Temperature Transition of Human Hemoglobin at Body Temperature: Effects of Calcium

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ABSTRACT We studied the effects of calcium ion concentration on the temperature dependence of rheological behavior of human red blood cells (RBCs) and concentrated hemoglobin solutions. Our previous study (G. M. Artmann, C. Kelemen, D. Porst, G. Büldt, and S. Chien, 1998, *Biophys. J.*, 75:3179–3183) showed a critical temperature (T_c) of 36.4 ± 0.3°C at which the RBCs underwent a transition from non-passage to passage through 1.3-μm micropipettes in response to an aspiration pressure of −2.3 kPa. An increase in intracellular Ca^{2+} concentration by using the ionophore A23187 reduced the passability of intact RBCs through small micropipettes above T_c; the micropipette diameter needed for >90% passage increased to 1.7 μm. Viscometry of concentrated hemoglobin solutions (45 and 50 g/dl) showed a sudden viscosity transition at 36 ± 1°C ($T_{c\eta}$) at all calcium concentrations investigated. Below $T_{c\eta}$, the viscosity value of the concentrated hemoglobin solution at 1.8 mM Ca^{2+} was higher than that at other concentrations (0.2 μM, 9 mM, and 18 mM). Above $T_{c\eta}$, the viscosity was almost Ca^{2+} independent. At 1.8 mM Ca^{2+} and 36 ± 1°C, the activation energy calculated from the viscometry data showed a strong dependence on the hemoglobin concentration. We propose that the transition of rheological behavior is attributable to a high-to-low viscosity transition mediated by a partial release of the hemoglobin-bound water.

INTRODUCTION

Red blood cells (RBCs) of healthy human adults contain ~0.2 μM Ca^{2+}, which is ~10^4-fold smaller than the plasma Ca^{2+} concentration of 1.8 mM. Minor shifts in intracellular calcium ion concentration, $[Ca^{2+}]_i$, produce significant changes in the biological properties of RBCs (Edmonson and Li, 1976; Allan and Thomas, 1981a,b; Allan et al., 1989; Friederichs et al., 1989; Hagelberg and Allan, 1990), and excessive elevations of $[Ca^{2+}]_i$ are deleterious to cell function and survival. The $[Ca^{2+}]_i$ of intact RBCs is controlled by the inward diffusion of Ca^{2+} across the plasma membrane and the outward extrusion of Ca^{2+} by a highly efficient Ca^{2+} pump controlled by calmodulin (Lake et al., 1977; Cheung, 1980; Schrier et al., 1980). The human RBC membrane, which normally has a very low Ca^{2+} permeability, can be rendered more permeable to Ca^{2+} by using the ionophore A23187, a divalent cation transport antibiotic (White, 1976; Lew and Simonsen, 1980; Lew and Garcia-Sancho, 1985; Friederichs et al., 1992). During Ca^{2+} loading, the ATP content of RBCs drops; as a result, Ca^{2+} extrusion is inhibited and a net Ca^{2+} uptake results. Increased intracellular Ca^{2+} levels are associated with various concomitant chemical and structural alterations: 1) losses of K^+ and cell water, leading to a cell shrinkage by 20% to 40% (Gardos, 1958; Weed et al., 1969; Clark et al., 1981); 2) polyphospholipid breakdown to diacylglycerol, whose accumulation in the inner lipid layer leads to exovesiculation (Allan and Michell, 1975); 3) changes in membrane polypeptide and phospholipid organization; 4) spheroechinocytic shape transformations; and 5) decrease of cell membrane deformability (Kuettner et al., 1977; O'Rear et al., 1982; Shiga et al., 1985).

An alteration of $[Ca^{2+}]_i$ leads to a change in K^+ permeability (Gardos effect), but the exact mechanism for this is not known. Possibly a Ca^{2+}-dependent change in the state of aggregation of the major RBC membrane skeletal protein, spectrin, leads to a reorientation of membrane proteins and/or a Ca^{2+} association with membrane phospholipids. As a result, the lipid environment of ion channels is altered, thus modulating the rate of K^+ efflux (Lake et al., 1977). Furthermore, an elevated $[Ca^{2+}]_i$ has been found to be associated with increases of catalase and band 8 in the RBC membrane, as well as the membrane-bound hemoglobin fraction (Allen and Cadman, 1979). The aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS), which are mainly located in the inner leaflet of normal RBC membranes, tend to translocate partially into the outer leaflet in response to an increase in $[Ca^{2+}]_i$ (Wagner et al., 1985). Up to 20% of the total RBC membrane lipids are consumed to form microvesicles following an elevation of $[Ca^{2+}]_i$ (Allan et al., 1976; Bütikofer et al., 1989). These vesicles are small hemoglobin-containing spherical bodies (diameter ≅ 100 nm) depleted of spectrin (bands 1 and 2), actin (band 5), and glycophorin, but enriched with diacylglycerol. The phospholipid and cholesterol compositions of the microvesicles are quite similar to those in the parental RBCs. An external Ca^{2+} level of 1 mM induces maximum microvesiculation in the presence of 5 μM A23187. This is assumed to be at least partially due to a direct physical interaction of Ca^{2+} with PS, the major anionic phospholipid of human RBC membrane. Microvesiculation is calcium

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specific. Although Mg^{2+} also interacts with negatively charged lipids, it differs from Ca^{2+} in that it does not induce microvesicles.

