to the escape of the bacteria from early phagosomal compartments, and it is likely that multiple toxins may be used for membrane lysis.**

Rickettsia prowazekii, the causative agent of epidemic typhus and Brill-Zinsser disease, is a gram-negative obligately intracellular bacterium. Rickettsiae can infect a wide range of host cells, ranging from human endothelial cells to louse gut epithelial cells and rodent cells (36). Adherence to these cells takes place through as-yet-uncharacterized receptors on the host cell surface, and upon adherence, the rickettsiae are believed to induce their own phagocytosis (34). Shortly after entry into the host cell, the bacteria are observed within the cell cytoplasm, where they proliferate before exiting and infecting other cells.

Electron microscopic studies on host cell penetration by *R. tsutsugamushi* and *R. conorii* show that bacteria in the process of being phagocytized can be detected within 3 min (17, 22–24). As rickettsiae are found in the cytoplasm within 15 min or less after adhesion (17, 22, 24, 35), one may assume that they escape from the phagosomes at an early stage of phagosome formation, before fusion with lysosomes and most likely before substantial acidification of the vacuole, which (for particles unable to leave phagosomes under normal circumstances) begins within minutes and can ultimately produce a pH below 5 (11). The time course of *R. prowazekii* escape from phagosomes has yet to be characterized by electron microscopy. However, consistent with the rapid escape of rickettsiae from internal vacuoles, fibroblasts infected with *R. prowazekii* have never shown the bacteria to be enclosed within phagosomal membranes (28), suggesting that escape for these rickettsiae may take place at mildly acidic pHs.

The hemolytic activity of *R. prowazekii* has been extensively characterized at neutral pHs for over 4 decades (4, 15, 16, 21, 34) and appears to be associated at least in part with the action of a phospholipase A_2 (PLA_2), which cleaves membrane phospholipids into lysophosphatides and free fatty acids, leading to membrane lysis. Similarly, phospholipase activity has been observed when cytochalasin-treated fibroblasts (L929 cells) are incubated with *R. prowazekii* or when untreated fibroblasts are incubated with large numbers of rickettsiae (33). On the basis of these results, it has been proposed that rickettsiae elaborate or activate a phospholipase that is first involved in inducing rickettsial phagocytosis and is then used by rickettsiae to escape from host cell phagosomes (29). However, the enzyme responsible for the phospholipase activity has not been identified, and it has yet to be properly demonstrated that it is of bacterial rather than host cell origin or that it is in fact used by the bacteria to escape from phagosomes.

Furthermore, as bacterial escape from phagosomes presumably occurs under the acidic or mildly acidic conditions most likely found in phagosomes normally, the rickettsial membranolytic activity most relevant for survival within nucleated cells should be expressed at acidic rather than neutral pHs. We have therefore characterized the membranolytic activity of *R. prowazekii* as a function of pH, using hemolysis as a convenient readout system. Since most known secreted PLA_2s also contain a calcium-binding site and display a hydrolytic activity that is strongly dependent on calcium (7, 10, 12, 19, 25, 26), we also studied the effects of calcium on hemolysis. These studies were complemented by measurements of PLA_2 activity, as a function of pH and in the presence of divalent cations, in order to ascertain whether the PLA_2 alone could account for all the characteristics of hemolysis.

MATERIALS AND METHODS

Materials and cells. *R. prowazekii* (strain Madrid E) and *R. typhi* (*R. mooseri*, Wilmington) were obtained from the American Type Culture Collection (Rockville, Md.). Sheep erythrocytes were purchased from Sanofi Diagnostics Pasteur (Marnes-la-Coquette, France). PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and purified PLA_2 from *Naja mossambica mossambica* were from Sigma (St. Louis, Mo.), and dithiothreitol (DTT; Cleland's reagent) was purchased from Boehringer GmbH (Mannheim, Germany). All pyrene-labelled phospholipids were from Interchim (Montluçon, France).

