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Biochimica et Biophysica Acta 1612 (2003) 154–163
BIOCHIMICA ET BIOPHYSICA ACTA
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# Temperature dependence of fluid phase endocytosis coincides with membrane properties of pig platelets

Willem F. Wolkers[a,b,*], Sheri A. Looper[a,c], Ray A. Fontanilla[a,c], Nelly M. Tsvetkova[a,b], Fern Tablin[a,c], John H. Crowe[a,b]

[a] Biostabilization Program, University of California, Davis, CA 95616, USA
[b] Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA
[c] Department of Anatomy, Physiology, and Cell Biology, University of California, Davis, CA 95616, USA

Received 6 February 2003; accepted 4 April 2003

**Abstract**

In previous studies we have shown that platelets take up low molecular weight molecules from the medium by fluid phase endocytosis, a phenomenon that we previously have used to load trehalose into human platelets, after which we have successfully freeze-dried them. We now extend those findings to a species to be used in animal trials of freeze-dried platelets:pigs. Further, we report results of studies aimed at elucidating the mechanism of the uptake. Temperature dependence of fluid-phase endocytosis was determined in pig platelets, using lucifer yellow carbohydrazide (LY) as a marker. A biphasic curve of marker uptake versus temperature was obtained. The activation energy was significantly higher above 22 °C (18.7 ± 1.8 kcal/mol) than below that critical temperature (7.5 ± 1.5 kcal/mol). The activation energy of fluid phase endocytosis in human platelets was 24.1 ± 1.6 kcal/mol above 15 °C. In order to establish a correlation between the effect of temperature on fluid phase endocytosis and the membrane physical state, Fourier transform infrared spectroscopy (FTIR) and fluorescence anisotropy experiments were conducted. FTIR studies showed that pig platelets exhibit a main membrane phase transition at approximately 12 °C, and two smaller transitions at 26 and 37 °C. Anisotropy experiments performed with 1,6 diphenyl-1,3,5 hexatriene (DPH) complemented FTIR results and showed a major transition at 8 °C and smaller transitions at 26 and 35 °C. In order to investigate the relative roles of known participants in fluid phase endocytosis, the effects of several chemical inhibitors were investigated. LY uptake was unaffected by colchicine, methylamine, and amiloride. However, disruption of specific microdomains in the membrane (rafts) by methyl-β-cyclodextrin reduced uptake of LY by 35%. Treatment with cytochalasin B, which inhibits actin polymerization, reduced the uptake by 25%. We conclude that the inflection point in the LY uptake versus temperature plot at around 22 °C is correlated with changes in membrane physical state, and that optimal LY internalization requires an intact cytoskeleton and intact membrane rafts.
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*Keywords:* Cholesterol; Cytoskeleton; Fluid phase endocytosis; FTIR; Membrane; Pig platelet

## 1. Introduction

Platelets are chilling-sensitive, and as a result they are blood banked at room temperature for a maximum of 5 days. During hypothermic storage they change shape and secrete α-granules and lysosomal constituents [1,2]. These chilling-induced events start below 22 °C, coincident with the temperature at which the platelet membranes undergo a lipid phase transition [3]. Studies in our laboratory have shown that chilling damage in platelets can be inhibited by antifreeze glycoproteins isolated from Atlantic cod [3]. In addition, we have shown that human platelets can be stored in the freeze-dried state using trehalose as a lyoprotectant [4]. Trehalose was introduced in millimolar concentrations in the platelets by a mechanism primarily based on fluid phase endocytosis.

In order to evaluate different storage techniques that would increase storage times in blood banks, platelets must be validated in a series of animal models as well as in humans. Earlier studies suggested that pig platelets are a good model for human platelets because their membrane properties are very similar to those of humans [5]. The aim of the present work is to establish a correlation between fluid phase endocytosis and membrane lipid properties in pig platelets, used as a model for human platelets. Fluid

* Corresponding author. Section of Molecular and Cellular Biology, University of California, 1 Shields Avenue, Davis, CA 95616, USA. Tel.: +1-530-752-1094; fax: +1-530-752-5305.
*E-mail address:* wfwolkers@ucdavis.edu (W.F. Wolkers).

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doi:10.1016/S0005-2736(03)00114-7

phase endocytosis was studied using lucifer yellow carbohydrazide (LY) as a marker. Membrane properties were studied using Fourier transform infrared spectroscopy (FTIR) and fluorescence anisotropy.

Cells can internalize fluid through receptor-mediated or fluid phase endocytosis. Receptor-mediated endocytosis requires a specific receptor–ligand interaction and is saturable with respect to the external ligand concentration, whereas fluid phase endocytosis is not saturable with respect to the external concentration [6]. Although the pathways are different from each other, they are thermodynamically equivalent [7]. A characteristic feature of fluid phase endocytosis is that it is inhibited at low temperatures [8]. LY has been extensively used as a marker for fluid phase endocytosis [8,9]. The molecule is highly soluble in water, lipid-insoluble, and fully dissociated at physiological pH. The two negative charges prevent passage of the molecule through the membrane by diffusion [10].

Phase behavior in cellular membranes of intact cells and tissues can be measured by FTIR using the lipid symmetric $CH_2$ stretching vibration around 2850 $cm^{-1}$ [11–13]. The wavenumber position of this band is a measure of membrane fluidity. FTIR studies on human platelets showed that they have a major phase transition at 15 °C and a smaller transition at 30 °C, which were assigned to phase transitions of the major phospholipid fraction and of sphingomyelin-enriched microdomains (rafts), respectively [14].

The lipid order of the platelet membranes can also be measured using steady state fluorescence anisotropy. The lipid-associated fluorescent dyes DPH and TMA–DPH can be used to assess the membrane order in the core of the membrane as well as on the lipid–water interface. DPH inserts in the core of the membrane [15] whereas TMA–DPH has a charged group and is located at the membrane/water interface [16].

In this work we show that the uptake of LY has all the characteristics of fluid phase endocytosis; i.e., uptake is not saturable with respect to the external concentration, proceeds linearly with time, and is inhibited at low temperatures. The effect of temperature on uptake of LY was correlated with the membrane properties of the cells, as reported by FTIR and fluorescence anisotropy. The involvement of membrane rafts in the uptake of LY was demonstrated using methyl-β-cyclodextrin, which is known to disrupt these microdomains. The role of actin polymerization in LY internalization was investigated using cytochalasin B.

