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Mechanism of hemolysis of human erythrocytes exposed to monosodium urate monohydrate crystals. Preliminary characterization of membrane pores

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Abstract

Microcrystals of monosodium urate monohydrate (MSUM) have the ability to cause rapid hemolysis of erythrocytes. The nature of the initial MSUM crystal–erythrocyte membrane binding interaction was investigated over a range of different ionic strength media. There was negligible binding of MSUM to erythrocyte ghost membranes in low ionic strength media such as isotonic mannitol but binding was dramatically increased in isotonic NaCl/mannitol solutions or isotonic mannitol containing 1 mM Ca^{2+}. Hemolysis induced by MSUM crystals was preceded by the leakage of K^+ from the cells suggesting a colloid-osmotic mechanism of hemolysis. The inclusion of large (oligosaccharide) molecules in the extracellular media or the modulation of the extracellular solution tonicity inhibited both the rate and extent of hemolysis supporting the concept of MSUM-induced pores followed by colloid osmotic hemolysis.

Keywords: Sodium urate crystal; Urate monohydrate crystal, monosodium; Hemolysis; Erythrocyte; Membrane pore

1. Introduction

Microcrystals of monosodium urate monohydrate (MSUM) produce the inflammatory reaction characteristic of acute gouty arthritis. Crystal-induced inflammation involves the interaction of the crystals primarily with polymorphonuclear leukocytes or neutrophils within the synovial fluid of the joint. The crystal–neutrophil interaction has been proposed to proceed as follows [1,2]. MSUM crystals are phagocytozed by neutrophils and lie within a phagolysosomal sac. The crystal then binds to the phagolysosomal membrane which results in membrane lysing or membranolysis, neutrophil cytolysis and inflammation. An understanding of the mechanism of the crystal–phagolysosomal membrane interaction is of major importance to understanding the process of crystal-induced inflammation.

Microcrystalline materials such as MSUM, calcium pyrophosphate dihydrate, silica, calcium oxalate and hydroxyapatite have the ability to cause hemolysis of erythrocytes and therefore crystal-induced membranolysis has been extensively studied using the erythrocyte plasma membrane as a model membrane [3–7].

In previous work, we have studied the nature and extent of the binding interaction of MSUM with erythrocyte membranes [8–10] and have demonstrated that MSUM crystals produce rapid and extensive hemolysis of erythrocytes which is sensitive to the effects of membrane permeable and impermeable protein crosslinking agents [11,12]. We have proposed a mechanism of MSUM crystal-induced membranolysis in which negatively charged MSUM crystals interact with membrane proteins inducing the lateralization or redistribution of transmembrane proteins into clusters or aggregates, leading to pore formation and colloid osmotic lysis [10–12].

The mechanism of erythrocyte hemolysis by melittin [13,14], complement [14], diamide [15] and ethanol [16] has been described and characterized in terms of pore size and intracellular ion and hemoglobin release [16–18].

In this study we have investigated the MSUM crystal–erythrocyte membrane interaction and a colloid-osmotic mechanism for MSUM crystal-induced hemolysis.

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2. Materials and methods

2.1. Materials

Melittin (bee venom), diphenylhexatriene (DPH), D(+)-melezitose, stachyose, raffinose, uric acid and all buffer salts were obtained from Sigma, St. Louis, MO. D-Mannitol and sucrose were from BDH (Canada), Toronto, Ont.

All distilled water and buffers were presaturated with MSUM at 37°C to prevent subsequent dissolution of crystals during hemolysis experiments. Buffers used were isotonic Hepes (10 mM)/NaCl buffer (pH 7.4), isotonic 10 mM phosphate-buffered saline (pH 7.4) (PBS) and isotonic 10–80 mM phosphate/NaCl buffers. Unbuffered isotonic solutions were used in studies involving different inorganic and organic salts. The pH of these solutions was always within the range 6.5 to 7.4.

Blood was obtained from healthy adult volunteers by venipuncture using heparin as an anticoagulant and used within 3 days of collection. PBS was used in all erythrocyte wash procedures prior to suspension in the final buffer solutions.

2.2. Methods

2.2.1. Preparation and characterization of crystals

MSUM crystals were prepared as previously described [4], characterized using X-ray diffraction and scanning electron microscopy [4] and used unheated in all studies. The size distribution of crystals was approx. 75% of crystals less than 10 μm, 16% between 10 and 20 μm and 8% greater than 20 μm (crystals measured along the long needle axis).