SPG buffer consisted of 218 mM sucrose–3.76 mM KH_2PO_4–7.1 mM

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K_2HPO_4–4.9 mM glutamate, pH 7.0 (3), to which was added 10 mM $MgCl_2$ to give SPG-Mg. Hemolysis buffer solutions at different pH values were made with 200 mM sucrose, 5 mM glutamate, 10 mM $MgCl_2$, and the following buffers (50 mM) at the indicated pHs: sodium acetate, pH 5; sodium acetate, pH 5.5; PIPES, pH 6; PIPES, pH 6.5; PIPES, pH 7; and HEPES, pH 7.5.

Growth and isolation of rickettsiae. The rickettsiae were grown in egg yolk sacs, and they were purified essentially as previously described (15), except that the 20% suspensions of infected yolk sacs were first incubated in SPG-Mg with 2 M KCl at 4°C for 2 h (conditions under which there is not a significant loss of rickettsial viability [3]) in order to remove most of the egg yolk, and the mixture was homogenized in a blender. The suspension was centrifuged for 10 min at 600 × *g*, the yolk sac-derived top fatty layer was discarded, and the remaining supernatant was centrifuged for 10 min at 12,000 × *g*. The resulting pellet was homogenized, resuspended to the original volume of the 20% egg yolk sac suspension with SPG-Mg, and centrifuged for 10 min at 120 × *g*. The pellet was discarded, the supernatant was centrifuged again for 10 min at 12,000 × *g*, and the new pellet was resuspended in SPG-Mg to one-fourth of the volume of the 20% suspension, giving the final 80% rickettsial suspension used in subsequent experiments. This suspension typically had an optical density (at 600 nm) of about 2.0 and contained approximately 1 mg of protein per ml, and the purity of the suspension was checked by light microscopy with Gimenez stain (8). All centrifugation steps were carried out at 4°C, and the 80% suspension was kept on ice until ready for use in the hemolysis experiments. Because of the high lability of the hemolytic activity (4), the rickettsiae were used within 1 to 2 h after their isolation.

Hemolysis experiments. Sheep erythrocytes were washed by centrifugation three times in phosphate-buffered saline (PBS), pH 7.4, and the packed erythrocytes were resuspended to a 25% (vol/vol) suspension in SPG-Mg. The indicated buffers (100 μl per well) were introduced into 96-well plates (Costar) in triplicate. To the buffer was added 50 μl of the 80% bacterial (*R. prowazekii* or *R. typhi*) suspension, followed by 20 μl of the 25% erythrocyte suspension, and the resulting suspension was thoroughly mixed. The plates were placed in an incubator at 34°C for 2 h, and the samples were then fixed by the addition of 50 μl of PBS containing 0.8% formaldehyde. After a 1-h incubation at room temperature, the fixed samples were centrifuged and 100 μl of each supernatant was transferred to another plate and placed in a Dynatech MR5000 plate reader for measurement of hemoglobin release after the A_{545} was read. The level of spontaneous hemolysis was determined by adding 20 μl of the 25% erythrocyte suspension to 150 μl of the indicated buffers without bacteria, incubating for 2 h at 34°C, and measuring hemoglobin release. Complete hemolysis was measured by adding the erythrocytes to 150 μl of distilled water; typically, lysis of 100% of the erythrocytes corresponded to an absorbance reading of 1.0.

Measurement of phospholipase activity. Immediately after bacterial isolation, an 80% suspension of *R. prowazekii* was incubated with 4% erythrocytes (final concentration) for 5 min at 25°C and then kept on ice until ready for use in the PLA_2 activity assays. Typically, all measurements were performed within 3 h after the end of bacterial isolation. As preliminary experiments indicated that purified PLA_2 from *N. mossambica mossambica* venom is insensitive to treatment with 0.2% formaldehyde for 3 h, the rickettsial PLA_2 activity was measured after incubation with this concentration of formaldehyde for 30 min in order to permit fluorescence measurements outside of P3 facilities. Because formaldehyde treatment may inhibit expression of the rickettsial PLA_2, these measurements may underestimate the amount of phospholipase activity in rickettsiae. The experiments were also repeated with rickettsiae alone which had never been preincubated with erythrocytes.