## 2. Materials and methods

### 2.1. Isolation of platelets

Blood was collected in acid citrate dextrose from commercial gilts and barrows housed at the University of California, Davis swine facility using an approved institutional protocol. Ages varied between 4 and 6 months old. Blood samples were pooled in 15-ml falcon polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at $327 \times g$ for 15 min. Platelet-rich plasma (PRP) was decanted and pooled into clean polypropylene tubes. Platelets were then pelleted ($481 \times g$ for 15 min) and washed in Buffer A (100 mM NaCl, 10 mM KCl, 10 mM EGTA, 10 mM imidazole, 10 μM prostaglandin-E1, pH 6.5) to obtain washed cell concentrates. Platelets were counted on a Coulter counter T890 (Coulter, Inc., Miami, Fl).

### 2.2. Loading of lucifer yellow CH into platelets

Loading studies with the fluorescent dye lucifer yellow dilithium salt (Sigma, St. Louis, MO) were performed as described previously [4] with slight modifications. Washed platelets at a concentration of approximately $1 \times 10^9$ platelets/ml were incubated in the presence of the dye (10 mM). Incubation temperatures and incubation times were chosen as indicated. After incubation, the platelets were washed twice in buffer A (by centrifugation for 20 s at $14\,000 \times g$ in a microfuge). Platelets were then counted on a Coulter counter and pelleted in 1.5-ml tubes by centrifugation for 45 s at $14\,000 \times g$ in a microfuge. Platelet associated LY was extracted by lysing the pellet in 1% Triton X-100 buffer (10 mM TES, 50 mM KCl, pH 6.8). The fluorescence of the lysate was measured on a Perkin-Elmer LS50 B spectrofluorometer with excitation at 428 nm (slid width 4 nm) and emission at 530 nm (slid width 4 nm). Uptake was calculated for each sample as nanograms of LY per cell using a standard curve of LY in lysate buffer. Standard curves of LY were found to be linear up to 2000 ng $ml^{-1}$. The internal LY concentration and the loading efficiency were calculated as previously described [4] by assuming a mean platelet volume of 8.6 and 7.0 fl for pig and human platelets, respectively.

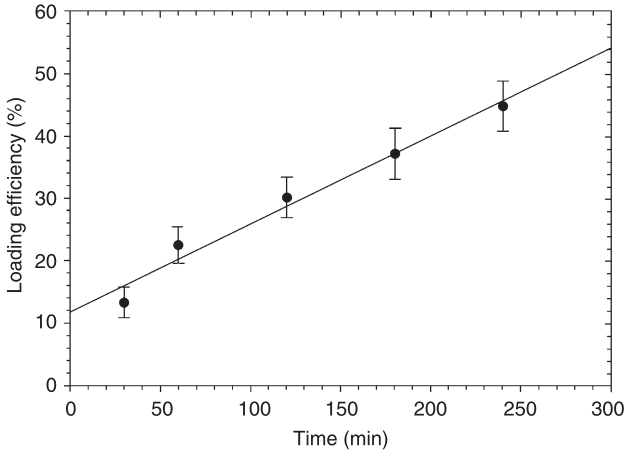


Fig. 1. Time course of LY uptake at 37 °C, in the presence of 10 mM external LY. The error bars reflect the standard error ($n = 4$).

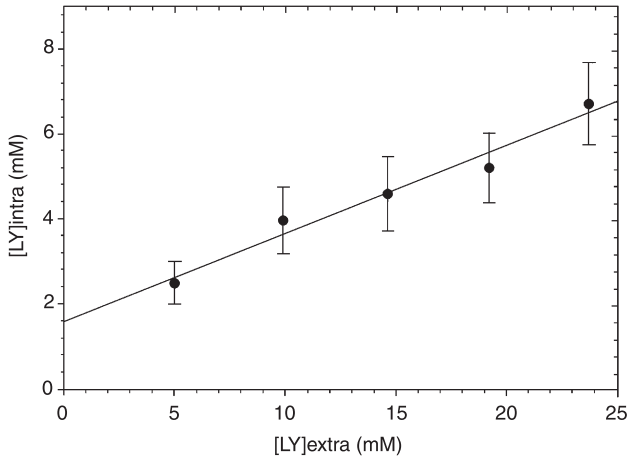


Fig. 2. LY uptake versus external LY concentration at 37 °C after a 4-h incubation period. The error bars reflect the standard error ($n=4$).

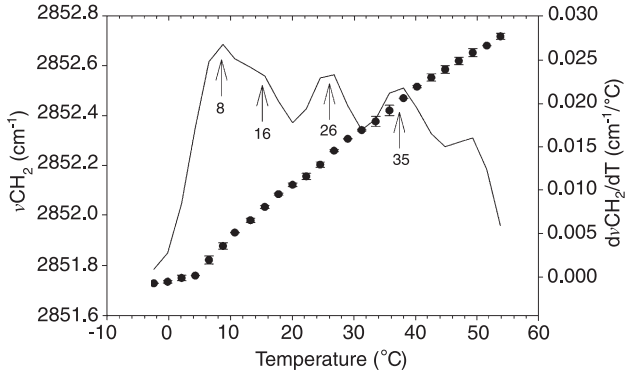


Fig. 4. Wavenumber versus temperature plots of the symmetric $CH_2$ stretching band of pig platelets (closed circles). First derivatives ($d\nu CH_2/dT$) of the wavenumber versus temperature plots are also shown (solid lines). The error bars reflect the standard error ($n=5$).

### 2.3. FTIR

Infrared spectra were recorded on a Perkin-Elmer 2000 Fourier transform IR-spectrometer equipped with a liquid nitrogen-cooled Mercury/Cadmium/Telluride (MCT) detector as described previously [12,13]. Platelet pellets were spread between two $CaF_2$ IR windows in a temperature-controlled cell. Spectra were recorded over a range of 0 to 50 °C, at a rate of 2 °C/min. Membrane fluidity was monitored by observing the band position of the $CH_2$ symmetric stretch vibration around 2850 $cm^{-1}$. The first derivative of the wavenumber versus temperature plots was taken to show inflections coinciding with lipid phase transitions more clearly.