2.2.2. Hemolysis

Erythrocyte suspensions were washed in PBS [4], centrifuged at $400 \times g$ for 5 min (Beckman GPR) and packed cells added to 25 ml of buffer containing MSUM crystals in 50 ml Erlenmeyer flasks incubated at 37°C with orbital shaking at 200 rpm. Final concentrations were MSUM, 22 mg/ml and erythrocytes, 0.5% (v/v). Control flasks contained cells in buffer (crystals absent). At given time intervals, 900-μl samples of the incubation media were transferred to 1.5 ml Eppendorf tubes and centrifuged at $10\,000 \times g$ for 5 s in a microfuge (Eppendorf). In experiments using hypertonic solutions, erythrocytes (0.5% v/v) were allowed to equilibrate in the buffer for 5 min, then MSUM (22 mg/ml) was added to the erythrocyte suspension. Quantitation of hemoglobin in the supernatants was carried out by visible spectroscopy at 540 nm [4]. Absorbance values for 100% hemoglobin release were obtained by the addition of Triton X-100 to tubes containing cells in buffer (crystals absent). % hemolysis values were calculated [4].

The efflux of K^+ from the cell-crystal suspensions was measured with a K^+ ion-selective electrode (Microelectrodes, Londonderry, New Hampshire). The total amount of K^+ was determined by the addition of Triton X-100 to the samples.

All experiments were repeated at least three times except those involving melittin which were performed only once. Repeat studies always showed the same effect and representative time-course data from one experiment are reported.

2.2.3. Crystal-erythrocyte membrane binding

Determination of the binding of MSUM crystals to erythrocyte membranes was based on a fluorescence method developed in our laboratory [10]. Briefly, a 1% (v/v) suspension of erythrocyte ghosts in PBS was prepared by the method of Dodge [19] and incubated with the fluorescent membrane probe DPH at 1 μM for 30 min at room temperature. The ghost suspension was centrifuged at $15\,000 \times g$ for 10 min and the ghosts were washed in 50 ml PBS before final resuspension at 50% (v/v) in 5 mM phosphate buffer, pH 7.4. Binding experiments were performed in quadruplicate in 3 ml capped tubes containing MSUM at 22 mg/ml and ghosts at 0.5% (v/v) in the appropriate buffer solution. Quadruplicate control tubes contained 0.5% (v/v) ghosts in buffer (crystals absent). Tubes were tumbled end-over-end at 37°C for 30 min, then centrifuged at $150 \times g$ for 2 min to sediment MSUM crystals and MSUM crystals bound to ghosts but not unbound ghosts which remained in the supernatant. The supernatants were transferred to another tube for quantitative fluorescence analysis at 355 nm excitation, 428 nm emission (Shimadzu RF 540 spectrofluorophotometer). The extent of binding of ghosts to MSUM crystals was determined using the following equation:

$$\%\ \text{ghosts bound to crystals} = \left(1 - \frac{F_c}{F_o}\right) \times 100$$

where F_c is the fluorescence intensity of the supernatant from the crystal/ghost incubation and F_o is the fluorescence intensity of the supernatant from the control ghost incubation.

3. Results

3.1. Effect of ionic strength on binding and hemolysis

Fig. 1 shows the % hemolysis values determined after 2 h MSUM-erythrocyte incubations in isotonic solutions of differing NaCl concentrations. Fig. 1 also shows the % ghosts bound to MSUM in the NaCl/mannitol solutions. Previous work [10] showed that low-speed centrifugation ($150 \times g$) was sufficient to sediment ghosts bound to crystals and discrete crystals, but not free ghosts which therefore remained in the supernatant. The % of ghosts

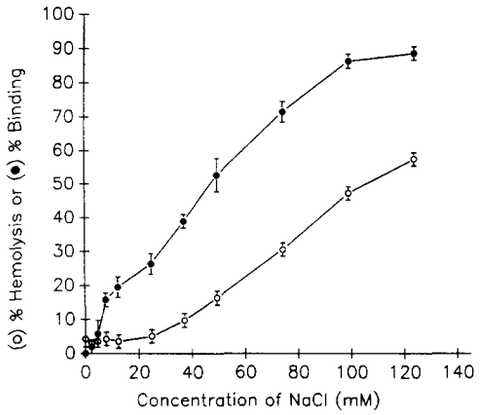

Fig. 1. Effect of increasing ionic strength of the suspension media on (●) % binding of erythrocyte ghosts (0.5% v/v) to MSUM (22 mg/ml) or (○) % hemolysis of erythrocytes (0.5% v/v) by MSUM (22 mg/ml) after a 2 h incubation. Ionic strength was increased by increasing the ratio of isotonic NaCl/isotonic mannitol and ranged from 0 to 0.12. All points are the mean of triplicate samples ± S.D.

