Influence of Calcium on Secretion and Activity of the Cytolysins of Actinobacillus pleuropneumoniae

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In vitro production of a secreted hemolytic cytolysin of Actinobacillus pleuropneumoniae has been reported to be dependent on the presence of calcium in culture media. This is not the case with Escherichia coli hemolysins, however, where calcium has been shown to be required only for activation and binding to target cells. Because the cytolysins of A. pleuropneumoniae have structural and functional similarities to those of hemolytic E. coli, we sought to reexamine the role that calcium plays in the secretion and activity of A. pleuropneumoniae cytolysins. A. pleuropneumoniae hemolytic strain S4074 secreted two major proteins into culture supernatants independent of the presence of calcium in growth medium. These proteins were identified with murine monoclonal antibodies as the 105-kDa cytolysin I and the 103-kDa cytolysin II. It was found that both cytolysins required calcium for binding to erythrocyte membranes. Culture fluids from bacteria grown with calcium lysed porcine erythrocytes even after free calcium in the fluid was removed prior to the hemolytic assay. When bacteria were grown in medium depleted of calcium, no lysis of erythrocytes was detected unless calcium was added to assay buffers. Culture supernatants from A. pleuropneumoniae nonhemolytic strain 1421 grown with or without calcium contained two predominant proteins, which were identified with mouse monoclonal antibodies as the 103-kDa cytolysin II and the 120-kDa cytolysin III. Binding to erythrocytes (without hemolysis) by cytolysin II was dependent on calcium. Cytolysin III did not bind to erythrocytes. These results indicate that the ability of strain S4074 to lyse swine erythrocytes (and the inability of strain 1421 to do so) was directly correlated with the presence of cytolysin I. Cytolysins I, II, and III bound to swine neutrophils and purified neutrophil membranes only in the presence of calcium. When calcium was depleted, cytolysin I of strain S4074 had a reduced binding and cytolysis II and III of strain 1421 did not bind at all. The data suggest that regardless of the target cell involved, calcium plays an integral role in the function but not the production of A. pleuropneumoniae cytolysins.

Actinobacillus pleuropneumoniae is the etiologic agent of swine pleuropneumonia (19). Lung lesions caused by instilling sterile culture fluids into the lungs of pigs appear similar both macroscopically and microscopically to those occurring early in natural disease (18, 22). It is therefore postulated that cytolytic and hemolytic proteins secreted by the bacteria in vitro contribute to lung lesions in vivo. A. pleuropneumoniae strains secrete heat-labile cytolysins that differ antigenically among serotypes (4, 13, 14). Some secreted cytolysins that lyse erythrocytes, polymorphonuclear cells, and macrophages in vitro, while others secrete cytolysins that are weakly hemolytic but still toxic to neutrophils and macrophages (13, 14, 21). Recently, three different cytolytic proteins (Cly) secreted by A. pleuropneumoniae strains have been identified and designated as Cly I (105 kDa), Cly II (103 kDa), and Cly III (120 kDa). Cly I is associated with strong hemolytic and cytotoxic activity, Cly II is associated with weak hemolytic and moderate cytotoxic activity, and Cly III is associated with strong cytotoxic activity but not with hemolytic activity (13). It has been reported that the production of a 105-kDa cytolysin with hemolytic activity, referred to as Hly 1, is dependent on the presence of calcium in growth medium, while the expression of a nonhemolytic cytolysin, called Hly 2, is apparently calcium independent (7, 9).

Several laboratories have cloned and sequenced the hemolysin genes of A. pleuropneumoniae serotypes 1, 5, and 9. It appears that these hemolysins are related to the RTX family of cytotoxins produced by various gram-negative pathogens including Pasteurella hemolytica and Escherichia coli (3, 16, 19). In contrast to the reported calcium-dependent expression of the A. pleuropneumoniae hemolysin, it has recently been shown that the expression of the E. coli hemolysin (HlyA) is independent of calcium but requires calcium for hemolytic activity. HlyA requires calcium for binding to erythrocyte membranes in order to exert its cytolytic effect (1, 2).