### 2.4. Steady state fluorescence anisotropy

Platelets were labeled with DPH or TMA–DPH at 37 °C for 40 min in Tyrodes buffer (10 mM HEPES, pH 7.3, 2.9 mM KCl, 138 mM NaCl, 12 mM $NaHCO_3$, and 5.5 mM glucose) according to Ref. [17]. Labeled platelets were diluted to a concentration of $1 \times 10^7$ cells/ml to avoid light scattering of the emitted fluorescence. The anisotropy was measured from 4 to 40 °C on a Perkin-Elmer LS50 spectrofluorometer (Norwalk, CT) using 365 and 460 nm as excitation and emission wavelengths, respectively. First

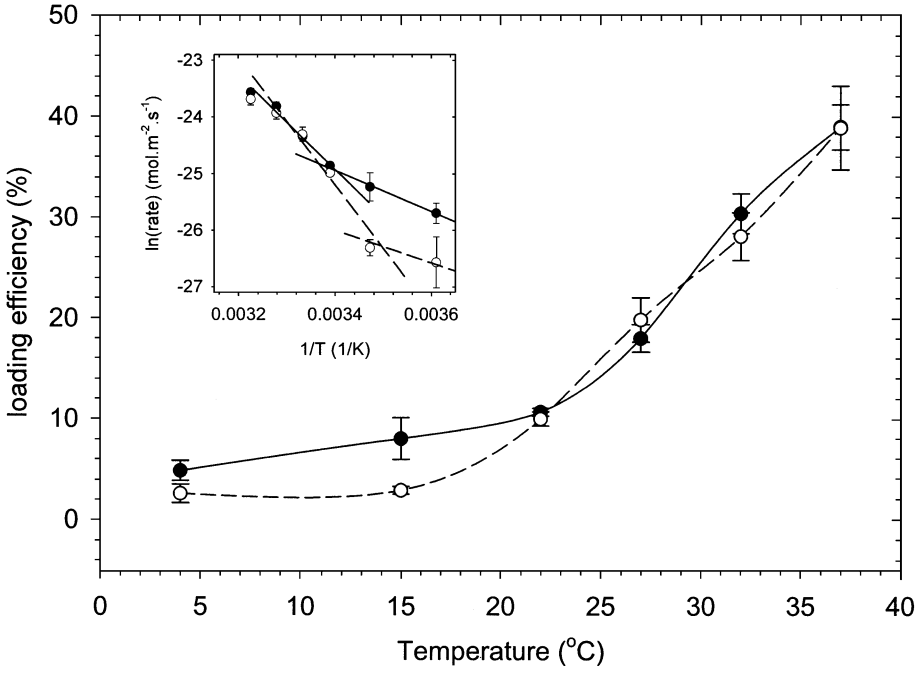


Fig. 3. Loading efficiency of LY in pig (filled circles) and human (open circles) platelets versus the incubation temperature after an incubation time of 4 h, in the presence of 10 mM external LY. The error bars reflect the standard error ($n=4$). The insert figure presents Arrhenius plots of the data.

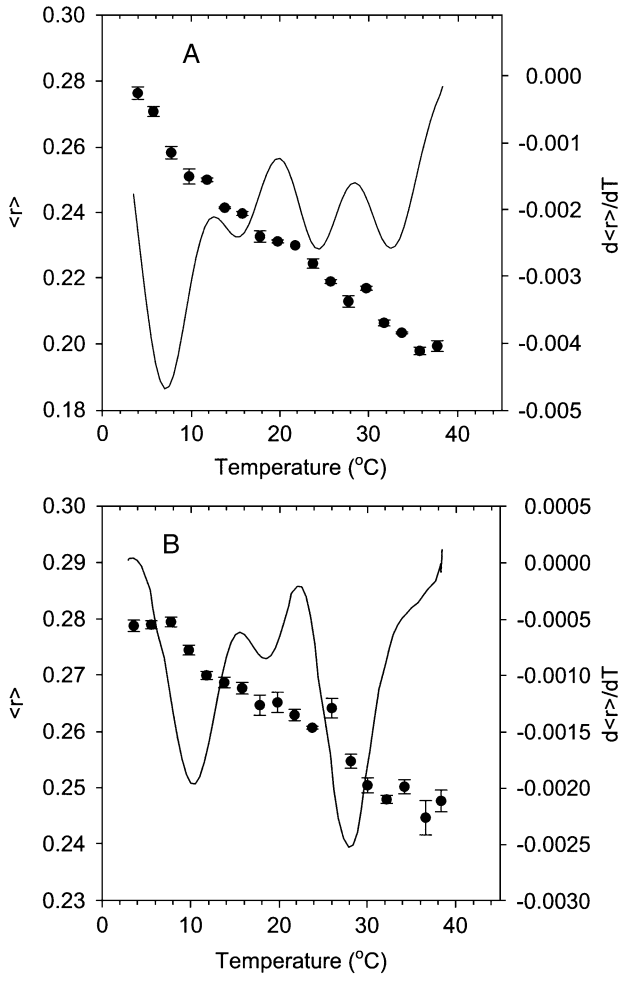


Fig. 5. Steady state fluorescence anisotropy decay of DPH (A), and TMA-DPH (B) labeled pig platelets (solid circles). First derivatives (d*r*/d*T*) of the anisotropy versus temperature plots are also shown (solid lines). The error bars reflect the standard error ($n=3$).

derivatives of the anisotropy versus temperature plots were calculated using Table Curve software (Jandel Scientific, San Rafael, CA).

### 2.5. Fluorescence microscopy

LY-loaded platelets were viewed on a fluorescence microscope (Olympus BX60, UplanFL 100X/1.3NA) employing a fluorescein filter set for fluorescence microscopy. Platelets were fixed with 1% paraformaldehyde in Buffer A for at least 1 h. Fixed cells were then cytocentrifuged onto ethanol cleaned slides with a ThermoShandon Cytospin 3 at 1500 rpm for 5 min.

