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Membrane Properties Involved in Calcium-Stimulated Microparticle Release from the Plasma Membranes of S49 Lymphoma Cells

Lauryl E. Campbell

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Membrane Properties Involved in Calcium-Stimulated Microparticle Release from the Plasma Membranes of S49 Lymphoma Cells

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The mechanism of microparticle shedding from the plasma membrane of calcium-loaded cells has been investigated in erythrocytes and platelets. Recent studies have revealed the physiological and clinical importance of microparticle release from nucleated cells such as lymphocytes and endothelium. The experiments of this study were designed to address whether simple mechanisms discovered in platelets and erythrocytes also apply to the more complex nucleated cells. Four such mechanisms were addressed: potassium efflux, transbilayer phosphatidylserine migration, cytoskeleton degradation, and membrane lipid order. The rate and amount of microparticle release in the presence of a calcium ionophore, ionomycin, was assayed by light scatter at 500 nm. To inhibit the calcium-activated potassium current, cells were exposed to 1 mM quinine or a high-potassium buffer. Both interventions substantially attenuated microparticle shedding induced by ionomycin. Microparticle release was also greatly reduced in a lymphocyte cell line deficient in the expression of scramblase, the enzyme responsible for calcium-stimulated phosphatidylserine migration to the cell surface. This result indicated that such phosphatidylserine exposure is also required for microparticle shedding. The importance of cytoskeletal rearrangement was evaluated through the use of E64-d, a calpain inhibitor, which appeared to have no affect on release. Thus, if cytoskeleton degradation is important for microparticle release, a different enzyme or protein must be involved. Finally, the effect of membrane physical properties was addressed by varying the experimental temperature (32–42 °C). A significant positive trend in the rate of microparticle release as a function of temperature was observed. Fluorescence experiments with trimethylammonium-diphenylhexatriene and patman revealed significant differences in the level of apparent membrane order along that temperature range. Ionomycin treatment appeared to cause further disordering of the membrane, although the magnitude of this change was minimally temperature-sensitive. Thus, it was concluded that microparticle release depends more on the initial level of membrane order than on the change imposed by calcium uptake. In general, mechanisms involved in particle release from platelets and erythrocytes appeared relevant to lymphocytes with the exception of the hydrolytic enzyme involved in cytoskeletal degradation.

Keywords: cytoskeleton, phosphatidylserine, TMA-DPH, patman, ionomycin, Raji, fluorescence, potassium gradient, membrane fluidity, nucleated cells, anisotropy, EGTA, calpain, gelsolin
ACKNOWLEDGMENTS

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Introduction

Microparticles are small vesicular structures (0.1-1µm diameter) produced and released by exocytic blebbing of the cell plasma membrane [1]. While circulating microparticles are typically derived from platelets, they may also be released from leucocytes, endothelial cells, erythrocytes, and lymphoma cells [2, 3]. They have also been shown to originate from epithelial, fibroblast, haematopoetic, immune, placental and tumor cells, with the structure pattern enclosed within the membrane of the microparticles specific to the parental cell that secretes them. The size of microparticles also strongly depends on the cell type secreting them [1].

Microparticles appear to function as mediators of intercellular communication. For example, there is evidence that microparticles are involved in macrophage activation resulting in upregulation of CD40 and the production of tumor necrosis factor [4]. Moreover, microparticles induce apoptosis in circulating angiogenic cells [5]. Because of their role in mediating cellular interactions, microparticles are a key component in the regulation of inflammation, coagulation, antigen presentation, and apoptosis [6]. Hence, they are a subject of interest in the pathogenesis of autoimmune diseases and inflammatory disorders [6, 7]. Detectable basal levels of microparticle are often found circulating in the blood of healthy individuals [2, 3]. Increased levels are typically seen in the blood of patients in a diseased state such as cerebral malaria, atherosclerosis, various cancers, and HIV [2, 3, 8].

While microparticles are typically shed due to activation signals and/or at the beginning of apoptosis, release is also observed following stimulation of platelets by thrombin. Furthermore, they are released during activation by
lipopolysaccharide and cytokines of monocytes, hepatocytes, endothelial cells, and smooth muscle cells [9, 10].