The study which we report here was undertaken to clarify the role that calcium plays in the secretion, activity, and binding to target cells of the cytolysins from A. pleuropneumoniae hemolytic strain S4074 and nonhemolytic strain 1471.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Cultures of A. pleuropneumoniae hemolytic strain S4074 (reference strain for serotype 1) and nonhemolytic strain 1421 (reference strain for serotype 3) were obtained from the American Type Culture Collection (Rockville, Md.). Bacteria were grown overnight at 37°C on blood agar supplemented with 0.05% NAD (Sigma Chemical Co., St. Louis, Mo.). Single colonies were picked and inoculated into 5 ml of Luria-Bertani (LB) broth supplemented with 0.05% NAD and either 5 mM CaCl₂ or 10 μM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA; Sigma). The cultures were incubated at 37°C while shaking. When bacteria

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reached the logarithmic stage of growth (about 4 h following inoculation), 1.5 ml of culture was transferred into Erlenmeyer flasks containing 50 ml of LB broth supplemented with NAD and either 5 mM CaCl₂ or 10 μM EGTA. The initial cell concentrations were adjusted to an optical density of 0.03 at an A₅₅₀ as determined on a Beckman DU64 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The cultures were incubated at 37°C while shaking, and samples for the following procedures were collected hourly. (i) Bacterial growth was monitored by measuring the A₅₅₀ of culture samples. (ii) Supernatant fluids were collected from samples after bacteria were pelleted at 1,650 × g for 4°C for 5 min. The fluids were sterilized by passage through 0.22-μm-pore-size syringe filters (Millipore, Bedford, Mass.) and assayed for hemolytic or cytolytic activity (see below). (iii) Proteins secreted into the medium during growth were precipitated from sterile culture fluids by adding trichloroacetic acid to a final concentration of 10% (wt/vol). Following incubation for 1 h at 0°C, the samples were centrifuged at 8,800 × g for 4°C for 15 min. The supernatant fluids were discarded, and the precipitated protein pellets were dissolved in a small volume of 1.0 M Tris (pH 9.06). After being mixed with an equal volume of 2× Laemmli buffer (15), the samples were heated for 5 min at 100°C and stored at −20°C.

Assays of binding of \textit{A. pleuropneumoniae}-secreted proteins to swine erythrocytes and purified erythrocyte membranes. Heparinized blood samples taken from 3- to 5-month-old pigs by puncture of the anterior caval vein were allowed to settle at room temperature. The plasma and buffy coats were discarded, and the remaining erythrocytes were washed five times by pelleting them at 440 × g for 10 min and then resuspending them in physiologic saline (PS). After the last wash, the cells were suspended in PS to a concentration of 10%. Aliquots (1 ml) were then washed three more times with either 10 mM CaCl₂ or 10 mM EGTA in PS, pelleted, and suspended in 0.8 ml of filter-sterilized culture fluid from strain S4074 or 1421 (each grown in the presence or absence of calcium). After incubation for 1 h at 37°C while shaking, the samples were centrifuged at 14,500 × g for 5 min and the pellets were washed three times with saline. To remove excess hemoglobin, erythrocytes were lysed hypotonically with 5.0 mM Na₂PO₄ (pH 8.0). After centrifugation (14,500 × g for 5 min), the pelleted erythrocyte membranes were mixed with an equal volume of 2× Laemmli buffer, heated for 5 min at 100°C, and stored at −20°C until subjected to polyacrylamide gel electrophoresis (PAGE) (see below).

Experiments to test the binding of secreted bacterial proteins to isolated erythrocyte membranes were also conducted. Erythrocyte pellets (from 1 ml of a 10% suspension) were lysed in 1.0 ml of 5.0 mM Na₂PO₄ (pH 8.0) and washed in saline until the pellets became colorless. The membranes were then washed three more times with either 10 mM CaCl₂ or 10 mM EGTA. One milliliter of log-phase sterile culture fluid from either strain, grown with or without calcium, was added to membrane pellets and incubated at 5 min at 37°C while shaking. Samples were centrifuged at 14,500 × g for 1 min and resuspended in fresh culture fluids. This procedure was repeated 10 times. Membranes were repeatedly incubated with culture fluids in order to increase the loading of bacterial proteins bound to erythrocyte membranes. The membranes were then washed five times with saline to remove any unbound proteins and prepared for PAGE as described above.

\textbf{Hemolytic assays.} Hemolytic titers were determined by combining twofold dilutions of sterile fluids from log-phase cultures with equal volumes of a 1% erythrocyte suspension in 10 mM CaCl₂ in PS and then incubating the mixture for 2 h at 37°C with gentle shaking. Cells and cell membranes were pelleted by centrifugation at 14,500 × g for 30 s, and the degree of hemolysis was determined by measuring the A₅₄₅ of released hemoglobin. The hemolytic titer was calculated as the reciprocal of the dilution that showed ≥50% hemolysis compared with a positive hemolytic control. To study hemolytic activity in the absence of calcium, sterile culture fluids were combined with a 1% erythrocyte suspension in 10 mM EGTA rather than 10 mM CaCl₂.