The shrinkage and subsequent crenation of cells in response to elevated extracellular Ca^{2+} concentration, $[Ca^{2+}]_o$, are in support of the concept of intramembrane interactions. The intrinsic transglutaminase (transamidase), which is essentially inactive within the RBC cytosol, mediates cross-linking of RBC membrane proteins whenever $[Ca^{2+}]$ exceeds 0.5 mM (Lorand et al., 1976). This transglutaminase-mediated membrane protein cross-linking can occur following the influx of Ca^{2+} in a variety of conditions, e.g., sickle cell disease (Eaton et al., 1973) or RBC senescence (Wagner et al., 1985; Clark, 1988). The elastic shear modulus of the RBC membrane ($\mu = 6 \times 10^{-3}$ dyn/cm) in normal RBCs is not significantly altered by the Ca^{2+}-triggered cross-linking, indicating that the Ca^{2+}-induced spherical shape change, rather than an altered membrane elasticity, causes the observed decrease in cellular deformability (Palek et al., 1978; Siefring et al., 1978; Nash and Wyard, 1981; Smith et al., 1981). It has also been shown that A23187 by itself mediates the cell shrinkage, rapid K^+ efflux, and ATP depletion (Kirkpatrick et al., 1975).

In this work we analyzed the temperature dependence of the passage behavior of calcium-loaded human RBCs through small micropipettes. As published previously, intact RBCs undergo a sudden change from blocking to passage through 1.3-μm pipettes under -2.3 kPa aspiration pressure at a transition temperature of $T_c = 36.4 \pm 0.3$°C (Artmann et al., 1998). Because Ca^{2+} ions can induce protein conformational changes, we studied the effects of elevated $[Ca^{2+}]_i$ on RBC passage through micropipettes and T_c.

Viscometric measurements of concentrated hemoglobin solutions showed a temperature transition of suspension viscosities at $T_{c\eta} = 36 \pm 1$°C (Artmann et al., 1998). We also assessed the effects of changing Ca^{2+} concentrations on the viscosity of hemoglobin solutions.

MATERIALS AND METHODS

Micropipette system

The micropipette aspiration technique involved the use of an inverted microscope (Carl Zeiss, Göttingen, Germany; Axiovert 100), a micromanipulator system (Luigs & Neumann, Ratingen, Germany), and a pressure system. Micropipettes with an inner diameter of 1.3–1.7 μm and a tip angle of 3° to 4° were pulled (Mecanex SA, Nyon, Switzerland; BB-CH-PC) from borosilicate glass tubes (World Precision Instruments, Berlin, Germany; 1B120–4). The inner diameters of the micropipettes at an accuracy of ± 0.5 μm were evaluated using light microscopy ($\times$500 magnification) and image analysis. The scale used had been calibrated before by scanning electron microscope (LEO 435VP, Oberkochen, Germany). The pipettes were pulled in series at constant air temperature and humidity to avoid fluctuating environmental conditions.

The pipette inner diameter chosen was smaller then the minimal cylindrical diameter (MCD) defined as the smallest size of pipette a RBC can enter without loss of volume. The mean MCD for middle-aged RBCs has been estimated to be 2.83 ± 0.14 μm (Linderkamp et al., 1993). We chose the pipette inner diameter to be deliberately smaller then the MCD to study the deformational cell behavior at extreme conditions.

All aspiration experiments were carried out at a constant aspiration pressure of -2.3 kPa. The preparation of RBCs and Hepes buffer followed a previously described protocol (Artmann et al., 1998). A RBC was considered blocking when no passage occurred within 50 s after entering the pipette. The video images obtained from the experiments were projected on a monitor and recorded on videotape.

Use of A23187 to increase calcium permeability through RBC membranes

Portions (0.5 ml) of packed RBCs were incubated for 3 min at 37°C in 4.5 ml of a medium containing 130 mM NaCl, 20 mM morpholinepropanesulfonic acid (pH 7.1 and <1 mM $CaCl_2$) together with 5 μM ionophore A23187 (Sigma Aldrich, Deisenhofen, Germany). Incubations were terminated by the addition of 200 ml of 100 mM EDTA, and the cells were immediately sedimented at $500 \times g$ for 5min (Allan and Thomas, 1981a; Hagelberg and Allan, 1990). The RBCs were than suspended in Hepes buffer (pH 7.4 ± 0.1, 290 ± 10 mosm/kg, 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 0.7 mM $MgSO_4$, 8.4 mM Hepes, 1 g/L glucose) at calcium concentrations of 0.2 μM, 0.5 mM, and 1.0 mM $CaCl_2$. To avoid RBC adhesion to the pipettes, 1g/L bovine serum albumin was added to the buffer.

Temperature control

For micropipette studies at higher than room temperature, an open-sided manipulation chamber (MPTC-1, University of Applied Sciences Aachen, Germany) was used with a stated precision of <0.5°C between 5°C and 50°C and a heating rate of 1°C/10 s. To enhance temperature accuracy, the microscope objective was heated and a temperature sensor (Farnell, Deisenhofen, Germany; Fluke-52) was placed next to the pipette tip.