Mechanisms of release may involve either of two processes. Small microparticles (less than 100 nm) are known as exosomes and have an endosomal origin. They are released via exocytosis of multivesicular bodies leaving the lysosomal pathway [11]. On the other hand, the majority of microparticles (100-1000 nm) are shed through the process of “reverse budding” [11]. In this case, microparticle release is initiated by elevation of the intracellular concentration of calcium through the use of ionomycin. Ionomycin, a calcium ionophore, is an amphipathic molecule with chemical groups that bind to the calcium ion in order to shield its charge and facilitate its crossing past the hydrophobic interior of the lipid membrane. Sustained increases in intracellular calcium result in three important events: reorganization of the cytoskeleton, translocation of phosphatidyserine (PS) to the outer face of the cell membrane, and enhanced permeability to potassium with associated osmotic effects. The action of calcium on the cytoskeleton is mediated by the hydrolytic enzyme calpain [12, 13]. Calcium influx can initiate the opening of potassium channels resulting in potassium ion efflux [14]. The asymmetric phospholipid distribution of the plasma membrane with PS located exclusively on the interior side is maintained by aminophospholipid translocase among other enzymes [11]. As a result of increased cytoplasmic calcium, inhibition of translocase and activation of another enzyme, scramblase allows phosphatidyserine to not be returned to the inner side of the membrane and therefore be exposed extracellularly [12, 13]. Consequently, microparticles typically display a majority of phosphatidyserine on the outer surface of their membranes [12, 13].
Alongside potassium gradient, translocation of phosphatidylserine, and cytoskeleton degradation, the membrane biophysical properties are another important component seen and evidenced in the release of microparticles in erythrocytes and non-nucleated cells. These membrane properties include lipid order, fluidity, and composition [15]. Calcium-stimulated microparticle release is dependent upon membrane fluidity together with the loss of phospholipid asymmetry [15]. Not only is release dependent on the fluidity, but also the disorder at the time calcium enters into the cell [15]. But, the calcium influx organizes and forces the membrane to become more ordered while microparticles are released [15]. While erythrocytes and platelets have shown the potassium gradient, phosphatidylserine translocation, lipid order, and cytoskeleton degradation to be pertinent in the release of microparticles, it is unknown if it is the same for nucleated complex cells as well.
**Methods**

**Reagents**

Ionomycin, 1-(trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene p-toluenesulfonate (TMA-DPH), and 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)methyl)amino)naphthalene chloride (patman) were all obtained from Invitrogen (Carlsbad, CA). Ionomycin was dissolved in dimethylsulfoxide (DMSO) as stock solutions, while TMA-DPH was suspended in dimethylformamide. Quinine and E64-d were both purchased from Sigma (St. Louis, MO).

**Cell Preparation**

S49 mouse lymphoma cells were obtained and grown in DMEM at 37°C in humidified air with a 10% CO₂ content. Raji human Burkitt’s lymphoma cells were obtained and grown at 37°C with a 5% CO₂ content in RPMI containing 10% fetal bovine serum and L-glutamine. Preparation of S49 and Raji cells are described in [16].

Prior to experiments, unless otherwise stated, cells were isolated through centrifugation then washed and suspended in MBSS (134 mM NaCl, 6.2 mM KCl, 1.6 mM CaCl₂, 18.0 mM Hepes, 13.6 mM glucose, pH 7.4 at 37°C) with a density of 0.4-3.0 x 10⁶ cells/mL. Samples were aliquotted and transferred to quartz fluorometer sample cells and allowed to equilibrate for 5 min in the fluorometer instrument for quantification and measurement of light scatter intensity, fluorescence, and/or anisotropy. Homogeneity and designated temperature of each sample were maintained through the use of magnetic stirring and heating of the sample chamber with circulated thermostated water. Most incubations were
done at 37°C. Some experiments were done at other temperatures in the range from 32-42°C.

*Standard Microparticle Release Assay*

Microparticles released from washed S49 cells suspended in MBSS were assayed by light scatter at 500 nm and 37°C using a fluorometer (excitation=500 nm, emission=510 nm). After temperature equilibration of the sample, data acquisition was initiated, and ionomycin (300 nM) a calcium ionophore, was added at 100 s. The rate of microparticle release was determined as the slope of increase in light scatter intensity.

*Blocking K⁺ Channels*

To assess involvement of osmotic shock created by the opening of calcium-activated potassium channels, the standard microparticle release assay was repeated in the presence and absence of quinine (1 mM), a potassium channel blocker. After initiating data acquisition, quinine was added at 100 s followed by ionomycin at 200 s. In order to determine if microparticle release was inhibited, the average total amount of microparticles released for the control was statistically compared to the average total amount of microparticle release for the quinine-treated samples.