\textbf{Isolation of swine neutrophils and purification of neutrophil membranes.} One volume of heparinized swine blood was gently mixed with two volumes of PS containing 6% dextran (mol. weight, 70,000) in silicon-coated tubes. The tubes were incubated for 1.5 h at 37°C, and the cells were pelleted for 10 min at 180 × g. Residual erythrocytes were isotonically lysed with ammonium chloride, and neutrophil-enriched supernatant fluids were washed with PS and layered onto 3 ml of Ficoll-Isoopaque (Sigma). The samples were centrifuged at 300 × g for 30 min. Pellets contained over 98% neutrophils with a viability of over 95% as determined by nigrosin dye exclusion (see below). The neutrophils were washed three more times with PS and suspended in PS to a final concentration of 10⁷ cells per ml until used for cytotoxicity and protein-binding assays.

The following procedures were used to purify neutrophil cell membranes (12). Neutrophils harvested after Ficoll gradient centrifugation were washed three times in 20 volumes of ice-cold 40 mM KCl–50 mM KPO₄ (pH 6.2). Cells were washed one time in 20 volumes of ice-cold 100 mM sorbitol–40 mM sodium acetate (pH 4.9) and resuspended to a final concentration of 50% in the same buffer. In preparation for membrane isolation, Affi-Gel 731 beads (Bio-Rad, Richmond, Calif.) were washed three times in 0.2 M NaCl and then three times in attachment buffer (20 mM sodium acetate–140 mM sorbitol, pH 4.9). A 50% bead suspension in attachment buffer was vortexed, and 0.3 ml was quickly removed and added slowly to 0.3 ml of the 50% neutrophil suspension. Beads were immediately neutralized by adding 0.5 ml of spermine (Sigma) (1 mg/ml in 100 mM sorbitol–20 mM Tris, pH 7.7), gently mixed, and allowed to settle. Supernatant fluids were discarded, and excess unattached cell membranes were removed by washing the beads three times with 130 mM sorbitol–10 mM MES (morpholineethanesulfonic acid, pH 6.0). The beads were pelleted between washes at 14,500 × g for 5 s at room temperature in a microcentrifuge. After the final wash, the pelleted beads were vortexed for 5 s, and 0.5 ml of sorbitol buffer (pH 6.0) was added. After the beads settled, they were vortexed a second time and placed on ice until used for binding assays.

\textbf{Binding of} \textit{A. pleuropneumoniae}-secreted proteins to neutrophils. Filter-sterilized supernatant fluids (0.8 ml) from log-phase cultures of strain S4074 or 1421 were added to 5 × 10⁷ pelleted neutrophils that had been previously washed with either 10 mM CaCl₂ or 10 mM EGTA as described above. The cells were incubated for 5 min at 37°C while shaking, pelleted at 14,500 × g for 10 s, and resuspended in fresh culture fluids. This procedure was repeated five times. The final pellets were combined with 2× Laemmli sample buffer. Lysed cell samples were viscous because of the presence of nuclear DNA and required several passages through a pipette tip before being loaded onto the gels.

\textbf{Binding of secreted proteins to purified neutrophil membranes.} Filter-sterilized culture fluids (1 ml) from either of the two strains grown to log phase were added to pelleted beads coated with neutrophil membranes (from 0.3 ml of a
50% neutrophil suspension; see above) and incubated at 37°C for 5 min while shaking. Beads were pelleted as described above, the supernatants were discarded, and fresh culture fluids were added. The procedure was repeated 10 times. After the last incubation and spin, 0.3 ml of 5% sodium dodecyl sulfate (SDS) in PS was added, and the samples were heated for 5 min at 80°C. The beads were pelleted, and the supernatants were mixed with an equal volume of 2× Laemml buffer, heated for 5 min at 100°C, and stored at -20°C until used for PAGE analysis.

**Cytocidal assay.** Undiluted filter-sterilized supernatant fluids (1.0 ml) from strain S4074 or 1421 cultured 4 h in the presence or absence of calcium were combined with 2 × 10⁵ neutrophils that had been washed with PS and pelleted. After incubation of the fluid for 6 h at 37°C while shaking, aliquots were removed and cell viability was determined by nigrosin (0.2%) dye exclusion (11).

