Calcium Enhances *Acanthamoeba polyphaga* Binding to Extracellular Matrix Proteins

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**Purpose.** To characterize better the ameba-host interactions that may be involved with the pathogenesis of *Acanthamoeba* keratitis, the role of calcium (Ca^{2+}) on the binding of *Acanthamoeba polyphaga* to extracellular matrix proteins was examined in vitro.

**Methods.** The binding of a metabolically labeled *A. polyphaga* (CDC:0187:1) isolate from a case of human keratitis to collagen type IV, laminin, and fibronectin was assessed through a range of calcium concentrations in the external fluid. Binding to collagen IV was studied in detail, with and without other divalent cations and calcium channel modulators.

**Results.** Calcium increased binding in a dose-dependent manner, with significant effects at 0.1 to 1.0 µM and near-maximal effects at 1 to 100 µM, depending upon the matrix protein. Magnesium alone had no effect on ameba binding to collagen IV but suppressed the action of calcium. Strontium enhanced ameba binding, with maximal effect at 100 µM. The calcium channel antagonists nifedipine and diltiazem-HCl and a calcium channel activator, Bay-K8644, had no effect on the action of calcium. However, the inorganic calcium antagonists, lanthanum and cobalt, suppressed the effect of calcium.

**Conclusion.** Low concentrations of calcium enhance the adhesion of *A. polyphaga* to extracellular matrix proteins. It remains uncertain whether calcium acts intracellularly or at the cell surface. Invest Ophthalmol Vis Sci. 1994;35:2421-2426.

We recently reported that *Acanthamoeba polyphaga* binds in vitro to the extracellular matrix proteins collagen IV, laminin, and fibronectin. The interaction of amebae with matrix proteins of the cornea could be an initial step in the binding and invasion steps of the pathogenesis process involved with *Acanthamoeba* keratitis. The aim of the present study was to characterize further the molecular mechanisms involved, especially the potential role of the external fluid in the binding process. Studies have suggested that the interactions of bacteria, yeast, and some other protozoa with extracellular matrix proteins can enhance their infectivity. Also, we and others have shown that α-methylmannonopyranoside inhibits *Acanthamoeba* binding to corneal cells, to extracellular matrix proteins, and to yeast; some types of mannose-dependent adhesins require calcium, so this class of lectin-mediated adherence might be decreased by lowering calcium concentrations. Finally, it is postulated that divalent cations play significant roles in the binding of cells to extracellular matrix proteins. Therefore, experiments were designed to examine the effects of divalent cations on the binding of *Acanthamoeba* to extracellular matrix proteins.

**MATERIALS AND METHODS**

An isolate of *A. polyphaga* (CDC:0187:1) from a case of human keratitis was grown axenically at 23°C in peptone-yeast extract-glucose medium. Amebae were harvested using a cell scraper at the late log phase of growth, harvested by centrifugation for 5 minutes at 1000g, and resuspended amebae were examined to confirm that at least 98% of the cells were trophozoites. Amebae pellets were suspended in Hepes buffer (0.85% NaCl, 10 mM Hepes, pH 6.8) and were counted using a hemocytometer. To quantitate bound ameba, 35S methionine (100 µCi of 1000 Ci/mM) was
used metabolically to label $5 \times 10^6$ amebae by incubation in 100 $\mu$l of Hepes buffer for 45 minutes, with gentle agitation every 15 minutes. Labeled amebae were washed three times with 1 ml of 0.1 mM EGTA in PBS (pH 7.0) and were resuspended in PBS (0.8% NaCl, 8.2 mM Na$_2$HPO$_4$, pH 7.4) at a density of $1 \times 10^5$ cells/ml.

**Protein Coating of 96 Well Plates**

Microtiter well plates were coated with 100 $\mu$l of 10 $\mu$g/ml concentrations of fibronectin, laminin, or collagen type IV, as previously described. The coated solutions were decanted and wells were rinsed twice with 0.1 mM EGTA in PBS to remove divalent cations.

**Preparation of Ion Solutions and Drugs**

Stock solutions (1 M) of MgCl$_2$, CaCl$_2$/MgCl$_2$, SrCl$_2$, and BaCl$_2$ were diluted to achieve final concentrations of 0.01, 0.1, 1, 10, 100, and 1000 $\mu$M in PBS in microtiter plate wells; 0.01 to 1000 $\mu$M free Ca$^{2+}$ was achieved as CaCl$_2$ with EGTA in PBS. Stock solutions of ion channel modulators (Research Biochemicals, Natick, MA) including two calcium channel antagonists, nifedipine (1 mM in acetone) and diltiazem-HCl (10 mM in water), and the calcium channel activator BAY-K8644 (1 mM in ethanol), were diluted in PBS to achieve final concentrations of 1 and 10 $\mu$M in PBS with cells. A sugar analog that also inhibits ameba adherence, $\alpha$-methylmannopyranoside, was prepared as a 500 mM stock in PBS.