## 3. Results

### 3.1. Uptake of lucifer yellow CH by pig platelets

The uptake of LY by pig platelets was studied as a function of time, extracellular concentration, and temperature. Intracellular LY was determined by measuring the platelet associated LY on a fluorometer and the loading efficiency was defined as the ratio between intracellular LY and the LY concentration in the loading buffer. The effects of colchicine, methylamine, amiloride, methyl-β-cyclodextrin, and cytochalasin B on the uptake of LY were also studied.

#### 3.1.1. LY uptake versus time

Fig. 1 depicts the time course of LY uptake at 37 °C and an extracellular LY concentration of 10 mM. An initial rapid uptake of LY within the first 30 min (the first time point) was followed by a linear uptake. The intracellular LY concentration approached the extracellular level, at a rate of approximately 8.5%/h. At an external concentration of 10 mM, the flux rate after 30 min was $4.7 \pm 0.7 \times 10^{-11}$ mol/m$^2$ s.

#### 3.1.2. Uptake of LY versus concentration

Variations in the extracellular concentration on uptake of LY at 37 °C were determined after 4-h incubation (Fig. 2). The intracellular dye concentration reached millimolar lev-

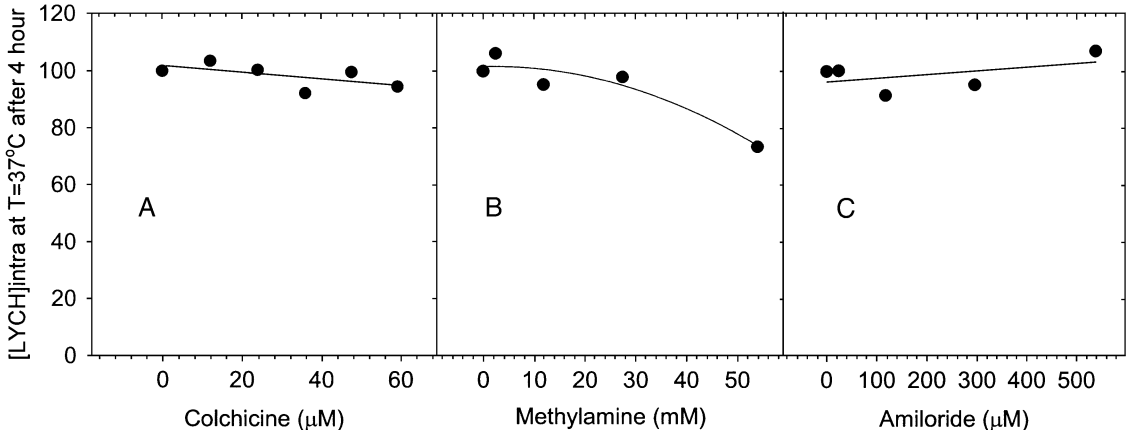


Fig. 6. Effect of colchicine (A), methylamine (B), and amiloride (C) on LY internalization by pig platelets after an incubation period of 4 h at 37 °C, in the presence of 10 mM LY.

els during the 4-h incubation. The uptake of LY was linear in the range of 0 to 30 mM external LY, showing that the uptake is not saturable with respect to the external concentration of the dye.

### 3.1.3. LY uptake versus temperature

When the uptake of LY was studied as a function of temperature, biphasic curves were observed for both pig and human platelets (Fig. 3). The uptake of the dye by the pig platelets is much more sensitive to temperature variations between 37 and 22 °C than between 22 and 4 °C. The uptake of LY by human platelets was found to be similar to that of pig platelets with a rapid increase in uptake of the dye above 15 °C. The activation energy of LY uptake was calculated using the Arrhenius relationship (Eq. (1)).

$$\ln(k) = \ln(A) + (-E_a/(RT)) \quad (1)$$

In this equation $k$ is the rate constant, $E_a$ the activation energy, and $A$ the frequency factor, the units of which are the same as those of the rate constant.

The activation energy can be obtained by plotting reciprocal temperature versus the natural logarithm of the rate of internalization (Fig. 3, insert). The Arrhenius plot of the pig platelets was fitted with two linear regressions in the temperature regions above and below the temperature at which the discontinuity apparently occurs, 22 °C. The activation energies were determined to be 18.7 ± 1.8 and 7.5 ± 1.5 kcal/mol above and below 22 °C, respectively. The activation energy of LY internalization by human platelets was determined to be 24.1 ± 1.6 kcal/mol above 15 °C.

## 3.2. Membrane phase behavior of pig platelets

The membrane properties of pig platelets were studied using FTIR and fluorescence anisotropy in order to establish a correlation between membrane phase behavior and the effect of temperature on the rate of LY internalization.

### 3.2.1. In situ FTIR assessment of membrane properties

Membrane phase behavior of intact pig platelets was assessed by FTIR. Phase transitions were derived from plots of the wavenumber of the symmetric $CH_2$ stretching vibration (around 2850 $cm^{-1}$) as a function of temperature (Fig. 4). The wavenumber increased from 2851.7 to 2852.7 $cm^{-1}$ with a temperature increase from 0 to 55 °C. The increase in wavenumber while heating denotes an increase in membrane fluidity, which is due to the sequential melting of the various lipid classes in the membrane. Three transitions at around 12, 26, and 37 °C are evident in the first derivative of the wavenumber versus temperature plot (solid line in Fig. 4). The first transition at approximately 12 °C (average of the transition at 8 °C and the shoulder at 16 °C) is in good agreement with previous results [5]. The other two smaller transitions at approximately 26 and 37 °C reflect high melting lipid components in the membrane, such as sphingomyelins. Previous studies in our laboratory have shown that similar high temperature transitions detected in human platelets are associated with membrane rafts [14].