**SDS-PAGE and immunoblotting.** Samples (5 to 10 μl) were electrophoresed in a Bio-Rad minigel apparatus at 15 mA constant current through 5% stacking and 7.5% resolving gels by the discontinuous buffer system of Laemmli (15). Prestained molecular weight standards (Sigma) were run simultaneously to determine the approximate molecular masses of samples. Proteins were stained with 0.1% Coomassie brilliant blue in 50% (vol/vol) methanol–10% acetic acid and destained in 25% methanol–7.5% acetic acid. Proteins analyzed by immunoblotting were electrophoretically transferred for 1 h at 100 V in 20 mM Tris–192 mM glycine–20% methanol (pH 8.3) to nitrocellulose membranes (Sigma; pore size, 0.4 μm) by using a Bio-Rad Miniblot unit (20). Protein-binding sites on the nitrocellulose membranes were blocked by overnight incubation in 5% horse serum in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl; pH 7.5). The membranes were probed with conavalens-stage sera from pigs that had each been sequentially infected with strains S4074 and 1421 by endobronchial inoculation (23). These sera neutralized the hemolytic activities of culture fluids from strain S4074 and neutralized the cytotoxic activities of fluids from both strains. The sera were diluted 1:300 in TBS containing 2% gelatin and incubated with membranes for at least 2 h. Membranes were washed four times with TBS and incubated with rabbit anti-swine immunoglobulin conjugated to horseradish peroxidase (Sigma) that had been diluted 1:1,000 in TBS. After a further 2-h incubation, the membranes were washed four times with TBS. Immunoblots were developed in 50 ml of TBS containing 25 mg of diaminobenzidine and 10 μl of 30% H₂O₂.

To further identify bound proteins secreted by either of both strains, proteins from gels were transferred to nitrocellulose as described above. Protein-binding sites on the nitrocellulose membranes were blocked by overnight incubation in 10% skimmed milk powder (DMV Campina, Amsterdam, The Netherlands) in TBS. The membranes were probed with 1:400 dilutions of monoclonal antibody (Mab) MAbCVI-Ap ply-9.2, MAbCVI-Ap ply-9.3, or MAbCVI-Ap ply-2.2, which have recently been described (13). MAbCVI-Ap ply-9.2 reacts with the 105-kDa cytolsin (Cly I), MAbCVI-Ap ply-9.3 reacts with the 103-kDa cytolsin (Cly II), and MAbCVI-Ap ply-2.2 reacts with the 120-kDa cytolsin (Cly III) (13). Membranes were washed four times with TBS and incubated with rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Sigma) that had been diluted 1:1,000 in TBS. Immunoblots were developed as mentioned above.

**RESULTS**

**Production of 105-kDa secreted protein of strain S4074 and 103- and 120-kDa secreted proteins of strain 1421 is independent of calcium.** Bacteria of strains S4074 and 1421 were grown in LB broth containing 5 mM CaCl₂ or depleted of calcium by the addition of 10 μM EGTA. Samples were removed from cultures every hour to determine cell density and to obtain trichloroacetic acid precipitates from sterile culture fluids for SDS-PAGE and Western blotting (immunoblotting) analysis. The major secreted proteins of both strains were produced in approximately the same quantities (as detected by Coomassie blue staining or immunoblotting) irrespective of whether calcium was added to or depleted from the culture medium (Fig. 1B). The major secreted proteins of strain S4074 had molecular masses of approximately 105 kDa. Strain 1421 produced two major secreted proteins with approximate molecular masses of 103 and 120 kDa. Two minor proteins with molecular masses of approximately 60 and 40 kDa were also detected in supernatant fluids from strain 1421 (Fig. 1).

**Hemolytic and cytolytic activities of A. pleuropneumoniae strains S4074 and 1471 are calcium dependent.** The hemolytic activity of strain S4074 cultured in LB broth with or without calcium is shown in Fig. 2. Culture supernatant fluids reached their maximum hemolytic activities at the late log phase when grown in the presence of 5 mM CaCl₂. Culture supernatant fluids from bacteria grown in LB broth depleted of calcium by the addition of 10 μM EGTA were nonhemolytic. Hemolytic activity could be restored by the addition of 10 mM CaCl₂ to assay buffers. Hemolytic activity of supernatant fluids from bacteria grown with calcium was not reduced by the addition of EGTA. These results demonstrate that hemolysin was secreted independently of calcium but that calcium was required for activity. As expected, culture supernatant fluids of strain 1421 always proved to be nonhemolytic, regardless of whether calcium was added to growth medium or assay buffers.