Preparation of concentrated hemoglobin solutions

Concentrated hemoglobin solutions were prepared from lyophilized human hemoglobin (Sigma Aldrich) dissolved in Hepes buffer (pH 7.4 ± 0.1, 290 ± 10 mosmol/kg) at calcium concentrations of 0.2 μM, 1.8 mM, 9.0 mM, and 18.0 mM. Based on spectrophotometric data, the following composition of hemoglobin solutions was calculated (Benesch et al., 1973): oxyhemoglobin, 2.4%; desoxyhemoglobin, 9.2%; and methemoglobin, 88.4%. Even though native and lyophilized hemoglobin contain predominantly different derivatives ($\sim$86% oxyhemoglobin versus 88% methemoglobin), we considered them equivalent in our studies. As shown by Perutz et al. (1974), methemoglobin has a tertiary and quaternary structure very similar to oxyhemoglobin.

Viscosimetric measurements

The viscosity measurements of hemoglobin solutions were carried out at a constant shear rate of 5 s^{-1} (Low Shear 30, Contraves, Germany). This corresponds to one of the smallest wall shear stresses occurring in the peripheral blood circulation. The temperature of the co-axial cylindrical chamber was automatically regulated by a temperature/voltage converter (accuracy 1 mV/°C) and recorded simultaneously with the rotational moment. To obtain the viscosity values, we multiplied the data with a device-specific factor. To prevent surface artifacts due to drying and/or protein denaturation, we put a thin film of mineral oil at the air-solution

interface. To derive activation energies from the viscosity data, Arrhenius plots of $\ln(\eta/\eta_0)$ versus $1/T$ were drawn, where η is the viscosity of the hemoglobin solution, η_0 is the reference viscosity of the Hepes buffer, and T is the absolute temperature. Reversing the experimental sequence (i.e., cold to warm versus warm to cold), although not carried out in all experiments, did not affect the viscosity temperature characteristics.

RESULTS

Previously we reported that RBCs of healthy human donors blocked a 1.3-μm pipette at temperatures up to 35°C and an aspiration pressure of −2.3 kPa (Artmann et al., 1998). When the temperature was raised above 36°C, all RBCs passed the pipette. This transition from blocking to passage took place within a narrow temperature range of ±0.3°C around a critical temperature T_c = 36.4°C, at which 50% of RBCs passed the micropipette. Blocking RBCs showed a trailing sphere whose diameter decreased slightly during the first 0.1 s of aspiration, but remained constant for the ensuing 50 s. The passage time decreased linearly (r = 0.97) from 10 s to 1.7 s as the temperature rose from 36.0°C to 38.5°C. In the experiments reported here we were interested in how and to what extent enhanced intracellular calcium content would affect this phenomenon. By increasing Ca^{2+} permeability through the RBC membrane with 5 μM A23187 and then suspending the cells in an isotonic medium containing different concentrations of $CaCl_2$, the RBCs underwent distinct morphological changes from the initial discocytic shape. With a low Ca^{2+} concentration (0.2 μM) in the suspending solution, 90% of the RBCs became echinocytes III and 10% echinocytes II (classification according to Bessis et al., 1973) (Fig. 1 *A*). At 0.5 mM Ca^{2+}, 90% of the RBCs turned spheroechinocytes I and 10% showed miscellaneous shapes (Fig. 1 *B*). At 1.0 mM Ca^{2+}, all RBCs showed spheroechinocyte II shapes (Fig. 1 *C*).