### 3.2.2. Steady state fluorescence anisotropy

Temperature dependence of steady state anisotropy of DPH-labeled pig platelets is depicted in Fig. 5A. The anisotropy decreases with increasing temperatures, which is indicative of a decrease in the molecular order of the lipids. The anisotropy of DPH dropped from 0.28 to 0.20 when the temperature was increased from 4 to 40 °C. The first derivative of the anisotropy versus temperature plot

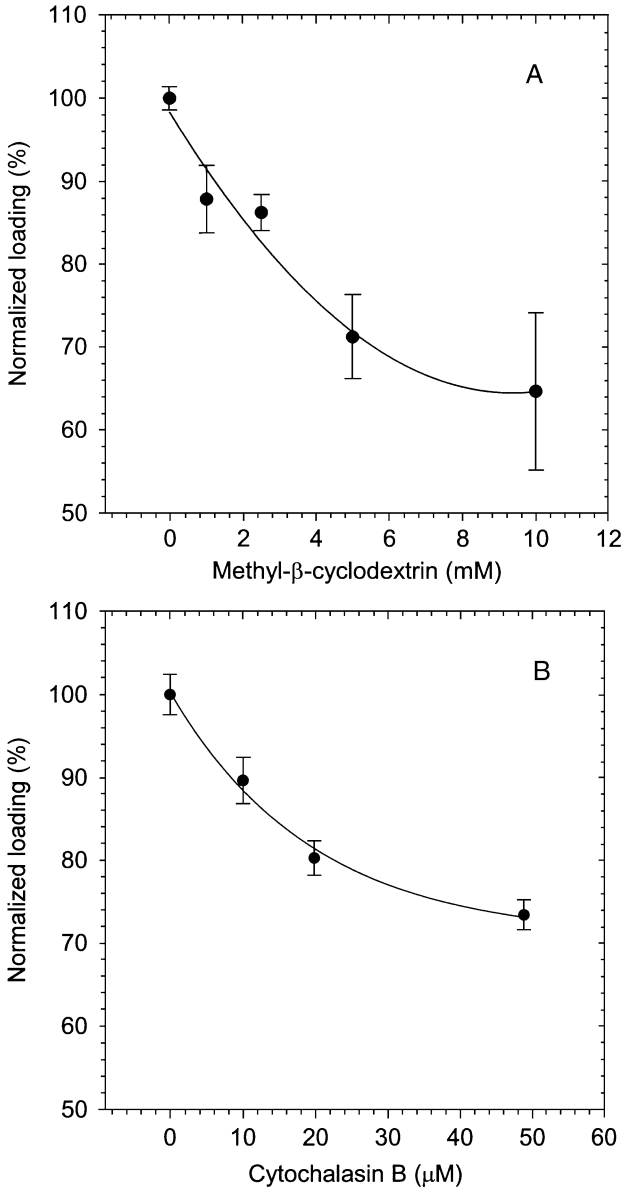


Fig. 7. Effect of methyl-β-cyclodextrin (A) and cytochalasin B (B) on LY internalization by pig platelets. The samples were incubated for 4 h at 37 °C in the presence of 10 mM LY. The error bars reflect the standard error ($n$ = 4).

showed the presence of a main phase transition at approximately 8 °C, and smaller transitions at 25 and 35 °C, in good agreement with the FTIR results.

The fluorescence anisotropy of the membrane surface probe TMA–DPH dropped from 0.28 to 0.25 with a temperature increase from 4 to 40 °C (Fig. 5B). First derivative analysis indicates the presence of two transitions at 10 and 28 °C.

### 3.3. Effect of chemical inhibitors of endocytosis on uptake of LY

To examine further the mechanism of LY internalization, pig platelets were treated with well-characterised general inhibitors of endocytosis such as colchicine and methylamine. Treatment with colchicine, a microtubule disrupting agent [18], up to 50 μM did not reduce LY uptake (Fig. 6A). Treatment with the vacuolar pH perturbant, methylamine, resulted in no reduction of LY uptake, except at the elevated concentration of 50 mM, which reduced uptake by about 20% (Fig. 6B). However, we suspect that at this high concentration intracellular pH was probably elevated out of the physiological range and that this effect is not due to alkalinization of the lysosomes per se. A third inhibitor, amiloride, which has been reported to reduce clathrin-independent pinocytic pathways specifically [19], had no effect on LY internalization (Fig. 6C).

### 3.4. Role of membrane rafts and cytoskeleton in LY internalization

The involvement of membrane rafts in the uptake of LY was tested by disrupting these microdomains with MβCD. MβCD has been widely used to remove cholesterol selectively from the plasma membrane of cells [20,21]. Removal of membrane cholesterol by MβCD particularly affects the cholesterol rich membrane rafts [22]. Treatment by MβCD reduced the capability of platelets to take up LY by approximately 35% (Fig. 7A). At 10 mM of MβCD, 98% of cholesterol is removed from the platelets [14]. We also attempted to hydrolyze sphingomyelin, one of the other major components in membrane rafts, by treatment with sphingomyelinase. However, treatment with the enzyme did not result in cleavage of sphingomyelin (based on thin layer chromatography experiments; data not shown). The enzyme was found to be effective only after cholesterol removal from the cells (data not shown).

The involvement of a functional cytoskeleton in the uptake of LY was tested using cytochalasin B, which is an