Neutrophils were incubated with filter-sterilized fluids from log-phase cultures of strains S4074 or 1421 grown with or without calcium, and their viability was determined by nigrosin dye exclusion. Neutrophils incubated in nonoccluded LB broth controls supplemented with either 5 mM CaCl₂ or 10 μM EGTA had a viability of 89 or 90%, respectively. Neutrophil viability in culture fluids from bacteria grown without calcium was 77 and 80% for strains S4074 and 1471, respectively. When neutrophils were incubated in culture fluids from either strain grown with calcium, the inviability dropped to 49% (strain S4074 fluids) and 40% (strain 1421 fluids).

**Calcium-dependent binding to erythrocytes by the 105-kDa Cly I.** Erythrocytes were incubated with culture supernatant fluids from strains S4074 and 1421 grown with or without calcium. Binding assays were performed with the addition of an equal volume of either 10 mM CaCl₂ or 10 mM EGTA to supernatant fluids. The samples were then subjected to Western blot analysis with conavalens-stage swine sera. Hemolytic activities of supernatant fluids from strain S4074 and attachment of the 105-kDa secreted protein occurred only when bacteria had been grown in the presence of calcium or if calcium was subsequently added to assay buffers (results not shown). Supernatant fluids from strain 1421 did not lyse erythrocytes, and the 103- and 120-kDa cytolsins did not bind to erythrocytes in the presence or absence of calcium. The experiments were repeated with isolated erythrocyte membranes rather than intact cells. In
erythrocyte membranes.

356 strains hemolytic trophils were associated calcium was added to the bacteria grown in LB broth supplemented with 5 mM CaCl₂ and assayed with calcium (○) or EGTA (×) or grown in LB broth depleted of calcium by the addition of 10 μM EGTA and assayed with calcium (△) or with EGTA (▲). Hemolytic assays were performed on sterile supernatant fluids supplemented with equal volumes of 10 mM CaCl₂ and 10 mM EGTA. HT, hemolytic titer.

FIG. 1. Western blot analysis of the major secreted proteins produced by A. pleuropneumoniae S4074 and 1421. (A) Growth curves. Bacteria were grown in LB broth which had been supplemented with 5 mM CaCl₂ or depleted of calcium by the addition of 10 μM EGTA. Symbols: *, S4074 with CaCl₂; ×, 1421 with CaCl₂; ○, S4074 with EGTA; △, 1421 with EGTA. (B) Western blots. Proteins precipitated with trichloroacetic acid from sterile culture supernatant fluids obtained at hourly intervals (lanes 1 to 4) were resolved by SDS-PAGE on 7.5% gels, transferred onto nitrocellulose filters, and incubated with convalescent-stage swine sera (1:300) followed by goat anti-swine immunoglobulin conjugated to horseradish peroxidase (1:1,000). The positions of the molecular mass markers are indicated by the arrowheads on the left of the blot and are as follows (top to bottom): α₂-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphatase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), and lactic dehydrogenase (36.5 kDa).

these assays, more protein could be loaded onto gels by repeatedly incubating cell membranes with supernatant fluids. Membrane-bound proteins were subjected to analysis by SDS-PAGE and immunoblotting (Fig. 3). As before, the 105-kDa Cly I bound to erythrocyte membranes only when associated with calcium; binding was greatly reduced if calcium was not available in both growth medium and assay buffer. The secreted cytolysins of strain 1421 did not bind to erythrocyte membranes.