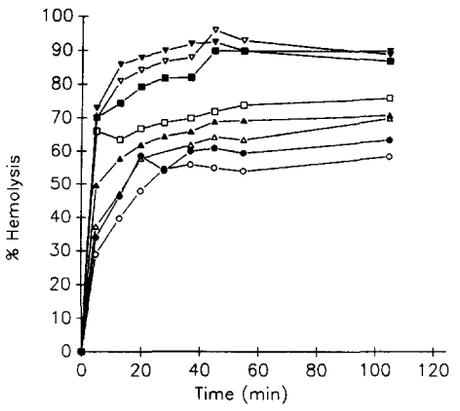

Fig. 2. Time-course of % hemolysis of erythrocytes (0.5% v/v) by MSUM (22 mg/ml) in 10 mM Hepes/saline pH 7.4 containing calcium chloride at a final concentration of: (○) zero, (●) 50 μM, (△) 100 μM, (▲) 500 μM, (□) 1 mM, (■) 5 mM (▽) 10 mM and (▼) 20 mM. Ionic strength ranged from 0.16 to 0.21.

remaining in the supernatant was obtained from the total DPH emission from the ghosts in the supernatant. Serial dilutions of a DPH labelled ghost suspension gave a linear decrease in fluorescence intensity with a correlation coefficient of 0.99. At low NaCl concentrations and low ionic strength binding and hemolysis were negligible. Subsequently, both the values of % binding of ghosts to MSUM and % hemolysis rose steadily with increasing ionic strength.

Since these studies were conducted in NaCl/mannitol solutions which were unbuffered, we determined the effect of changes in pH between 5.5 to 7.4 on the % hemolysis by MSUM. Hemolysis studies in isotonic 10 mM Hepes buffered NaCl at pH 5.5, 5.65, 5.8, 5.95, 6.1, 6.4 and 7.4 gave % hemolysis values of 45%, 45%, 43%, 45%, 45%, 47% and 44%, respectively (values determined in duplicate or triplicate). pH values of less than 5.5 could not be studied due to precipitation of uric acid. Within the pH range studied, pH had no effect on MSUM-induced hemolysis.

3.2. Effect of divalent cations and phosphate on binding and hemolysis

The time-courses of hemolysis by MSUM in Hepes-buffered saline alone and then in the presence of increasing concentrations of Ca^{2+} are given in Fig. 2. The values of the % hemolysis at a given time increased with increasing Ca^{2+} concentration.

Fig. 3A shows that there was negligible MSUM-induced hemolysis in the isotonic sugar solutions up to 15