*Removing K⁺ Gradient*

In order to further verify the importance of potassium current in microparticle release, the normal potassium gradient was removed by suspending treatment cells in a high potassium concentration MBSS (89 mM KCl,
51 mM NaCl, 1.6 mM CaCl₂, 18.0 mM Hepes, 13.6 mM glucose, pH 7.4 at 37°C). After a baseline was established, treatment with ionomycin followed, and microparticle release was detected by the standard release assay. In order to quantify microparticle release and determine if removing the potassium gradient inhibits it, the average total amount of microparticle release for the control was compared to that of the variable suspended in high potassium MBSS.

**Raji Cells**

To specify whether or not trans-bilayer migration of phosphatidylserine was necessary for microparticle release, Raji cells, human lymphoma cells incapable of flipping phosphatidylserine from the interior to the exterior of the cell [16], were used instead of S49 cells in repeats of the standard microparticle release assay described above. The average total amount of microparticles released in control experiments using the S49 cells were statistically compared to the average total amount of microparticles released from the Raji cells in order to determine and quantify if trans-bilayer migration is vital in the release of microparticles from lymphoma cells.

**Inhibition of Cytoskeletal Degradation**

Previous studies have that calpain plays a role in the cleavage of the cytoskeleton of erythrocytes and appears to be necessary for the release of microparticles in non-nucleated cells [15]. In order to establish if cytoskeletal degradation is equally important in the process of microparticle release in S49 nucleated cells, E64-d, a calpain inhibitor, was used. Cells were washed and isolated as explain above at 37°C and were allowed to establish a baseline. E64-d
was added (36 µM) and given 5 min to equilibrate followed by the addition of ionomycin, with light scatter being quantified for a total of 2100 s (excitation=500 nm, emission=510 nm) [16].

To verify the accuracy of the calpain inhibitor, E64-d, protocol from Smith et al. [16] was repeated using red blood cells (Figure 2).

**Temperature Variation**

Initially, to establish the importance of environmental temperature in the rate of microparticle release, S49 cells were isolated through a standard washing procedure and assayed at 500 nm using a fluorometer. The light scatter intensity was measured for 10 min (excitation=500 nm, emission=510 nm) for a control (37°C) and for each temperature over a varying range (32°C – 42°C), followed by the addition of ionomycin (300 nM). This temperature range was chosen to allow variation of membrane properties while still allowing the cells to remain alive and functioning. The trend of microparticle release rate was assessed by linear regression.

**Membrane Fluidity**

Membrane fluidity has shown to be significant in the process of microparticle release in non-nucleated cells [15]. So, in order to complete the possible criteria for nucleated cell microparticle release, membrane order was assessed in S49 lymphoma cells. First, TMA-DPH was added to S49 cells prepared in accordance as described above for cell preparation, anisotropy was measured and quantified. For patman, fluorescence emission was obtained and
quantified (excitation=350 nm, emission=452 nm) for 10 min to establish a baseline. This was followed by the addition of ionomycin.
Results

Effect of Ionomycin

To test whether lymphocytes react similarly to erythrocytes and platelets by releasing microparticles in response to elevated intracellular calcium, S49 lymphoma cells were treated with a calcium ionophore (ionomycin), and particle release was assayed by light scatter [16]. As shown in Figure 1, light scatter intensity increased within 50 s after addition of ionomycin and continued to rise for another 100 s. Figure 2 demonstrates that the difference in light scatter intensity before and after treatment with ionomycin was reproducible and statistically significant. Moreover, repetition of the experiment in the presence of a calcium chelator (EGTA) demonstrated that microparticle release was dependent on calcium and therefore not an artifact of the ionophore itself (Figure 2).

Role of Potassium Channels

To evaluate the importance of calcium-activated potassium ion channels in the process of microparticle release, the experiment of Figure 1 was repeated in the presence of a reduced potassium gradient. Initially, to remove the potassium gradient, S49 cells were washed and suspended in the high potassium concentration MBSS. Secondarily, calcium-activated potassium channels were blocked directly by treating cells with the drug quinine. Figure 2 shows a significant reduction in the amount of microparticles shed from the cells suspended in high potassium MBSS in comparison to the cells in normal MBSS. Figure 2 also demonstrates that the cells treated with the drug quinine previous to the addition of ionomycin showed an even greater reduction in the amount of
microparticles shed; indicating the importance of potassium current in the process of particle release.