**Acanthamoeba Binding**

To 96-well microtiter plates coated with extracellular matrix proteins, different volumes and concentrations of each cation or drug were added in quadruplicate or duplicate. Then to each well, 50 $\mu$l PBS with $1 \times 10^4$ metabolically labeled amebae cells were added (final volume, 100 $\mu$l/well). After 20 minutes' incubation, the wells were washed twice with 200 $\mu$l PBS to remove unbound cells. The remaining adherent amebae in each well were then solubilized in 100 $\mu$l of 1% sodium dodecyl sulfate for at least 5 minutes, and scintillation counting was used to determine binding as previously described. Student's t-test was used to determine statistical significance. Humans were not used in this study.

**RESULTS**

Figure 1 shows the effect of Ca$^{2+}$ on *Acanthamoeba* binding to fibronectin, laminin, and collagen type IV. The amebae binding to all three proteins increased significantly ($P < 0.05$) as the calcium concentration increased, with the binding saturation between 1 and 100 $\mu$M CaCl$_2$. Significant effects of Ca$^{2+}$ were observed at concentrations as low as 0.1 to 1.0 $\mu$M, and binding was near-maximal at 1 $\mu$M for collagen IV and laminin and at 100 $\mu$M for fibronectin (Fig. 1). In most subsequent experiments that focused on binding to collagen IV, the degree of binding (approximately

**FIGURE 1.** *Acanthamoeba* binding (CPM remaining in wells) to extracellular matrix proteins collagen type IV (A), fibronectin (B), and laminin (C) in 10-fold increasing concentrations of Ca$^{2+}$ (0 to 1000 $\mu$M). Ameba binding reached an initial plateau at 1 to 100 $\mu$M calcium. Each point is mean of duplicate observations, and the experiment was independently repeated twice with similar results.
Calcium and Acanthamoeba Adherence

FIGURE 2. Acanthamoeba binding to collagen IV in the presence of various divalent cations. The ameba bound (CPM) was significantly increased by CaCl₂ and SrCl₂, with saturation points at about 1 and 100 µM, respectively. Negligible binding was observed in the presence of 0.01 to 1000 µM MgCl₂ or BaCl₂ (A). Some additional binding was observed at high (10 mM) concentrations of CaCl₂ and BaCl₂ (B). Each point is the mean of duplicate observations.

60% of the applied counts) was relatively constant between 1.0 and 1000 µM CaCl₂, although a slight increase was sometimes observed above 1 mM calcium (Figs. 2 and 3).

The influence of Ca²⁺ on ameba binding to matrix protein was mimicked by higher concentration of SrCl₂ (saturation 100 µM), but not by 0.01 to 1000 µM BaCl₂ (Fig. 2A). At the same concentrations, MgCl₂ alone had no effect on the interaction of amebae with collagen IV, but it did suppress the effect of Ca²⁺ (Figs. 2A, 2B).

To examine the possibility that external Ca²⁺ enters Acanthamoeba and causes an intracellular (metabolic) change that leads to enhanced binding to matrix proteins, the effects of known blockers or activators of voltage-sensitive Ca²⁺ channels were examined. Nei-

FIGURE 3. Effects of calcium channel antagonists (nifedipine, diltiazem HCl) and activator (BAY-K8644) on Acanthamoeba binding to collagen IV. The calcium-dependent binding of Acanthamoeba to extracellular matrix proteins was not significantly affected by either BAY-K8644 or by nifedipine or diltiazem-HCl. Each point is the mean of duplicate observations.
ther the voltage-dependent Ca\(^{2+}\) channel blockers nifedipine and diltiazim-HCl (Fig. 3A) nor the calcium channel activator BAY-K8644 (Fig. 3B) had appreciable effects on the actions of Ca\(^{2+}\) on ameba binding to collagen IV.

Lanthanum chloride (LaCl\(_3\)), an inorganic calcium antagonist,\(^8\) blocked the effect of 10 \(\mu\)M Ca\(^{2+}\) in a dose-dependent manner (Fig. 4, 53% inhibition at 0.1 mM), as did cobalt chloride (CoCl\(_2\)), which showed 45% inhibition at 0.1 mM CoCl\(_2\) in the presence of 10 \(\mu\)M CaCl\(_2\). In the presence of 10 \(\mu\)M CaCl\(_2\), ameba binding to collagen type IV was decreased 30% and 50% in the presence of 1 and 50 mM alpha-methylmannopranoside, respectively.

**DISCUSSION**

Our results demonstrate clearly that extracellular Ca\(^{2+}\) is necessary for the binding of Acanthamoeba to collagen IV, laminin, and fibronectin. A better understanding of the mechanism and nature of interactions between ameba and the matrix proteins may suggest therapeutic or preventive measures for Acanthamoeba keratitis. Variable degrees of binding were observed for each matrix protein between 0.1 \(\mu\)M and 1000 \(\mu\)M CaCl\(_2\). Initial plateaus of near-maximal binding were observed at 1, 10, and 100 \(\mu\)M for collagen IV, laminin, and fibronectin, respectively, with a slight additional increase in binding above 1 mM CaCl\(_2\). These results may suggest the existence of several types of calcium-modulated adherence, or they reflect experimental variation. It is difficult to measure physiologically unbound calcium concentration, but because eye tissue fluid calcium ion concentrations are in the range of 0.09 to 5 mM,\(^9\) excess extracellular calcium is probably available for the majority of the calcium-mediated binding by Acanthamoeba to various components of the cornea in vivo.