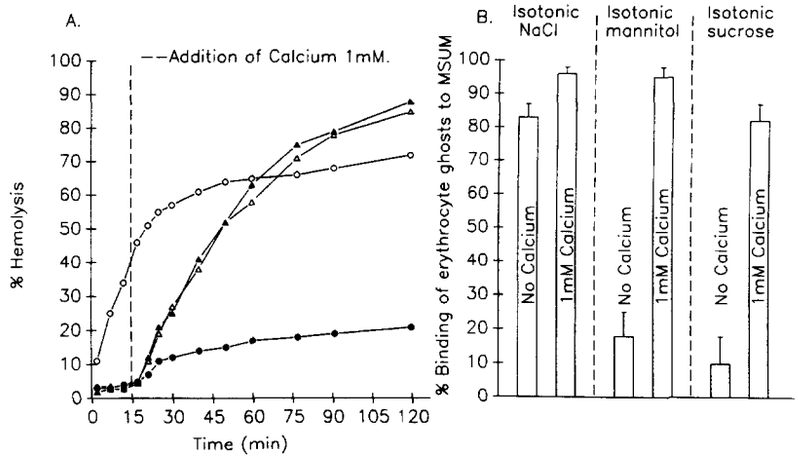

Fig. 3. (A) Effects of the addition of calcium chloride (final concentration 1 mM) on the time-course of % hemolysis of erythrocytes (0.5% v/v) by MSUM (22 mg/ml) in: (○) isotonic PBS, (▲) isotonic mannitol, (△) isotonic sucrose or (●) isotonic melezitose. (B) Effect of addition of calcium chloride (final concentration 1 mM) to suspension buffers on the % binding of erythrocyte ghosts (0.5% v/v) to MSUM (22 mg/ml) in isotonic NaCl, isotonic mannitol or isotonic sucrose. All solutions were buffered to pH 7.4 with 10 mM Hepes. Addition of 1 mM calcium chloride increased the ionic strength of the sugars from 0 to 0.003 and of PBS from 0.155 to 0.157.

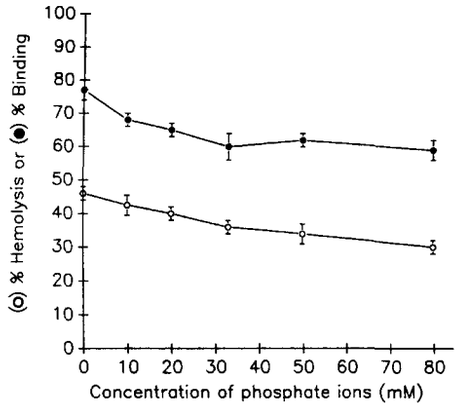

Fig. 4. Effect of increasing concentrations of phosphate ions in isotonic phosphate-buffered saline pH 7.4 on (●) % binding of erythrocyte ghosts (0.5% v/v) to MSUM (22 mg/ml) or (○) % hemolysis of erythrocytes (0.5% v/v) by MSUM (22 mg/ml). 0 mM phosphate experiments were performed in unbuffered isotonic saline. Ionic strength ranged from 0.16 to 0.48 (at 80 mM phosphate).

min but that after the addition of Ca^{2+}, hemolysis rapidly increased except for melezitose in which hemolysis was slower and less extensive. However the values of the % binding of ghosts to MSUM in isotonic mannitol, sucrose, melezitose and PBS were very similar, being $97 \pm 3\%$, $87 \pm 6\%$, $93 \pm 5\%$ and $95 \pm 2\%$, respectively.

The binding of ghosts to MSUM was greatest in the high ionic strength medium (saline) and was increased by the addition of 1 mM Ca^{2+} in all cases (Fig. 3B). Other divalent ions also showed similar effects. Concentrations of 1 mM of Mg^{2+}, Mn^{2+}, Ca^{2+} and Ba^{2+} gave values of % binding of $96 \pm 1\%$, $96 \pm 2\%$, $97 \pm 1\%$ and $96 \pm 2\%$, respectively, compared to $83 \pm 3\%$ for binding in isotonic NaCl alone (no added divalent cations).

Increasing concentrations of phosphate ions decreased % hemolysis and binding (Fig. 4). At 80 mM phosphate, binding was significantly lower than in NaCl ($P < 0.01$, Student's t-test).

3.3. Effect of different alkali chloride or sodium halide salts on binding and hemolysis

The rate and extent of hemolysis by MSUM was strongly influenced by the nature of the cation salt (Fig. 5A) and the binding of erythrocyte ghosts to MSUM in the presence of cesium, rubidium, lithium and potassium cation salts was significantly inhibited compared to sodium chloride control ($P \leq 0.0001$) (Fig. 5B). Changing either the inorganic anion (NaCl vs. NaF vs. NaBr vs. NaI) or the organic anion (sodium oxalate vs. sodium pyruvate) while keeping the sodium cation constant had no effect on the rate and extent of MSUM-induced hemolysis (data not shown).

3.4. Effect of organic cations on binding and hemolysis

Lysis and binding were significantly inhibited ($P < 0.01$) by organic cations in the extracellular media (Fig. 6A) but were increased by the addition of 1 mM Ca^{2+} to the extracellular solutions (Fig. 6B). Hemolysis in isotonic arginine/Ca^{2+} was significantly lower than control (isotonic sodium chloride/Ca^{2+} solution) at $P < 0.01$ (Fig. 6B).