The PLA_2 activity was measured with fluorescent phospholipid substrates, essentially as previously described (13). The substrates used were 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (PC); 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol (PG); and the phosphatidic acid analog 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-monomethyl phosphatidic acid (PA). For measurements of PLA_2 activity as a function of pH, either the SPG-Mg hemolysis media were used or the experiments were done in phospholipase buffer (137 mM NaCl, 27 mM KCl, and the buffers [15 mM] indicated above for the different pH values [without sucrose, glutamate, and magnesium]). The PLA_2 reaction medium was prepared by the sequential addition of 1 ml of buffer at room temperature, one of the fluorescent substrates (usually PA) (final concentration, 2 μM), 10 μl of a 10% fatty acid-free bovine serum albumin solution, and 25 μl of the 80% rickettsial suspension. The reaction was started by the addition of either 10 mM $CaCl_2$ or 10 mM $MgCl_2$, and the fluorescence increase during the first 5 min was used to calculate PLA_2 activity.

RESULTS

pH dependence of hemolysis. We observe that *R. prowazekii* displays high levels of lytic activity against sheep erythrocytes at neutral pHs (Fig. 1), which is in agreement with previous reports (15, 21, 32). Although the extent of hemolysis varied somewhat among different rickettsial preparations, *R. prowazekii* typically lysed 20 to 25% of the erythrocytes at neutral pHs under the conditions used in this study. Spontaneous hemolysis, in the absence of bacteria, was always less than 1%. The hemolysis due to the rickettsiae was extremely labile, however, and much of the activity was lost by subjecting the rickettsiae to a 1-h treatment at 37°C or by simply leaving the bacteria at 4°C overnight. Over 90% of the hemolysis was inhibited by pretreatment of the bacteria with 1 mM DTT for 1 h at 37°C at a neutral pH (data not shown).

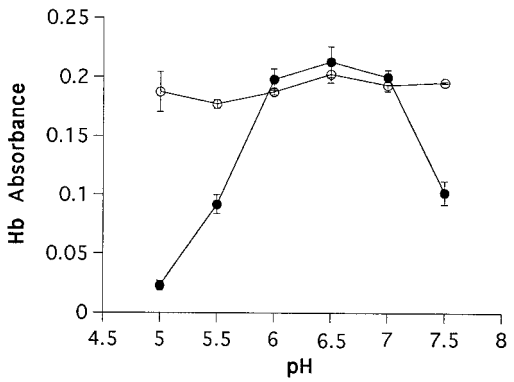

FIG. 1. Lysis of sheep erythrocytes by *R. prowazekii* in buffers at different pH values. The highest level of hemolytic activity represents lysis of 20 to 25% of the erythrocytes; spontaneous hemolysis was 1%. Measurements were performed in triplicate. Bars show standard errors. Hb, hemoglobin. ●, hemolysis buffer; ○, hemolysis buffer plus 6 mM $CaCl_2$ (final concentration).

In order to address the question of whether this membranolytic activity could extend to the acidic pH of phagosomes, we also measured hemolysis as a function of pH (Fig. 1). Extensive activity was in fact observed at a pH as low as 6; this activity decreased substantially by pH 5.5 and disappeared at pH 5. The optimal pH for hemolysis was between pH 6 and pH 7.0, consistent with an activity expressed in an early phagosomal compartment.

Effect of calcium on hemolysis. Hemolysis as a function of pH was also measured in the presence of calcium. As in previous studies (21, 32), calcium did not have a large effect on hemolysis at neutral pHs. However, large stimulatory effects, which were reproducible in all bacterial preparations, were observed with calcium at acidic pHs and at pH 7.5 (Fig. 1).

Hemolysis due to *R. typhi*. Hemolytic activity was previously observed when sheep erythrocytes were incubated with *R. typhi* at a neutral pH (21). We find that this activity is also present at acidic pHs, raising the possibility that other rickettsiae may also display hemolytic activity at acidic pH. This activity was observed reproducibly in different *R. typhi* preparations and was inhibited by 1 mM DTT, but in our hands, it was much lower than the activity observed in *R. prowazekii* preparations (data not shown). Mild-acid-dependent membranolytic agents may therefore also be present in other rickettsia species.