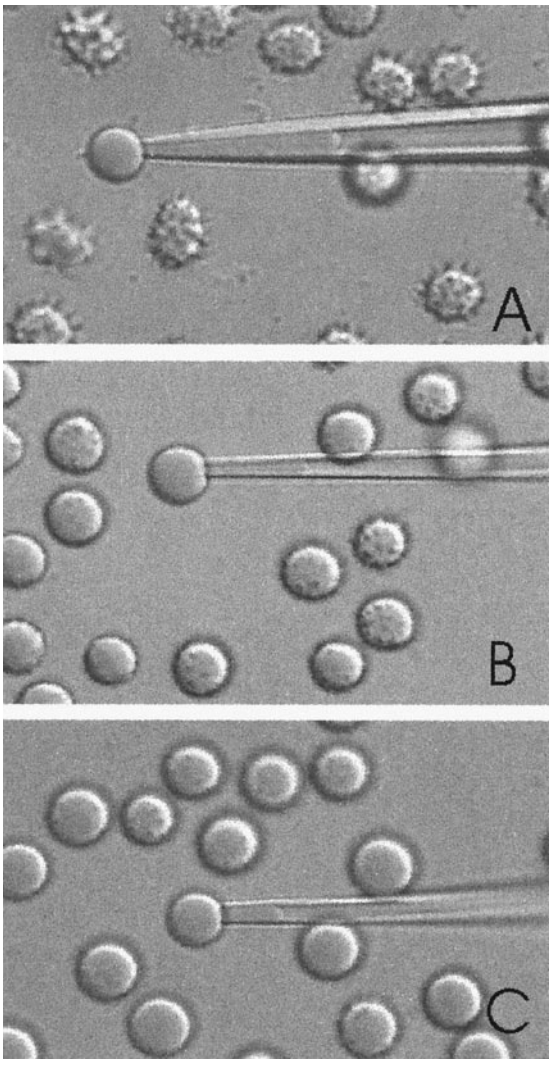

FIGURE 1 RBC shapes at 22°C following incubation with 5 μM A23187 and different concentrations of $CaCl_2$ (shape classification according to Bessis, 1973). (*A*) 5 μM A23187 and 0.2 μM extracellular calcium: 90% echinocytes E-III, 10% echinocytes E-II; (*B*) 5 μM A23187 and 0.5 mM extracellular calcium: 90% spheroechinocytes SE-I (crenated), 10% miscellaneous shapes; (*C*) 5 μM A23187 and 1.0 mM extracellular calcium: 100% spheroechinocytes SE-II (smooth).

RBC deformability was assessed by aspiration with a micropipette (inner diameter 1.3 μm). In control cells untreated with A23187 and at a $[Ca^{2+}]_o$ of 1.8 mM, the length of the aspirated cell segment (tongue) into the pipette at 10 s after the onset of the pressure increased as the temperature was raised from 28°C to 36°C. No cell passage occurred until the critical temperature (T_c) of 36.4 ± 0.3°C was reached. For cells treated with A23187, the tongue length of the aspirated RBCs increased with rising temperature, but it declined above 37°C (Fig. 2). This turning point in tongue lengths was the same for different $[Ca^{2+}]_o$ and was only slightly higher than the T_c observed for the passage of untreated RBCs. At all temperatures, the tongue length varied inversely with $[Ca^{2+}]_o$.

Because at −2.3 kPa and 1 mM $[Ca^{2+}]_o$ few of the A23187-treated RBCs passed through 1.3-μm pipettes, their inner diameter was increased in steps of 0.1 μm (see Materials and Methods; Fig. 3). As the pipette diameter increased from 1.3 to 1.4 μm, the Ca-loaded RBCs began to pass at 35.5°C. As the diameter was further increased to 1.7 μm, the temperature for the onset of passages decreased slightly toward 35°C. For each micropipette size, the percentage of RBC passage rose with the increase in temperature. At temperatures of 38–40°C, the percentage of RBC passage rose from 25% for 1.3-μm to 95% for 1.7-μm pipettes. For the 1.7-μm pipettes, where essentially all the A23187-treated cells passed, the T_c of 36.4°C was the same as that of normal untreated RBCs, but the transition from blocking to passage occurred in a much broader temperature range of ±1.6°C, as compared with the ±0.3°C for the untreated RBCs aspirated into 1.3-μm pipettes.

To assess the effects of $[Ca^{2+}]_o$ on the temperature transition of hemoglobin solutions, we performed low shear viscosity measurements. The viscosity of hemoglobin solutions at concentrations of 33, 45, and 50 g/dl was measured

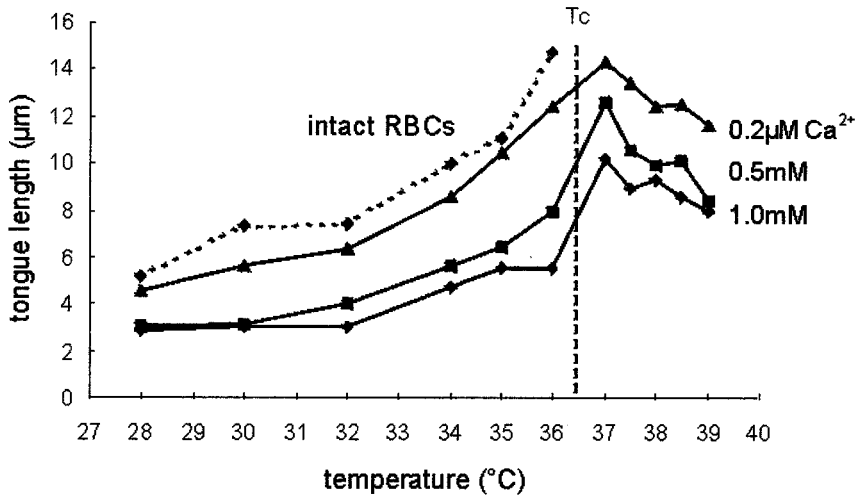

FIGURE 2 Tongue length of aspirated RBCs (1.3 ± 0.5 μm pipette inner diameter) treated with 5 μM A23187 (——) at 10 s after aspiration onset and at different external calcium concentrations (accuracy of tongue length: ±0.5 μm, $N = 15$). For comparison, the tongue lengths of normal untreated RBCs are included (— — —). At $T_c = 36.4$°C, normal RBCs passed the pipette.

over a temperature range of 25°C to 45°C at a shear rate of 5 s^{-1} (Fig. 4). At 33 g/dl hemoglobin, the viscosity showed an essentially linear decrease with increasing temperature (coefficients of correlation $r > 0.97$). At 45 and 50 g/dl, however, a relatively sharp drop of viscosity occurred at a $T_{c\eta}$ of 36°C ± 1°C for all [Ca^{2+}] up to the extremely high level of 18 mM. Below the transition temperature, the changes in viscosity with temperature and hemoglobin concentration were most pronounced at a [Hb] of 50 g/dl and a [Ca^{2+}] of 1.8 mM, next at 0.2 μM, and the least for 9 mM and 18 mM (Figs. 4 and 5). Above $T_{c\eta}$, the variation of viscosity with hemoglobin concentration declined and became essentially undetectable at 1.8 mM Ca^{2+}. Generally, the elevation of calcium level to 9 and 18 mM led to lower viscosities at all temperatures investigated.