3.5. Rate of K^+ efflux

Fig. 7A and Fig. 7B show that the K^+ efflux resulting from the MSUM crystal–erythrocyte interaction in both buffers occurred over a period of 200–300 s and preceded hemoglobin release from the erythrocyte. The appropriate binding data are given in Fig. 3B.

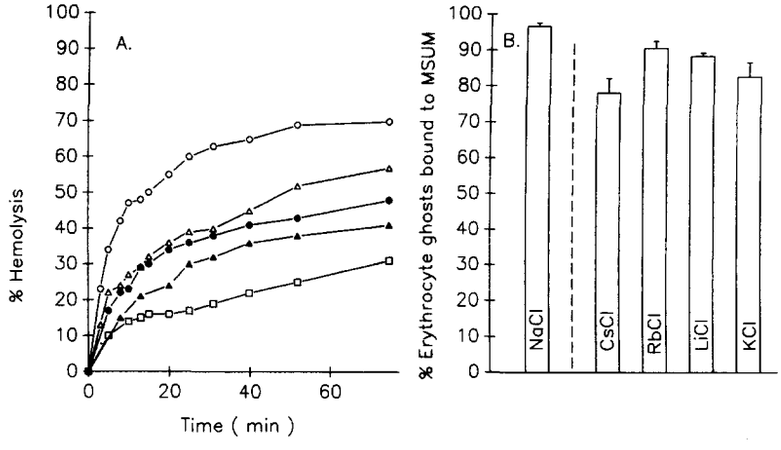

Fig. 5. (A) The time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) hemolysis in isotonic solutions of alkali chloride salts: (○) NaCl, (△) CsCl, (●) RbCl, (▲) LiCl, (□) KCl. (B) Percentage binding of ghosts to MSUM in isotonic solutions of organic cation chlorides and sodium chloride. All solutions have the same ionic strength.