**Phosphatidylserine Translocation**

The Raji cell line is deficient in the enzyme scramblase, which is responsible for flipping PS from the interior to the exterior of the cell [16]. Therefore, this cell line was used to determine the importance of the trans-bilayer migration of PS. Figure 3 compares the response to ionomycin in both the S49 and Raji cell lines. Trials using the S49 cells showed typical levels of microparticle release. The Raji cells showed significantly lower microparticle levels in the presence of ionomycin, implying that PS translocation is important in the process of microparticle shedding by lymphocytes.

**Calpain Activation**

Previous studies have shown that inhibition of the hydrolytic enzyme, calpain, impeded the release of microparticles in erythrocytes [16](Figure 4). This result suggests that cytoskeletal degradation by calpain may be required for microparticle shedding. This hypothesis was further studied using S49 lymphoma cells with the addition of the drug E64-d, a calpain inhibitor, prior to the addition of ionomycin. Figure 5 shows there was no effect on the amount of microparticles released in these cells indicating that if cytoskeletal changes are important, they must involve a mechanism other than calpain.

**Membrane Lipid Order**

Temperature variation (32-42°C) was used to understand the role of
membrane properties of S49 cells during the process of microparticle shedding. These membrane properties were assessed by fluorescence spectroscopy using patman generalized polarization (GP) and TMA-DPH anisotropy. Initial rates of release were quantified from the light scatter assays at the varying temperatures in response to ionomycin. Initial rates were calculated by determining the slope of the first 20 time points following the addition of ionomycin, and multiple trials at each temperature were then averaged. As shown in Figure 6A, the rate of microparticle release increased monotonically with temperature. Linear regression analysis demonstrated that this trend was statistically significant. However, the normalized total amounts of microparticles shed were not different among the varying temperatures. The average level of membrane order at the same temperatures was inferred from the values of patman GP and TMA-DPH anisotropy measured prior to ionomycin addition. The data with both probes (Figures 6B, C) showed a significant decrease in trend line slope based on linear regression. Overall, Figure 6 supports the idea that as temperature increases; membrane lipid order decreases, therefore allowing increasing rates of microparticle release.
Discussion

While little is known about the mechanism of microparticle release in erythrocytes, nothing is known about nucleated cells. Using lymphocytes to further understand this mechanism in nucleated cells, microparticle release was studied in response to a calcium ionophore. Based on the studies done, it can be concluded that lymphocytes release microparticles when intracellular calcium levels increase. A potassium gradient must be present and translocation of phosphatidylserines must occur in order for shedding. Also, as temperature increases, there is a decrease in membrane lipid order, allowing for an increase in microparticle release rates. While these criteria are consistent with what has been observed in red blood cells, lymphocytes did not require the action of calpain in the degradation of cytoskeleton for release as did the red blood cells [14-17].

Calpain was shown to be an important enzyme in the degradation of the cytoskeleton in red blood cells [16]. In this study, on the other hand, the calpain inhibitor (E64-d) did not affect the release of microparticles. We can now conclude, in reference to nucleated cells that the enzyme calpain does not have a direct relationship to the rearrangement of cytoskeleton in microparticle shedding. However, ongoing studies have evidenced that the cytoskeleton does in fact change significantly when releasing microparticles (Figure 7), but it is suspected that a different enzyme, protein, or other pathway may be involved.

Gelsolin, a protein, has been found to be an influencing factor on the cytoskeleton by severing the actin filaments and capping the newly severed ends in nucleated cells. Moreover, $\text{Ca}^{2+}$ regulates the binding of gelsolin to actin [18-21]. This protein not only may be directly related to microparticle release, but it is also said to have the ability to suppress growth and induce apoptosis in cancer
cell lines [22].

As evidenced in Figure 6, the rate of release depends on the environmental temperature. The release rate increased as temperature increased while membrane lipid order decreased. This may be due to increased membrane fluidity allowing for flexibility of the membrane to induce exocytosis of these microparticles. In relation, it can be deduced that as environmental pressure decreases, a higher rate of microparticle release may follow. This assertion may relate to a recent report of enhanced microparticle release associated with deep sea diving [23]. In that study, microparticle release followed the decrease in ambient pressure as the divers returned to the surface at the end of the dive.

Cancer as well as many other physiological diseases, medical applications, and intercellular communication can be better understood through studies of microparticle shedding and formation. For example, because microparticles are important in intercellular communication, harvested particles, having specific protein markers, have been used to treat metastatic melanoma patients. These microparticles are capable of promoting an immune response, tumor rejection, and tumor growth reversal [24, 25]. As another example, microparticles released from enterocytes can carry antigens, acting as communicators between the local immune system and digestive tract, providing a link between the immune response and food antigens [26]. Also, the epididymis releases microparticles that contain certain proteins essential for optimal reproduction. These proteins delivered to spermatocytes by the microparticles have been shown to exert an important influence on the maturation of the male gamete and participate in the binding of the egg during fertilization [27].