From Arrhenius plots of $\ln(\eta/\eta_0)$ vs. $1/T$, the activation energies were derived. The activation energies E_1, E_2, and E_3 in Table 1 correspond to the temperature ranges of 25–35°C, 35–37°C, and 37–45°C, respectively, for data obtained at a calcium concentration of 1.8 mM. The results for all [Ca^{2+}] are plotted in Fig. 6. The highest activation energy E_2 of 366.6 kJ/mol was seen in the temperature range of 35–37°C, for 50 g/dl hemoglobin with 1.8 mM Ca^{2+}. The next highest activation energy E_2 of 189.2 kJ/mol was found for 45 g/dl with 1.8 mM Ca^{2+} in the same temperature range. The ratios of the activation energies for 50 g/dl hemoglobin concentration vs. 33 g/dl, both at 1.8 mM Ca^{2+}, were 2.2 for 25–35°C, 14.2 for 35–37°C, and 2.3 for 37–45°C. For each of the three temperature ranges, the effects of calcium on activation energies were the highest at 1.8 mM, next at 0.2 μM and 9mM, and least at 18 mM.

DISCUSSION

Hypothesis on hemoglobin's free and bound water

The micropipette aspiration technique was generally used in the literature to study the mechanical properties of human

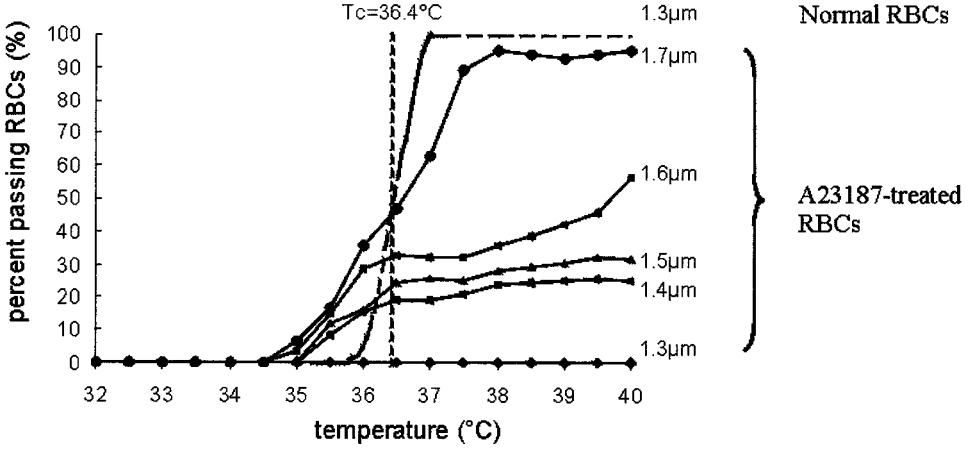

FIGURE 3 Effects of temperature on the passage of RBCs treated with 5 μM A23187 at 1 mM $[Ca^{2+}]_o$ (——); micropipette diameters between 1.3 μm and 1.7 μm (see Materials and Methods). For comparison, the passage curve of normal untreated RBCs through a 1.3-μm micropipette is included (— — —).