Calcium-dependent binding to neutrophils by cytolysins of hemolytic strains S4074 and nonhemolytic strain 1421. Neutrophils were tested for binding by proteins in sterile culture fluids from bacteria of strains S4074 and 1421 that had been grown in the presence or absence of calcium. Binding assays similar to those with erythrocytes were performed. Binding to neutrophils by the 105-kDa Cly I of strain S4074 and the 103- and 120-kDa proteins of strain 1421 was evident only when calcium was present in growth medium and assay buffer. The experiments were repeated with isolated neutrophil membranes (isolated as described in the text) were incubated with culture supernatant fluids and analyzed by Western blotting with convalescent-stage swine serum. Lane 1, molecular mass markers; lane 2, major secreted proteins of hemolytic strain S4074 grown with calcium; lane 3, membrane-bound protein (~105 kDa) from strain S4074 grown with calcium; lane 4, major secreted proteins of hemolytic strain S4074 grown without calcium; lane 5, membrane-bound protein (~105 kDa) from strain S4074 grown without calcium; lane 6, major secreted proteins of nonhemolytic strain 1421 grown with calcium; lane 7, erythrocyte membranes incubated with supernatant fluids from strain 1421 grown with calcium; lane 8, major secreted proteins from strain 1421 grown without calcium; lane 9, erythrocyte membranes incubated with supernatant fluids from strain 1421 grown without calcium. The positions of the molecular mass markers are indicated by the arrowheads on the left of the blot and are as follows (top to bottom): α₂-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphatase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), and triose phosphate isomerase (26.5 kDa).
membranes in order to improve the resolution of the attached bacterial proteins on SDS-polyacrylamide gels. Neutrophil membranes were repeatedly exposed to culture supernatant fluids from bacteria grown in the presence or absence of calcium. The samples were subsequently analyzed by Western blotting (Fig. 4). The 105-kDa (and 103-kDa) cytolysins secreted by strain S4074 bacteria bound to neutrophil membranes in the presence of calcium. Much less binding by the 105-kDa protein (and none by the 103-kDa protein) was seen in the absence of calcium. Attachment to neutrophil membranes by the 103- and 120-kDa cytolysins secreted by bacteria of strain 1421 occurred only in the presence of calcium. These results indicate that the major secreted proteins from hemolytic and nonhemolytic A. pleuropneumoniae strains require calcium for binding to neutrophils.

Identification of Cly I, II, and III. Isolated neutrophil or erythrocyte membranes were repeatedly exposed to supernatant fluids from cultures of strains S4074 or 1421 grown in the presence or absence of calcium. The samples were subjected to SDS-PAGE and transferred onto nitrocellulose paper. Blots were probed with MAbCVI-Apcly-9.2, a mouse MAb that reacts with the A. pleuropneumoniae 105-kDa Cly I (13). This MAb detected the 105-kDa secreted protein of strain S4074 that bound to erythrocyte and neutrophil membranes in the presence of calcium. Less binding was seen when strain S4074 was cultured in the absence of calcium (Fig. 5A). Blots were also probed with MAbCVI-Apcly-9.3, a MAb that reacts with the 103-kDa Cly II of A. pleuropneumoniae. Strains S4074 and 1421 both secreted the 103-kDa protein that bound to erythrocyte and neutrophil membranes when cultured in the presence of calcium (Fig. 5B). When blots were probed with MAbCVI-Apcly-2.2, a MAb that recognizes the 120-kDa Cly III (13), only the 120-kDa protein secreted by strain 1421 was detected. This 120-kDa protein bound to neutrophil membranes when strain 1421 was cultured in the presence of calcium (Fig. 5C).

DISCUSSION

Our studies indicate that secretion of the cytolysins of A. pleuropneumoniae S4074 and 1421 occurs independently of calcium in growth medium. These findings are in direct contrast with those of Frey and Nicolet (7, 9), who reported...
that the expression of a 105-kDa hemolysin (Hly) of strain S4074 is calcium dependent. Although the 103-, 105-, and 120-kDa secreted proteins were always present in supernatant fluids regardless of calcium, we found that hemolytic and cytolytic activities as well as the binding of these proteins to erythrocytes and neutrophils depended on calcium in growth medium or assay buffers. Cytolytic activities of supernatant fluids correlated directly with the binding of the 103- and 105-kDa major secreted proteins of hemolytic strain S4074 and the 103- and 120-kDa secreted proteins of nonhemolytic strain 1421 to erythrocytes and neutrophils. Although we did not directly demonstrate that hemolytic or cytolytic activity of culture supernatant fluids was the result of the binding of these proteins to cells, the fact that the major secreted proteins of these bacteria reacted with MAb against neutralized hemolytic and cytolytic proteins secreted by *A. pleuropneumoniae* serotypes 2 and 9 (13) makes it highly likely. In Western blot analysis, we used these neutralizing MAb to identify the erythrocyte- and neutrophil-bound proteins secreted by strains S4074 and 1421 as Cly I, Cly II, and Cly III. Because of similar molecular masses, the 103- and 105-kDa proteins of strain S4074 were difficult to distinguish on gels. Western blots probed with MAbCV1-Apicy 9.3 required blocking with skim milk in order to detect the 103-kDa protein. In future experiments, we plan to test MAb which neutralize the hemolytic and cytolytic activities of supernatant fluids for their abilities to block the attachment of the 105-, 103-, and 120-kDa proteins to target cells.