Phospholipase activity of *R. prowazekii*. The PLA_2 activity was measured by a sensitive fluorescent assay that has been previously described (13). Briefly, phospholipid substrates tagged with pyrene on the second acyl chain were incubated with purified PLA_2 or with rickettsiae. In the absence of phospholipase activity, the fluorescence of the pyrene is self-quenched in membranes or micelles made from the fluorescent phospholipids, but hydrolysis of the phospholipids causes the pyrene-labelled acyl chains to be released and, consequently, causes the fluorescence to increase.

Initial experiments with rickettsiae were performed in the hemolysis medium, i.e., SPG-Mg. The amount of PLA_2 activity was variable from one bacterial preparation to the other; under the best conditions, the highest values varied between 0.4 and

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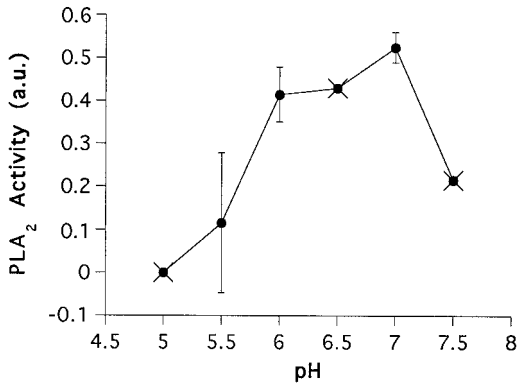

FIG. 2. PLA_2 activity as a function of pH. Activity in the hemolysis buffers containing sucrose, glutamate, and magnesium (●) is shown. Addition of 6 mM calcium (×) to the hemolysis buffers had no effect on PLA_2 activity. Three different *R. prowazekii* preparations prepared at different weeks gave qualitatively similar pH dependence profiles. Bars show standard errors. a.u., arbitrary units.

0.9 nmole/min/mg of rickettsiae. The activity was completely inhibited by treatment with 1 mM DTT for 1 h at 37°C.

The pH dependence of the PLA_2 activity due to *R. prowazekii*, measured in hemolysis buffers, is shown in Fig. 2 and resembles the profile for hemolytic activity in the absence of calcium described above (Fig. 1). No cation dependence was observed when the measurements were performed in the hemolysis medium, i.e., SPG-Mg. However, when a standard buffer (containing K^+ and Na^+ but no sucrose or Mg^{2+}) was used alone, essentially no PLA_2 activity was measured, but the activity increased considerably in the presence of either of the two divalent cations tested (Mg^{2+} or Ca^{2+}). Thus, the divalent-cation dependence was masked by the high concentration of Mg^{2+} typically used in hemolysis buffers but was observed in the standard phospholipase buffer devoid of Mg^{2+}. Unlike the calcium dependence displayed by hemolysis, though, there was no calcium-induced enhancement of PLA_2 activity at any of the pH values when SPG-Mg was used as the assay buffer, and under no circumstances did either of the two divalent cations have any effect on the lack of phospholipase activity at pH 5.

The experiments above were done with rickettsiae that had been preincubated for 5 min at room temperature with erythrocytes. Nonetheless, similar values for PLA_2 activity were also obtained with purified rickettsiae alone, implying that the phospholipase activity is expressed on the bacterial surface or in the extracellular space even in the absence of target membranes.

Given the possibility that the PLA_2 activity might be due to contaminants from the yolk sacs used to culture bacteria, over 10-fold quantities of uninfected yolk sacs were subjected to the purification procedure used for bacteria, in order to identify the behavior of potential contaminants in the bacterial preparation. An artifactual activity, probably due to fatty acids, was in fact observed with the fluorescence assay, but it was measured even in the absence of divalent cations and was not affected by DTT. As no PLA_2 activity was detected with the rickettsial preparations in the absence of Mg^{2+} or Ca^{2+} and the bacterial activity was completely inhibited by treatment with 1 mM DTT for 1 h, one can safely assume that no residual activity due to the yolk sacs remained at the rickettsial concentrations described above.

The substrate specificity for the rickettsial PLA_2 was also measured. Similar to the specificity observed with many extracellular phospholipases (10, 13, 25), the rickettsial PLA_2 has a preference for the negatively charged phospholipids PA and PG. The PLA_2 activities (expressed in nanomoles per minute per milligram of whole bacterium ± standard deviation) for PA and PG were 0.9 ± 0.04 and 0.73 ± 0.09, respectively. Hydrolysis of the zwitterionic PC was below the level of detection of the fluorescence assay (i.e., PLA_2 activity of <0.1) at the rickettsial concentrations used in this study.