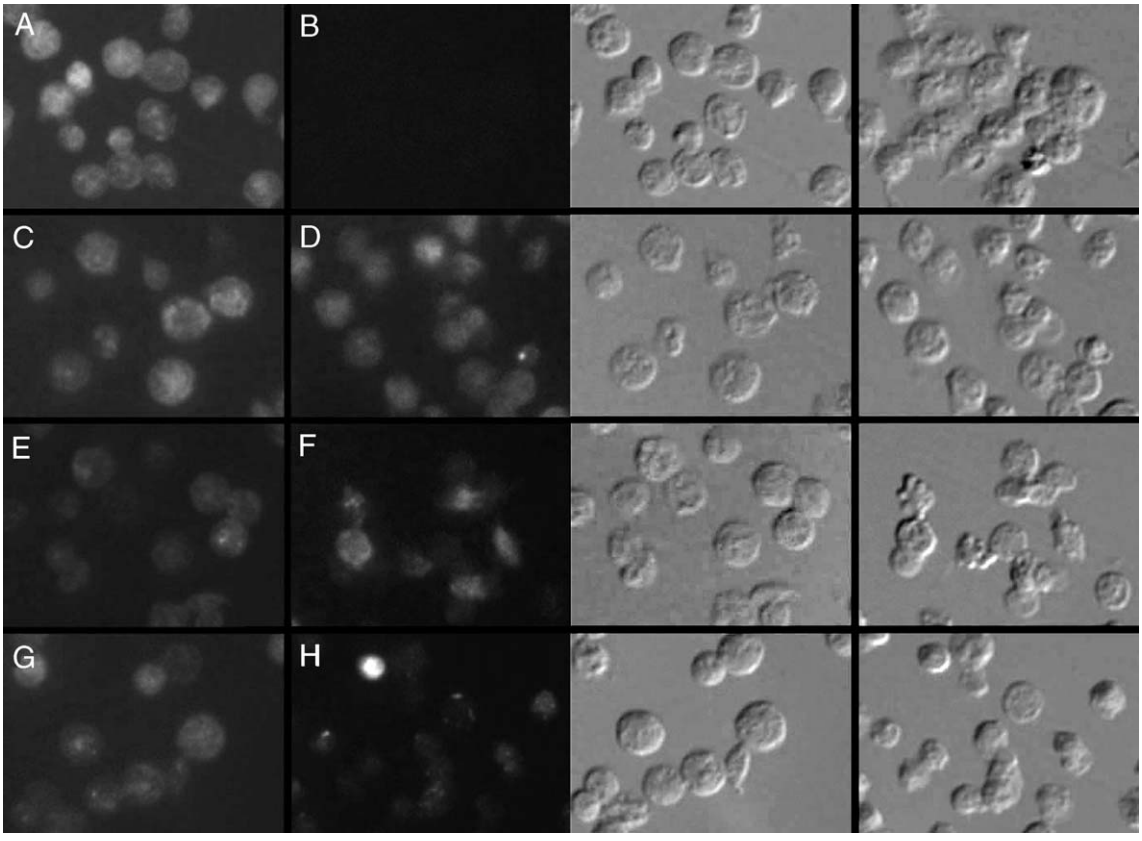


Fig. 8. Fluorescent (left panel) and phase contrast (right panel) images of LY loaded platelets. Platelets were incubated in 10 mM LY for 4 h. Platelets were incubated at 37 (A) or 4 (B) °C; incubated at 37 °C and treated with either cytochalasin B at 10 (C), 20 (E), 50 (G) μM, or treated with MβCD at 1 (D), 5 (F), 10 (H) mM. The left panel represent the phase contrast images corresponding to the fluorescent images.

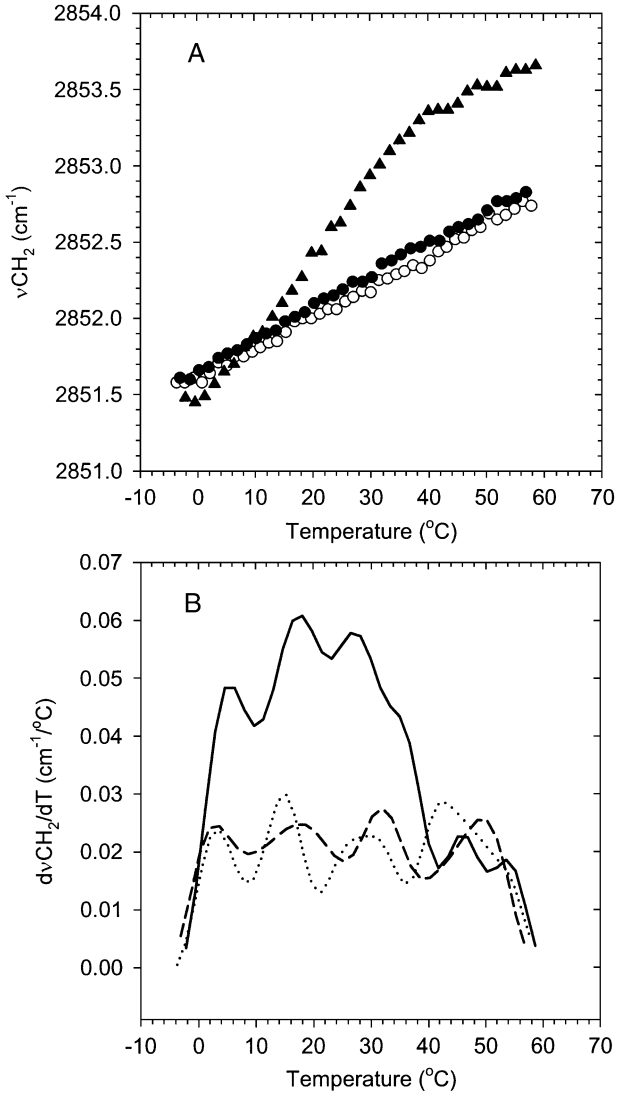


Fig. 9. (A) Wavenumber versus temperature plots of the symmetric $CH_2$ stretching band of control pig platelets (open circles), platelets treated with 50 mM cytochalsin B (closed circles), and platelets treated with 10 mM MβCD (closed triangles). (B) First derivatives ($d\nu CH_2/dT$) of the wavenumber versus temperature plots of control (dotted lines), cytochalasin B-treated (dashed lines), and MβCD-treated (solid lines) cells are also shown (solid lines).

inhibitor of actin polymerization [23]. Treatment of the platelets with cytochalasin B reduced LY internalization in a dose-dependent manner, with a reduction of 25% at 50 μM (Fig. 7B).

Fluorescence microscopy showed a more or less homogeneous distribution of LY in the platelets (Fig. 8). The fluorescence intensity of control platelets increased with time during incubation at 37 °C, and virtually no fluorescence was detectable in platelets that were incubated at 4 °C (Fig. 8A and B). The fluorescence intensity of platelets treated with cytochalasin B and MβCD showed a more reduced fluorescence intensity compared to control cells with increasing inhibitor concentrations, in agreement with Fig. 7.

### *3.4.1. Effect of cytochalasin B and methyl-b-cyclodextrin on membrane properties*

In order to test whether the inhibiting effects of cytochalasin B and MβCD on LY internalization are associated with changes in membrane properties, the effects of these treatments on membrane fluidity were determined using FTIR. Treatment with cytochalasin B did not affect the membrane fluidity, as shown in Fig. 9. The wavenumber excursion from 0 to 55 °C was virtually identical to that of control cells. First derivative analysis revealed minor differences between cytochalasin B-treated and control cells; however, these differences fall within the normal range observed for control cells.