The calcium concentration (5 mM) used in binding experiments was considerably higher than the physiological Ca²⁺ concentration (90 nM) that is present in the cytosol of both prokaryotic and eukaryotic cells (10). The total extracellular calcium concentration in swine plasma ranges between 1 and 2 mM, corresponding to a concentration of free Ca²⁺ of approximately 700 µM (17), which is closer to the calcium levels used in our experiments.

*A. pleuropneumoniae* serotypes secrete similarly sized proteins with an apparent molecular mass of approximately 105 kDa (4, 9, 13). These proteins all react to a variable degree on Western blots with rabbit sera raised against the 105-kDa hemolysin purified from strain S4074 (8). However, differences in hemolytic activities for various *A. pleuropneumoniae* serotypes exist (7, 9, 14). Pig convalescent-stage serum against serotype 2 neutralizes cytolsinys produced by serotypes 2, 3, 4, 7, 8, and 12 but does not neutralize cytolsinos produced by serotypes 1, 5, 9, 10, and 11 (14). It appears, therefore, that variations in activity between related cytolsinos are the result of different functional rather than structural sites on the molecules. Nonhemolytic strain 1421 produces a 120-kDa protein that binds only to neutrophils. The 105-kDa protein from hemolytic strain S4074 binds to both erythrocytes and neutrophils. The 103-kDa protein secreted by both strains binds to erythrocytes and neutrophils. Our study provides direct evidence that the differences in hemolytic activities in the two serotypes is based on different target cell specificities of the cytolsinos.

The *E. coli* hemolysin (HlyA) has repeated amino acid sequences responsible for calcium binding, a reaction which is also essential for the attachment of HlyA to erythrocytes (2). Deletions in the DNA sequence coding for these amino acids prevents calcium association and eliminates the ability of the molecule to bind and lyse erythrocytes. It is also known that active HlyA requires posttranscriptional modification by HlyC, a protein coexpressed on the *E. coli* hemolysin operon. However, this modification, as yet not characterized, does not involve the calcium-binding site of HlyA (2). The hemolysin operon of *A. pleuropneumoniae* serotype 5 (Cly II) has recently been cloned and partially sequenced (3). A 53.3% homology exists between the gene encoding the hemolysin of *A. pleuropneumoniae* and the *E. coli* hemolysin gene. Both the *E. coli* and *A. pleuropneumoniae* hemolysins show homology in the glycine-rich hexapeptide repeats found to be responsible for calcium binding by the *E. coli* hemolysin (2, 3). The *A. pleuropneumoniae* operon contains a gene immediately 5’ to the hemolysin structural gene that has 61% homology to *E. coli* hlyC (3). Mechanisms of calcium binding and activation may be similar in both molecules.

As stated previously, a family of proteins with molecular masses of approximately 105 kDa are associated with hemolysis and cytolsis and are reported to be produced by all *A. pleuropneumoniae* serotypes (7, 9, 13). Immunoserological comparison of these proteins revealed that they are closely related but not identical. We have demonstrated that differences exist in the secreted proteins of serotypes 1 and 3 with respect to binding to target cells.

Erythrocytes can apparently bind relatively large amounts of the *E. coli* 110-kDa hemolysin without becoming saturated. It has been shown that the amount of cell-bound toxin increases as a linear function of the toxin dose applied (5). A similar situation probably exists for the binding of *A. pleuropneumoniae* cytolysins to target cells. When erythrocyte or neutrophil cell membranes were repeatedly incubated with *A. pleuropneumoniae* culture fluids containing calcium, the amount of cell-bound protein increased. A faint 105-kDa band was detected by immunoblotting when cell membranes were repeatedly incubated with strain S4074 culture fluids without calcium. This could be the result of a calcium-independent binding mechanism, but it is more likely that a small amount of calcium-activated Cly I from our initial culture was transferred to the final calcium-depleted LB medium. We have found that EGTA does not reverse the effects of calcium on hemolysin activity (Fig. 2) and binding (results not shown). A similar situation occurs with the *E. coli* hemolysin (1).

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