Because little was known about the release of microparticles in nucleated
cells, these studies have provided a further understanding about the mechanism of release. Aspects such as calcium entry initiating release, the requirement for a potassium gradient, migration of phosphatidylserines, and involvement membrane lipid order are consistent with erythrocytes. However, further studies are needed in order to develop the understanding and importance of cytoskeletal rearrangement during release.
Figures

**Figure 1**

**Ionomycin stimulated microparticle release.** Microparticle release was assayed by light scatter at 500 nm at 37°C. Ionomycin (300 nM) was added at the dotted line. An increase in scatter intensity indicates particle release.

**Figure 2**

**Ionomycin-stimulated microparticle release requires calcium-activated potassium current.** Cells were washed and suspended in normal MBSS or in MBSS that contained EGTA (2 mM) instead of calcium, high potassium (see Materials and Methods), or quinine (1 mM) at 37°C. The normalized intensity was calculated by subtracting the average initial intensity immediately prior to ionomycin addition (20 points) from the average intensity at the highest point in the plateau after ionomycin (see Figure 1). This difference was then divided by the average initial intensity to standardize among trials. Differences in the normalized intensity among groups were significant by one-way analysis of variance ($p < 0.0001$, $n = 2-5$ per group). A post-test (Dunnett’s) revealed that the group treated with normal MBSS was distinguishable from each of the other three ($p < 0.01$)
Microparticle release was impaired in a cell line (Raji) that is deficient in its ability to expose phosphatidylserine. Both cell types (S49 and Raji) were washed and suspended in a normal MBSS solution. Light scatter was assayed at 37°C before and after addition of ionomycin (see Figure 2 for explanation of the normalized intensity). The difference between cell lines was significant by unpaired t-test ($p = 0.001$, $n = 3$).

Inhibition of microparticle release in response to E64-d, a calpain inhibitor, in erythrocytes. Light scatter was assayed at 37°C of red blood cells in the presence or absence of E64-d prior to addition of ionomycin.

Effect of the presence or absence of E64-d (calpain inhibitor) in S49 lymphoma cells. Light scatter intensity was quantified using S49 lymphoma cells in the absence (control) or presence of E64-d prior to ionomycin addition. The normalized change in intensity between the two treatments showed to not be significant using a two-tailed t-test ($p = 0.1957$, $r^2 = 0.3757$, $n = 3$).
Figure 6
Relationship between the rate of microparticle release and membrane order as a function of temperature. (A) The initial rate of particle release upon addition of ionomycin was calculated from experiments such as that shown in 1. Cells were equilibrated at 37°C and then adjusted to the indicated temperature and equilibrated for 10 min prior to adding ionomycin. Average rate of release was determined by the slope of 20 time points following ionomycin addition. Based on linear regression, the data showed a significant increasing trend with temperature ($p = 0.0002, r^2 = 0.81, n = 3-11$ per temperature). (B-C) The experiments of (A) were repeated with cells labeled with patman (B) or TMA-DPH (C). Values of patman GP or TMA-DPH anisotropy were averaged from 20 points prior to addition of ionomycin. The negative trends in both cases were significant by linear regression ($p < 0.009, r^2 > 0.55, n = 3$ per temperature).
Figure 7
Confocal photographs of actin cytoskeletal without (A) or with (B) ionomycin treatment. These experiments were performed by Elizabeth Gibbons. Data represent staining of actin skeleton labeled with FITC-labelled phalloidin.


Curriculum Vitae

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• Western Dental Services, Registered Dental Assistant/Supervisor 2006-2010

Publications/Presentations

• Biophysical Society, Poster Presentation February, 2012
• Membrane Properties Involved in Calcium-stimulated Microparticle Release from the Plasma Membranes of S49 Lymphoma Cells, Master's Thesis June, 2012

Research Experience

• Brigham Young University, Dr. John D. Bell 2010-2012
  o Molecular pharmacology; membrane signal transduction mechanisms, and biophysics of
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### Awards/Honors

- **Department Grant**
  
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  - 2 Research Assistantships: $5,280

- **Dean’s Honors List**
  
  - California State University, Fullerton
  
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- **Governor’s Math and Science Award**
  
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### Affiliations/Memberships

- **AAAS Science**
  
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- **Biophysical Society**
  
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- **Graduate Student Society**
  
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