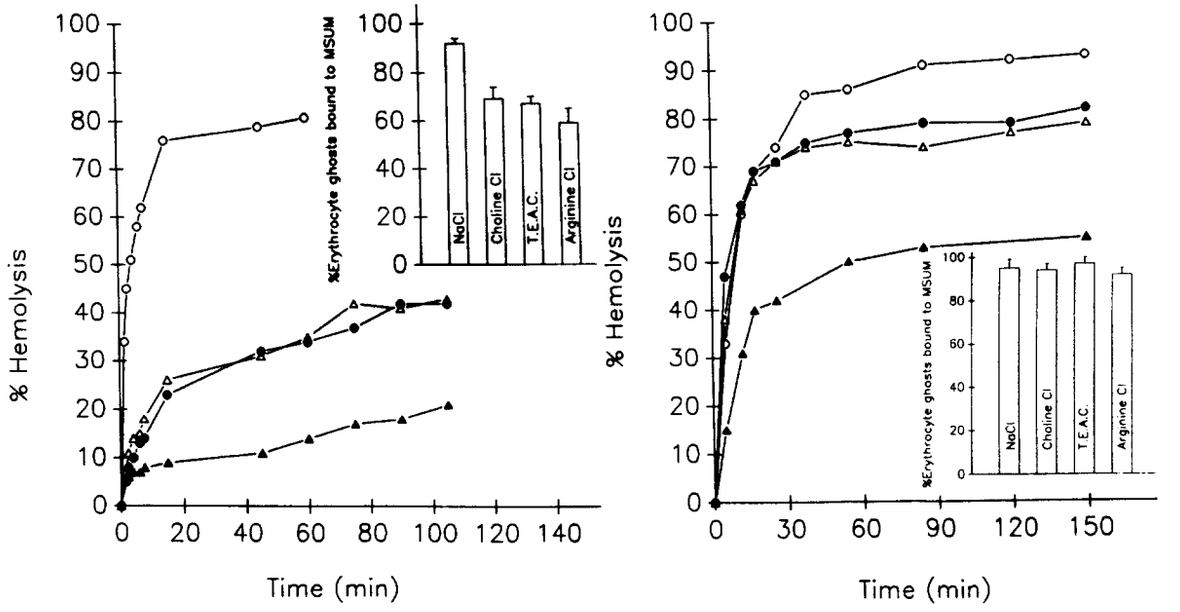

Fig. 6. (A) Time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) hemolysis in isotonic solutions of organic cations; (▲) arginine chloride, (△) tetraethylammonium chloride, (●) choline chloride, and (○) sodium chloride. (Inset) Percentage binding of ghosts to MSUM in isotonic solutions of organic cation chlorides and sodium chloride. (B) Time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) hemolysis in isotonic solutions of organic cations containing 1 mM Ca^{2+}; (▲) arginine chloride, (△) tetraethylammonium chloride, (●) choline chloride, and (○) sodium chloride. (Inset) Percentage binding of ghosts to MSUM to isotonic solutions of organic cation chlorides and sodium chloride all containing 1 mM Ca^{2+}. Ionic strength of the solutions varied between 0.15 to 0.17.

3.6. Effect of hypertonic buffers

The rate of MSUM-induced hemolysis was reduced for dehydrated erythrocytes incubated in increasingly hypertonic buffers of phosphate/NaCl and Hepes/mannitol (Fig. 8A and Fig. 8B). For Fig. 8A, the % binding of ghosts to MSUM in 10 mM phosphate buffers containing NaCl at 0.15 M, 0.3 M, 0.46 M, 0.46 M, 0.62 M and 0.77 M were 78 ± 3%, 88 ± 4%, 86 ± 1%, 83 ± 1% and 81 ± 1%, respectively. For Fig. 8B, the % binding of ghosts to MSUM in 10 mM Hepes buffers containing 1 mM Ca^{2+} and mannitol at 0.3 M, 0.6 M, 0.75 M and 0.9 M were 81 ± 5%, 84 ± 2%, 82 ± 2% and 79 ± 2%, respectively. There were no differences between the values of the % binding of ghosts to MSUM in the increasing hypertonic solutions of either phosphate/NaCl or Hepes/mannitol and control % hemolysis values were always below 5%.

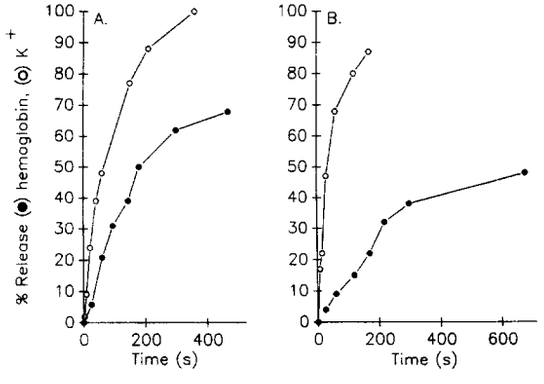

Fig. 7. Time-course of the % release of (●) hemoglobin or (○) K^+ ions from erythrocytes (0.5% v/v) incubated with MSUM crystals (22 mg/ml) in: (A) 10 mM Hepes (pH 7.4) buffered isotonic mannitol containing 1 mM calcium chloride or (B) 10 mM Hepes (pH 7.4) buffered saline. Solutions have the same ionic strength.

By incubating erythrocytes in PBS made slightly hypertonic by the inclusion of 35 mM oligosaccharides it was possible to induce only minor dehydration of the erythrocytes. This is the same method as that used by Chi et al. [16] to demonstrate pore formation and colloid osmotic hemolysis caused by ethanol. Upon addition of MSUM to these cells the rate and the extent of hemolysis was reduced in order of the molecular size of the sugar molecules (Fig. 9), mannitol (0.35 nm) to stachyose (0.62 nm) [20,21].

4. Discussion

Quantitation of crystal-membrane binding is essential in these studies. The extent of binding directly influences the extent of hemolysis and it is therefore necessary to be able to distinguish between factors which affect initial crystal-membrane binding and factors which directly influence membrane perturbation and lysis. There was very little binding of MSUM to ghost membranes in low ionic strength buffered sugar solutions but binding could be dramatically increased by the addition of divalent cations to the medium (Fig. 3). In hemolysis studies using incuba-