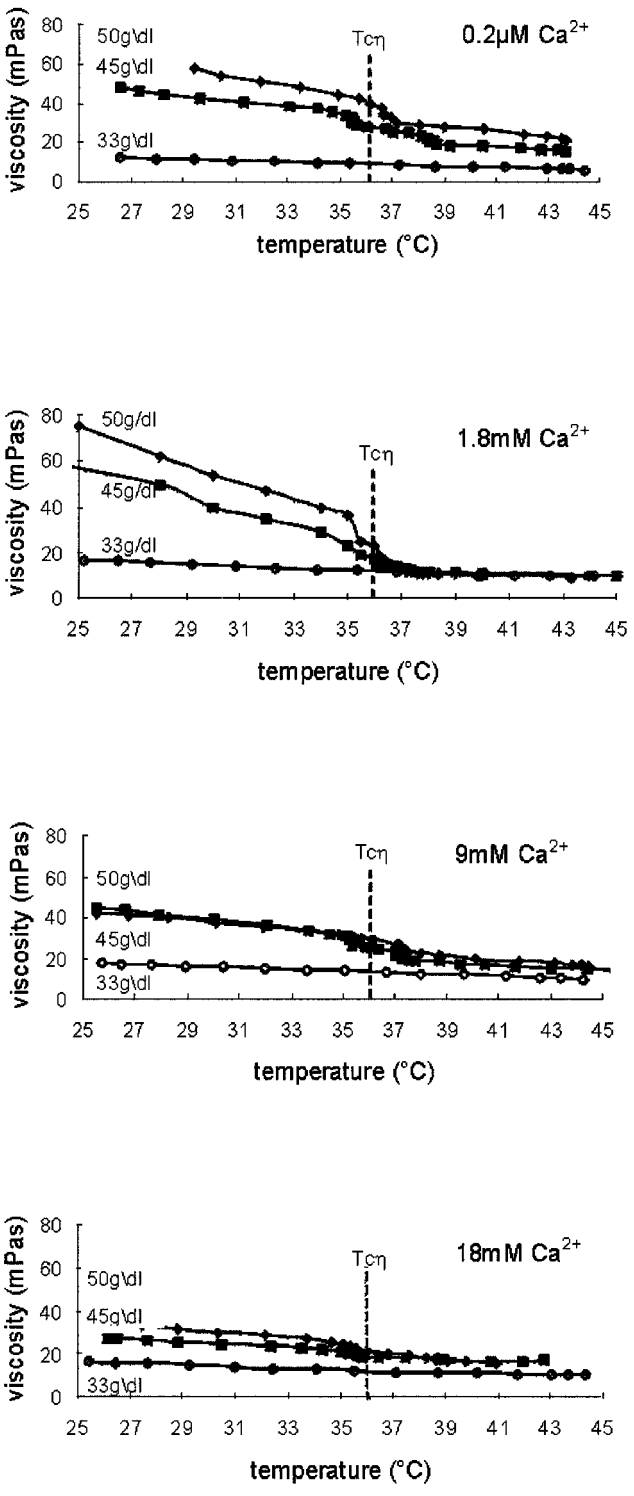

FIGURE 4 Temperature dependence of the viscosities of highly concentrated hemoglobin solutions (33, 45, and 50 g/dl in Hepes buffer) at various Ca^{2+} concentrations (0.2 μM, 1.8 mM, 9.0 mM, and 18.0 mM). Each point represents the mean of 10 repeats (typical SD = 0.75 mPas).

erythrocytes at room temperature (Hochmuth et al., 1980; Evans, 1989). When entering a narrow glass pipette, the RBCs show a projection inside the pipette and an outer spherical trail (Fig. 1). In our previous paper we estimated the hemoglobin concentration in the trail end to be very high, ~50g/dl (Artmann et al., 1998). At these conditions, the RBCs blocked the pipette unless the cytosolic hemoglobin viscosity could be reduced. The temperature transition in both the micropipette and the viscosity experiments was limited to high hemoglobin concentrations at which the average intermolecular distances between neighbors are short (Kelemen, 1999). Hydration of proteins is one of their important properties. At room temperature, the fraction of bound water to hemoglobin was 0.339 g/g hemoglobin (Drabkin, 1950; Perutz, 1985). The hydration fraction of human hemoglobin, however, is 2.29 g/g (20.0°C) hemoglobin (Cameron and Ord, 1988). The water bound to hemoglobin is osmotically inactive as compared with the free osmotically active one. We postulate that when the temperature is raised above the critical level $T_{c\eta}$, the concentrated hemoglobin solution in the trailing sphere undergoes a high-to-low viscosity transformation mediated by a partial loss of hemoglobin-bound water. Thus, the sudden RBC passage observed above T_c (Fig. 3) can best be explained by a decrease of cytosolic viscosity (Figs. 4 and 5). In a simple approach, we considered a compact arrangement of spherical hemoglobin molecules and calculated the relative amounts of the two distinct water fractions (Fig. 7) corresponding to the hemoglobin concentrations used in our experiments. The distance between two neighboring hydrated hemoglobin molecules would decline from 26 Å at 33 g/dl to 12 Å at 50 g/dl. At 50 g/dl, two hydrated hemoglobin molecules might even come as close as the length of a few hydrogen bonds (18.8 kJ/mol; typical length 1.7 Å). These data together with the temperature-dependent passage times (see Results) allowed us to estimate the rate of water loss of a normal RBC of 11.6 ± 0.5 $\mu m^3/s$ within the first 0.75 s of aspiration.

Effects of calcium loading on RBC passage

The passage of calcium-loaded RBCs required higher aspiration forces, e.g., the aspiration increased to 1.7-fold when $[Ca^{2+}]_o$ was raised to 1 mM (Fig. 3). Calcium loading leads to cellular dehydration and a decrease in deformability, due to condensed hemoglobin and enhanced spectrin-hemoglobin interactions (Shiga et al., 1985). Although an increase in extracellular calcium causes an upward shift of the aspiration force at all temperatures, the transition temperature itself remained essentially unchanged at 36.4°C (Fig. 3). The observed broadening of the temperature transition range to ±1.6°C in calcium-loaded RBCs might be related to RBC age-deformability distribution. Aged RBCs display a lower energy metabolism and, thus, are more sensitive to calcium-induced changes in overall deformability (Weed et al., 1969; Nash and Wyard, 1980, 1981; Wagner et al., 1985; Sutera et al., 1985; Clark, 1988; Waugh et al., 1992).