Cholesterol removal by MβCD had a drastic effect on the thermotropic response of the membranes. The wavenumber excursion from 0 to 55 °C increased from 1.2 to 2.1 $cm^{-1}$ upon cholesterol removal. The effect is particularly visible at high temperatures, when the membranes are in a disordered phase; the wavenumber of the lipid band increased after cholesterol depletion of the cells, indicative of an increased membrane fluidity, compared to control cells. Four transitions at 5, 16, 27, and 35 °C were visible after cholesterol removal from the plasma membranes, and, as expected, $d\nu/dT$ of the transitions was greater compared to the control cells. The derivative of the wavenumber versus temperature plot, expressed in $d\nu/dT$ ($cm^{-1}$/°C), can be considered as a measure for the cooperativity of the transition. The cooperativity of the transition at 15 °C was increased by 0.036 $cm^{-1}$/°C after cholesterol removal. The transition at 27 °C exhibited the greatest enhancement in cooperativity after cholesterol removal; 0.045 $cm^{-1}$/°C.

### *3.5. Uptake of LY in autologous plasma*

For comparison, uptake of LY was also determined in autologous plasma. Fig. 10 shows that the uptake of LY by

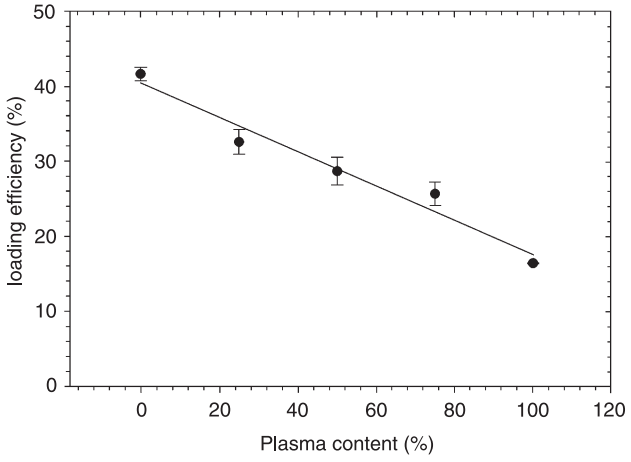


Fig. 10. LY internalization in autologous plasma compared to that in buffer. Loading efficiency of LY is depicted as a function of the buffer plasma *v/v* ratio (%) after an incubation time of 4 h at 37 °C, in the presence of 10 mM LY. The error bars reflect the standard error ($n=3$).

platelets is inhibited with increasing fractions of autologous plasma in the incubation buffer. Uptake of LY in plasma is reduced by approximately 50% compared to the uptake in buffer.

## 4. Discussion

In this paper we have shown that the uptake of LY by pig platelets has all the kinetic features of fluid-phase endocytosis, i.e., the amount taken up is proportional to the concentration in the medium, the uptake proceeds continuously with time and exhibits a biphasic curve as a function of temperature. This biphasic behavior of LY internalization is possibly related to the membrane physical state. Several general inhibitors of fluid phase endocytosis did not inhibit LY internalization in pig platelets. Disruption of the cytoskeleton and membrane rafts resulted in a reduction of LY uptake.

The inhibition of LY uptake at low temperatures, observed here for both pig and human platelets, is one of the characteristic features of fluid phase endocytosis [8]. The uptake of LY by pig and human platelets increased drastically above 22 and 15 °C, respectively. The rate of LY internalization did not show Arrhenius behavior in the 4–37 °C temperature range. The activation energy drastically increased above 22 and 15 °C for pig and human platelets, respectively. Such biphasic behavior in the effect of temperature on LY uptake has been observed in other cell types. Fluid phase endocytosis in renal epithelial cells occurs with an activation energy of 15–22 kcal/mol above and 9–12 kcal/mol below a critical temperature of 27 °C [24]. Rat hepatocytes internalize LY with an activation energy of 25.8 kcal/mol [7]. The activation energies that we report here for pig and human platelets are in good agreement with these results and further confirm that LY is internalized through fluid phase endocytosis. The uptake of LY proceeds linearly with time after an initial rapid uptake of the dye during the first 30 min. The initial rapid uptake of the dye followed by a slower uptake has also been observed in macrophages and has been assigned to a small, rapidly turning over compartment, and the slower phase has been assigned to filling up of lysosomes [9]. The internal LY concentration increased linearly with respect to the external concentration. Unlike receptor-mediated endocytosis, fluid phase endocytosis is not saturable with respect to the external concentration.

Fluid phase endocytosis requires vesicle formation and fusion events, and hence it can be expected that the membrane fluidity plays a crucial role in this event. FTIR and fluorescence anisotropy studies were conducted to test the role of membrane fluidity in endocytosis. Pig platelets were found to exhibit a major transition at 12 °C, in good agreement with previous results [5]. FTIR studies on human, horse, and mouse platelets show that the main phase transition in platelets typically falls between 10 and 15 °C [3,13,17,25]. In addition, two transitions were observed at 26 and 35 °C, which we tentatively assign to membrane rafts. We base this on previous studies with human platelets, which showed that a second high temperature transition at around 30 °C could be linked to the main transition in isolated membrane rafts of the platelets [14]. Cholesterol depletion of the cells showed that the membrane phase behavior in platelets is strongly modulated by cholesterol. The transition at 26 °C was most affected by the cholesterol depletion, indicating that this transition is possibly associated with the cholesterol rich membrane rafts. Cholesterol fluidizes the lipid bilayer in the gel state and reduces motional order in the fluid state [26,27]. The membrane properties of platelets were also studied using the fluorescent probes DPH and TMA–DPH to corroborate the FTIR studies. The fluorescence anisotropy of the fluorescent dyes reports on the lipid order of the membrane. DPH partitions in the core of the membrane, whereas TMA–DPH interacts with the polar lipid headgroups and, thus, is more sensitive to the structural order at the membrane surface. The greater anisotropy excursion between 4 and 40 °C of DPH compared to that of TMA–DPH implies that the lipid order in the core of the membrane is more affected by temperature variations than the lipid order at the membrane surface. As expected, the membrane phase transitions that can be deduced from the anisotropy decay in the core of the membrane more closely resembled the FTIR results than the transitions derived from the anisotropy decay at the membrane surface.