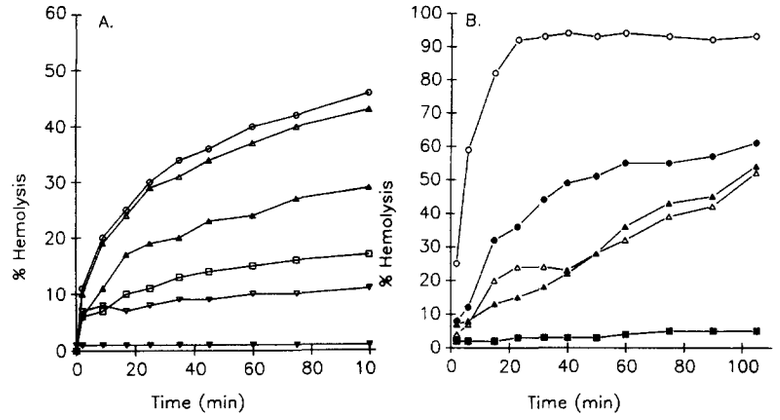

Fig. 8. (A) Time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) % hemolysis in 10 mM phosphate buffers (pH 7.4) containing sodium chloride at (○) 0.15 M (isotonic), (△) 0.3 M, (▲) 0.46 M, (□) 0.62 M or (▽) 0.77 M. % hemolysis for controls (MSUM absent) in (▼) phosphate buffer with 0.77 M NaCl. All control % hemolysis values for erythroytes in phosphate buffer with 0.15–0.6 M NaCl were less than 4%. Ionic strength ranged from 0.15 to 0.77. (B) Time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) % hemolysis in 10 mM Hepes buffers (pH 7.4) containing 1 mM calcium chloride and mannitol at (○) 0.3 M (isotonic), (●) 0.6 M, (△) 0.75M or (▲) 0.9 M. % hemolysis for controls (MSUM absent) in (■) Hepes buffer with 0.9 M mannitol. All control % hemolysis values for erythrocytes in phosphate buffer with 0.3–0.75M mannitol were less than 3%. Solutions have the same ionic strength.

tion media with a low ionic strength, low concentrations of Ca^{2+} were routinely added to ensure high levels of binding.

The mechanism of binding of MSUM to membranes has been proposed to involve hydrogen bonding [3,22] or an electrostatic interaction [8,9]. We observed that binding of the crystals to ghost membranes in low ionic strength buffers was decreased and that binding could be enhanced by either increasing the ionic strength of the buffer or by adding divalent cations to buffers containing strong electrolytes. MSUM crystals possess high negative surface and zeta potentials when dispersed in water [4] and the addition of counter ions such as Na^+ or Ca^{2+} to the dispersion medium results in the movement of these ions into the electric double layer surrounding the crystal and a reduction in the zeta potential. The charge screening effect of the positively charged counter ions may permit the close approach of the charged surfaces of the crystals and the membranes to a distance where strong forces of attraction (such as Van der Waal's forces) predominate.

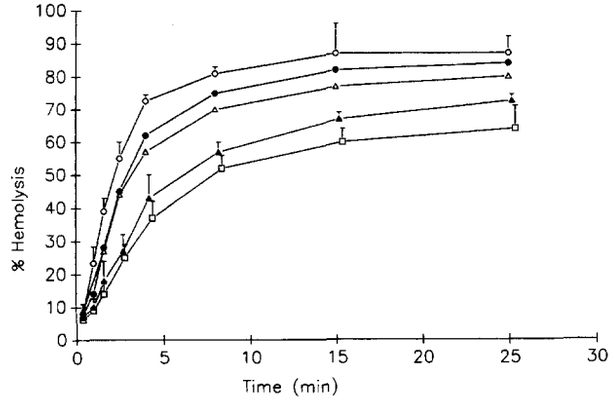

Fig. 9. Time-course of MSUM (22 mg/ml)-induced erythrocyte (0.5% v/v) % hemolysis in isotonic phosphate buffered saline containing: (○) no sugars, (●) 35 mM mannitol, (△) 35 mM raffinose, (▲) 35 mM melezitose or (□) 35 mM stachyose. Solutions have the same ionic strength.

The interactions of a variety of soluble hemolytic agents with erythrocyte membranes have been well characterized over the last decade. Melittin [13,14], complement [14], diamide [15], ethanol [16], *Pseudomonas aeruginosa* cytotoxin (PACT) [23] and treatment with carbodiimide with shear stress [24], voltage pulsation [17], or low pH [25] are all potent membranolytic agents that cause hemolysis in the same manner. This common mechanism involves pore formation in the erythrocyte membrane, the movement of solutes through the pore and colloid-osmotic bursting of the cell. Treatments of erythrocytes with carbodiimide and mild shear stress [24] or ethanol at 3.4 M and 4.1 M [16] caused a rapid efflux of K^+ preceding the slower hemolysis process. These investigators also found that both the K^+ efflux and hemolysis processes were biphasic, the first phase being rapid followed by a much slower phase. Fig. 7 shows that K^+ efflux from MSUM treated erythrocytes preceded the slower hemolysis process. These data strongly support the concept of MSUM-induced hemolysis occurring via the formation of membrane pores and colloid-osmotic hemolysis.