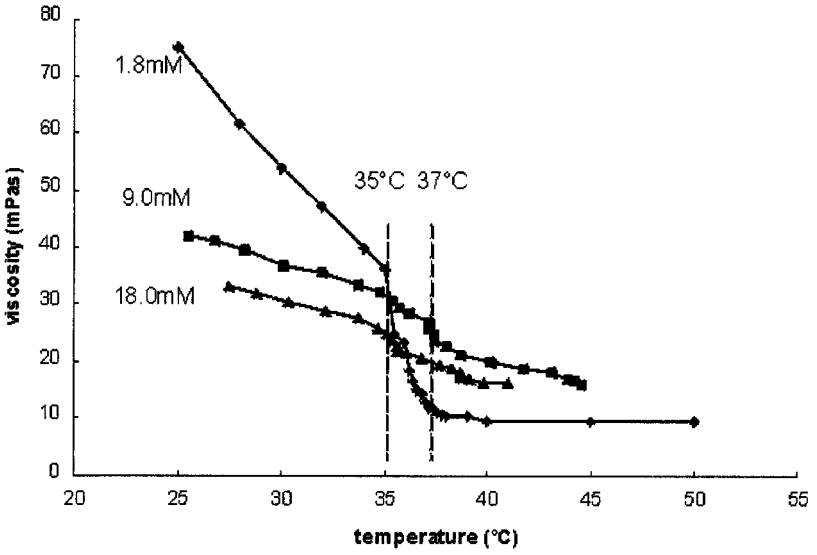

FIGURE 5 Composite plot showing the temperature dependence of the viscosities of highly concentrated hemoglobin solutions (50 g/dl) at various Ca^{2+} levels.

Effects of calcium loading on RBC morphology and tongue length

At room temperature, ionophore-treated (calcium-loaded) RBCs showed distinct spheroechinocytic morphological changes (Fig. 1), as previously described by other investigators (Gardos, 1958; Weed et al., 1969; Beaven and Gratzer, 1980; Clark et al., 1981; Friederichs et al., 1992). The tongue length of blocking RBCs within a 1.3-μm micropipette monotonously increased with temperature, displaying a maximum at 37°C, and declined when temperature was raised to 39°C (Fig. 2). This turning point of the tongue length occurred at body temperature and almost coincided with the critical temperature T_c where the onset of RBC passages was observed. Enhanced Ca^{2+} content did not affect the temperature at which a maximum tongue length appeared. It led to generally shorter tongue lengths, being the shorter the higher the extracellular calcium level (Fig. 2) was. This reduction of tongue lengths might be due to a Ca^{2+}-mediated formation of spectrin-hemoglobin complexes, making the cytoskeleton more resistant to extensional forces (Shaklai et al., 1977; Beaven and Gratzer, 1980; Eaton et al., 1980; Mikkelsen et al., 1984; Low, 1986; Friederichs et al., 1992; Stryer, 1996). But why did the tongue length rise below and decline above body temperature? Below 37°C, according to the hypothesis above, the spectrin-hemoglobin complex would contain an appreciable amount of bound water, and this in turn may partially block calcium-mediated spectrin cross-links. Temperature might also modify the calcium binding affinity to hemoglobin (Harrison and Long, 1968; Long and Mouat, 1971; Blum and Hoffman, 1972; Cohen and Solomon, 1976). Whereas above T_c, the spectrin-bound hemoglobin fraction would lose partially bound water and allow calcium molecules to fully develop protein cross-links and in turn cause stiffening and shorter tongue lengths.

TABLE 1 Activation energies as derived from low shear viscosity data of hemoglobin solutions in Hepes buffer at 1.8 mM calcium

	Activation energies (kJ/mol)		
Hemoglobin concentration	E_1 25–35°C	E_2 35–38°C	E_3 38–45°C
33 g/dl	25.7	25.7	25.7
45 g/dl	49.5	189.2	26.8
50 g/dl	55.1	366.6	58.1

For comparison, thermal energy at 25°C is 3.7 kJ/mol.

Effects of calcium on the viscosity of hemoglobin solutions

The viscosity of highly concentrated hemoglobin solutions at various calcium concentrations displayed a sudden viscosity drop at $T_{c\eta} = 36 \pm 1$°C (Fig. 4). Below $T_{c\eta}$, the suspension viscosities varied inversely with the calcium concentration. This might be due to a competitive process in which calcium replaces the water molecules bound to hemoglobin. Above $T_{c\eta}$ calcium only slightly affected the viscosity.

Briefly, $T_{c\eta}$ remained unaffected by calcium. Hence, the transition of intact RBCs at T_c is attributable to a calcium-independent fluidity change of hemoglobin molecules.