DISCUSSION

The adsorption of rickettsiae to erythrocytes and the subsequent hemolysis, as well as the infection of nucleated cells, are dependent on the metabolic state of the bacteria (6, 16, 21). The energy requirement for hemolysis suggests that either toxic factors must be secreted from the bacteria following contact with membranes or toxins bound on the bacterial surface must change conformation through an energy-consuming step in order for toxicity to be revealed.

Previous studies have determined that a phospholipase activity accompanies both the hemolysis and infection of nucleated cells (27, 30, 32, 33). Analysis of the phospholipid degradation products suggests that a PLA_2 may be responsible for this activity, and it is widely believed that this enzyme may be involved in both rickettsial entry into the host cell and escape from the phagosomes (34). Although it remained to be shown directly whether the phospholipase activity was due to the bacteria or to the host cell, recent studies have shown that host cell uptake of *R. rickettsii* is reduced when the bacteria are pretreated with phospholipase inhibitors or antiserum, suggesting that the rickettsiae themselves may produce this enzyme (20). Our experiments showing PLA_2 activity in purified *R. prowazekii*, in the absence of any target membranes, further strengthen the view that a bacterial enzyme is responsible for the phospholipase activity.

All studies to date report on the hemolytic activity of *R. prowazekii* at neutral pHs, even though the rickettsiae would presumably require toxins that are effective at the acidic pH of phagosomes. While it remains to be verified whether phagosomes harboring rickettsiae are in fact acidic, we find that rickettsiae lyse erythrocytes at acidic pHs and that the pH dependence of hemolysis is consistent with a membranolytic activity expressed in early phagosomal compartments. For other bacteria, such as the *Shigella* species, it has previously been shown that there is a tight correlation between hemolytic activity and the ability to invade nucleated cells (5).

It has been previously reported that calcium does not have a significant effect on rickettsia-mediated hemolysis at neutral pHs (21, 32). We observe that calcium has at best only a minor effect at a neutral pH but that hemolysis is much greater at an acidic pH when calcium is included in the hemolysis assay. Two possible explanations could be invoked to explain the enhanced hemolysis. First, calcium could increase the activity of a single toxin, such as PLA_2, at only acidic and basic pH values and not at a neutral pH. However, this explanation would seem to be at odds with our observation that the rickettsiae do not display any calcium-dependent PLA_2 activity at pH 5. Second, calcium may induce the activity of one or several toxins other than the hemolytic toxin previously characterized at neutral pHs. Both pore-forming proteins (1, 2, 14, 37), which bind to membranes and polymerize into transmembrane ion-conducting pores, and secreted phospholipases (7, 10, 12, 19, 25, 26) have been identified in other systems with membranolytic activities dependent on calcium, and our data at this point do not discriminate between pore-forming proteins and phospholipases other than PLA_2.

Besides requiring divalent cations for its activity, the rick-

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ettsial PLA_2 displays a marked preference for negatively charged phospholipids (PA and PG) compared with the zwitterionic PC. This is in accord with the specificities previously measured for extracellular PLA_2, which generally hydrolyzes PG and phosphatidylserine much better than it does PC (10, 13, 25). Previous studies on PLA_2 activity in the presence of *R. prowazekii* have measured the cleavage of PC and phosphatidylethanolamine (31–33), but a comparison of these specificities with those of the negatively charged headgroup was not reported.

As previously pointed out (32), pure PLA_2 does not cause hemolysis unless additional factors such as other lipases are added to the hemolysis mixture (9, 18). This suggests that rickettsiae may elaborate cofactors besides the phospholipase previously characterized at neutral pHs, and this possibility must be kept in mind when hemolytic assay results are interpreted. The data presented above are consistent with the possibility that additional cofactors may contribute to the escape of rickettsiae from early phagosomal compartments. Elucidation of the molecular basis for escape from phagosomes will have to await the identification and characterization of the membrane toxin(s) produced by rickettsiae.

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