The rate and extent of hemolysis was greatly reduced when MSUM/cell incubations were carried out in isotonic melezitose (Fig. 3). This effect was not due to decreased binding, as after 15 min of incubation, Ca^{2+} was added and binding experiments showed that the % binding of ghosts to MSUM in isotonic melezitose with Ca^{2+} was 93% ± 5%.

4.1. Effect of different extracellular ions on hemolysis

Membrane pores induced by crosslinking agents such as diamide have been shown to be slightly cation selective [26]. Alkali chlorides passed the leak more rapidly in the sequence of decreasing hydrated radii. The permeability sequence of the halides were similar [26]. Although the rate and extent of MSUM-induced hemolysis depended on the nature of the extracellular cationic milieu (Fig. 5A) there was no observed leak selectivity correlating with hydrated ion radii, similar to that found by Deuticke et al. [26]. It is likely that the differences in MSUM-membrane binding in the presence of the different ions (Fig. 5B) contributed significantly to the differences in the rate and extent of hemolysis with different halides or organic anions.

Deuticke et al. [26] found that the rate of oxidative injury induced hemolysis in arginine chloride solutions was 1/100 the rate in NaCl solutions indicating that the large cation arginine was essentially impermeable to the pore. In our studies with organic cations, when the observed differences in MSUM crystal-membrane binding (Fig. 6A) were eliminated by the addition of Ca^{2+} to the extracellular solution (Fig. 6B), the rate and extent of MSUM-induced hemolysis in arginine/Ca^{2+} solution was significantly less than in NaCl/Ca^{2+} solution. Nevertheless there was still significant hemolysis of erythrocytes in the arginine/Ca^{2+} solution. Thus a likely explanation is that the MSUM-induced pore is somewhat larger than the diameter of the hydrated arginine ion. The lack of any discrimination for other ions may therefore be due to the unrestricted passage of these ions through a relatively large pore.

The effect of suspending erythrocytes in hypertonic buffers is to dehydrate the cells and concentrate hemoglobin by osmosis. We have shown that cells suspended in hypertonic buffers undergo a much slower MSUM-induced hemolysis than cells in isotonic buffers (Fig. 8A and B). A possible explanation for this might be that a cell in a dehydrated state would have a much greater capacity to allow the equilibration of small intra and extracellular solutes through an MSUM-induced pore before the entry of water into the cell burst the cell. Since hemoglobin is normally present at high concentrations in the erythrocyte it might alternatively be argued that further concentration in hypertonic buffers might partially precipitate the protein and reduce the ability of hemoglobin to exit the cell, whatever the mechanism of hemolysis. However resuspension of such hypertonic cells in distilled water or the addition of Triton X-100 to hypertonic buffers resulted in an immediate 100% hemolysis of cells suggesting that the concentrated hemoglobin maintained a high degree of mobility and solubility. Studies using melittin (2 μg/ml) to induce hemolysis in similar hypertonic buffers showed the same pattern of reduced lysis as the tonicity of the extracellular buffer increased (data not shown). Hence we suggest that MSUM induces hemolysis by the same colloid osmotic mechanism as described for melittin-induced hemolysis [13].

The partial protection against MSUM-induced hemolysis offerred by low concentrations of sugar molecules (Fig. 9) indicates that larger molecules such as melezitose or stachyose have difficulty equilibrating across the erythrocyte membrane under lytic conditions. Presumably,as molecules move through an MSUM-induced membrane pore the size of the sugar molecule restricts their movement through the pore and the osmotic imbalance is reduced.

Based on our findings, we propose the following mechanism for MSUM-induced hemolysis: MSUM crystals bind to erythrocyte membranes and induce an alteration of membrane proteins leading to the formation of pores or aqueous leaks. Colloid-osmotic lysis of the cell then follows.

Acknowledgements

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