At first sight there is no clear correlation between membrane fluidity and LY internalization (Fig. 11). The increased LY internalization above 22 °C coincides with a threshold membrane fluidity of 2852.2 $cm^{-1}$, which is indicative of an intermediate membrane fluidity between the gel and the fluid phase. Fig. 11 shows that this critical membrane fluidity corresponds with the end of the first

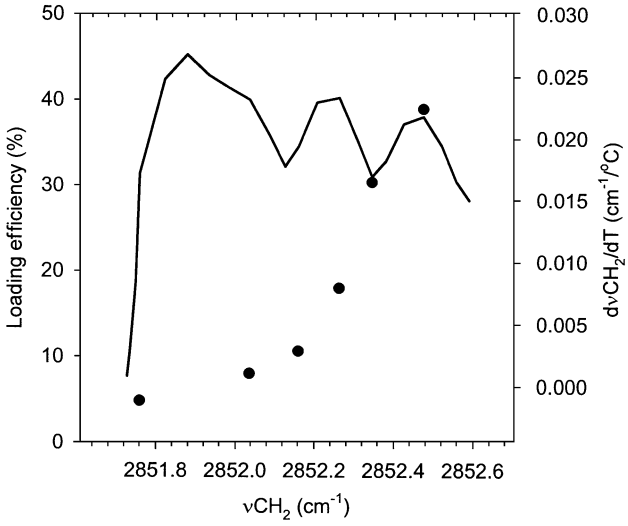


Fig. 11. Correlation between membrane fluidity ($\nu CH_2$) and LY internalization of pig platelets. A plot of the $d\nu CH_2/dT$ versus $\nu CH_2$ is also shown to indicate the phase transitions.

major phase transition of the membranes from 2851.6 to 2852.2 $cm^{-1}$ (the transition at 12 °C in Fig. 4). Further fluidization of the membrane after completion of the first transition results in a drastic increase in LY internalization.

Several general inhibitors of fluid phase endocytosis were tested to study their effects on LY uptake. The microtubule inhibitor, colchicine, did not inhibit LY uptake, suggesting that the microtubular system is not involved in the uptake. In resting platelets, microtubules assume an array in the form of a coil [28]. The microtubule coil helps to maintain the discoid shape of the resting platelet. Apparently, perturbation of the tubular coil does not affect endocytosis. The vacuolar pH perturbant, methylamine, showed only a moderate inhibition at 50 mM, which is well above the physiological range. The use of such substances acting on low pH intracellular compartments have been reported to inhibit endocytosis [19,29]. The use of a more specific inhibitor of fluid phase endocytosis, the $Na^+H^+$ channel blocker amiloride, did not inhibit LY internalization in pig platelets. Amiloride was shown to inhibit the uptake of *Coxiella burnetii* by a host cell through fluid phase endocytosis [19] and it blocks fluid phase endocytosis in human carcinoma cells, without affecting the receptor-mediated endocytosis of proteins [30].

Actin filaments are the essential contractile elements of the platelet cytoskeleton. They regulate shape change, extension of pseudopodia, platelet spreading and adhesion, platelet aggregation, secretion and clot retraction [31]. The connection between the cytoskeleton and the membranes in platelets is thought to be mediated primarily through vinculin [32] as well as through numerous other actin binding proteins [33]. The polymerization of actin filaments that are forming the membrane cytoskeleton can be inhibited by cytochalasin B [23,34]. Phagocytosis of liposomes by human platelets is inhibited by cytochalasin B, suggesting that a functional cytoskeleton is required for optimal vesiculation [35]. The results presented here demonstrate that a functional cytoskeleton is also required for optimal pinocytosis in platelets. Gousset [36] showed that treatment of human platelets with cytochalasin B prevents raft aggregation at low temperatures, suggesting that in addition to disruption of the cytoskeleton the molecule also affects the membrane phase behavior. The present FTIR data suggest that cytochalasin B does not affect the thermotropic response of the platelet membrane lipids (Fig. 10), which implies that the effect of cytochalasin B treatment on LY internalization is not mediated through an effect on the membrane phase behavior of platelets.

The involvement of membrane rafts in the uptake of LY was demonstrated using methyl-β-cyclodextrin, which is known to disrupt the microdomain structure of membranes [22]. Membrane rafts are enriched in sphingolipids and cholesterol and have a high degree of lipid structural order [37]. Rafts are present at physiological temperatures, but are generally too small to be detected by light microscopy. In platelets, membrane rafts can be visualized by chilling, which results in aggregation of rafts coincident with activation of the cells. These events start below 22 °C [3,14]. Disruption of the microdomain structure of the membranes by treatment with MβCD resulted in inhibition of LY internalization. FTIR studies showed that cholesterol depletion coincides with an increase in cooperativity of the transitions. It is worth noting that the increase in cooperativity of the transitions upon cholesterol depletion does not coincide with an increased uptake of LY. It is well established that cholesterol decreases the permeability of the lipid bilayer in model membranes [38]. The fact that cholesterol depletion of platelets reduces LY uptake confirms that vesiculation rate rather than membrane permeability dictates the rate of LY internalization. Esfahani et al. [39] have shown that cholesterol is required for optimal fluid phase endocytosis in a monocyte-like cell line. Ilangumaran and Hoessli [22] showed that both receptor-mediated and fluid phase endocytosis are affected by cholesterol depletion. Recently it was shown that lipid trafficking of alkyl-lysophospholipid in a tumor cell line occurs through raft-dependent endocytosis. Cholesterol, and hence intact rafts, were found to be required for optimal uptake [40].

The strong inhibition of LY internalization in autologous plasma compared to the one in buffer was also observed in studies with human and mouse platelets (W.F. Wolkers, unpublished data). We speculate that the inhibition of LY uptake in plasma can be attributed to plasma lipids or proteins that are interfering with the vesiculation rate.

We conclude that LY internalization in pig platelet occurs predominantly through fluid phase endocytosis. The uptake is dependent on the physical state of the membrane and requires a functional cytoskeleton and intact membrane rafts.

## Acknowledgements

This work was supported by grants HL57810 and HL61204 from NIH, and 981711 from DARPA.

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