The effect of Ca\(^{2+}\) appears to be specific because Mg\(^{2+}\), another divalent cation that is a component of body fluids, had no effect on the binding of ameba to matrix proteins. The molecular nature of the interaction between Acanthamoeba and the extracellular matrix glycoproteins remains unknown, but the present data indicate that Ca\(^{2+}\) is specifically and intimately involved in the process. Co\(^{2+}\) and La\(^{3+}\), which are known to compete with Ca\(^{2+}\) for binding sites in tissues, cells, and macromolecules, significantly suppressed the effects of Ca\(^{2+}\) on the binding of Acanthamoeba. In addition to direct effects on cell surface components, La\(^{3+}\) and Co\(^{2+}\) can block Ca\(^{2+}\) transport through specific channels and subsequently inhibit Ca\(^{2+}\)-dependent intracellular (metabolic) processes in many types of cells.\(^8\) Although Ba\(^{2+}\) is more permeable to known Ca\(^{2+}\) channels and, therefore, mimics Ca\(^{2+}\) in adrenal gland catecholamine release,\(^10\) it had little or no effect on ameba binding. Neither specific blockers (nifedipine and diltiazim) nor a specific activator (BAY K-8644) of many classes of voltage-sensitive calcium channels affected the process. These data are unable to resolve whether calcium effects are exerted at the cell surface or are exerted intracellularly. Indirect effects on binding could be due to activities on targets such as calcium-dependent ATPase.\(^12\)

Several distinct and nonexclusive classes of macromolecules have been shown to require calcium for binding to tissue components. Like higher eukaryotes, bacteria, yeast, and protozoa appear to possess cell surface integrins or receptors.\(^13\)--\(^15\) These integrins are a family of integral membrane glycoproteins made up of noncovalent \(\alpha\beta\) heterodimer.\(^7\)--\(^9\) These proteins span the plasma membrane and are involved in cell-to-cell and cell-to-matrix interactions. A variety of integrins has been reported from the cornea.\(^21\) Because the \(\alpha\)-subunit of integrins contains several divalent cation binding sites,\(^7\) a possible explanation of our data is that the Acanthamoeba also possesses receptors (integrins) that require the presence of Ca\(^{2+}\) for binding to matrix proteins.

Another class of macromolecules that has been implicated in cell migration and whose action requires calcium are the calcium-dependent cysteine proteases.\(^22\) We and others have shown that these Acanthamoeba contain both acidic and neutral proteases,\(^23\) including cysteine proteases;\(^24\) typical collagenase activity was not observed.\(^25\) We have recently shown that the degree of ameba binding to collagen IV is corre-

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933178/ on 11/19/2018)
lated with the amount of neutral protease secretion (Wang et al, data not shown, 1993), which suggests a relationship between adherence and protease release. Proteases may also degrade matrix proteins to metabolically active peptides, and enriched protease fractions hydrolyze these extracellular matrix proteins. However, it remains to be seen whether altering adhesion, protease release, or both will alter disease pathogenesis.

Additional roles of divalent cations in the interaction of ligands with intact cells or cell membranes have been reported. Ca$^{2+}$, Mg$^{2+}$, or both are necessary for binding follicle-stimulating hormone, angiotensin, and growth hormone to their specific target cell receptors. Similarly, Ca$^{2+}$ and Mg$^{2+}$ are required for the binding of asialoglycoprotein, ferritin, low-density lipoprotein, sex steroid hormone-binding globulin, and corticosteroid-binding globulin to cell membranes. In those studies, significant effects of Ca$^{2+}$ and Mg$^{2+}$ were observed when their concentrations were in the millimolar range. Most of the binding effects on amebae were near maximal in micromolar concentrations, which suggests a different mechanism than is observed for hormones.

The lectins are another class of adhesin that bind carbohydrates, and some classes of lectins require calcium. We have previously shown that mannose inhibits Acanthamoeba binding to corneal cells and extracellular matrix proteins, suggesting a role for a lectin-like molecule. Because both the absence of calcium and the presence of α-methylmannopyranoside inhibit ameba binding, it is tempting to speculate that a single major calcium-dependent lectin adhesion mediates ameba binding. However, the data are consistent with role(s) for integrins, proteases, and lectins in the binding process. This type of defined binding assay should be helpful in efforts to define better the molecular nature of the putative calcium-dependent ameba adhesin(s).

**Key Words**
Acanthamoeba, calcium, adhesion, extracellular matrix, drugs

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