Activation energies derived from low shear viscosity data

Non-monotonous slopes of the Arrhenius plots in general are related to phase transitions (Glaser, 1996). In the context of this paper, the activation energy E_A is defined as the energy required for the transition from a high viscosity

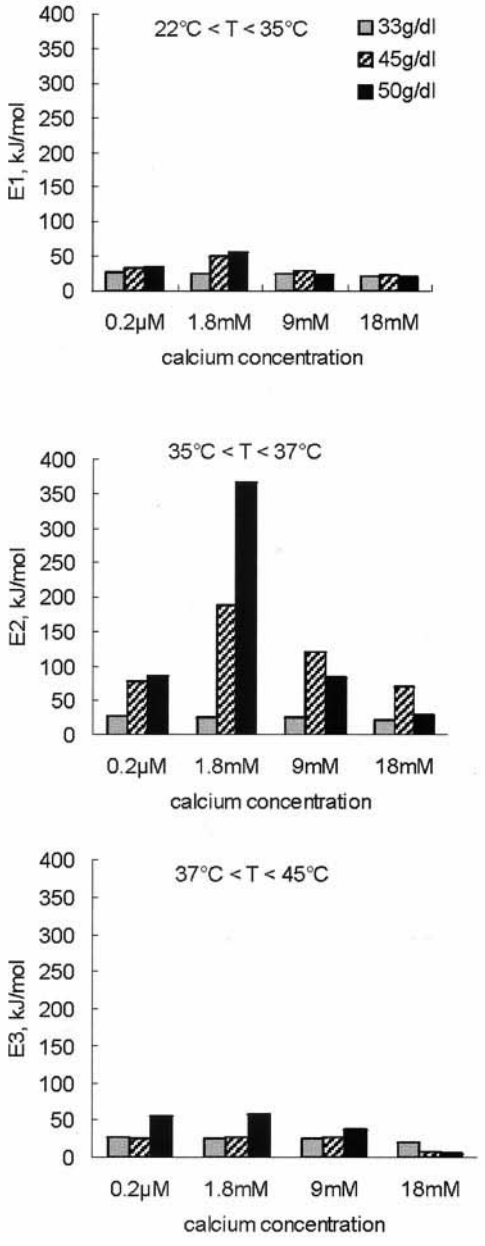

FIGURE 6 Activation energies derived from viscosity data of hemoglobin solutions (Fig. 4).

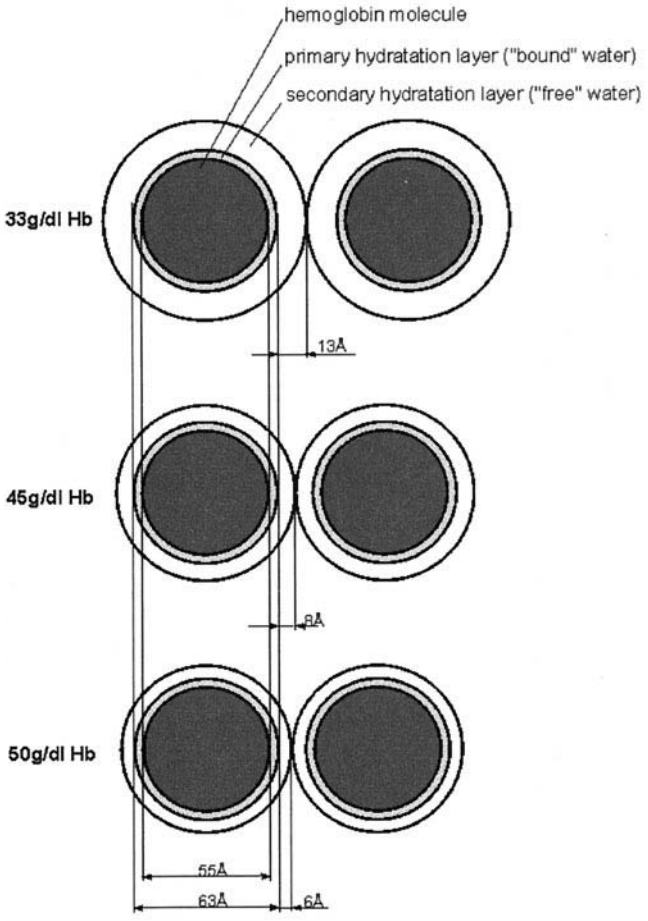

FIGURE 7 A spherical model of neighboring hemoglobin molecules at the high concentrations encountered in the trailing end of an aspirated RBC, showing the hypothesized shell thicknesses of bound water and free water surrounding the molecules.

below a critical temperature to a low viscosity above it. An obvious finding was the extremely high activation energy of 366.6 kJ/mol within the temperature range of 35°C to 38°C for 50 g/dl hemoglobin with 1.8 mM Ca^{2+}. The next highest activation energy of 189.2 kJ/mol was found at 45 g/dl and the same calcium content (Fig. 6; Table 1). A temperature increase of 1°C represents a thermal energy difference of 12.5×10^{-3} kJ/mol, which is much too small to explain the activation energy needed to enable the transition from high viscosity below $T_{c\eta}$ to low viscosity above.

Where do these differences in activation energies come from? Our hypothesis is based on the assumption that at $T_{c\eta}$, a part of the bound water is suddenly released from the hemoglobin molecule. Calcium ions may compete with water molecules for binding sites to hemoglobin. At $T_{c\eta}$, Ca^{2+} might replace bound water and the viscosity declines rapidly. The strong hemoglobin concentration dependency might, thus, result from the release of bound water and the reduction of high molecular packing density, which in turn would decrease the suspension viscosity. In the suggested replacement mechanism, the Ca^{2+} concentration of 1.8 mM might be optimal and thus cause the strongest temperature dependence at this concentration (Fig. 5). Interestingly, 1.8 mM represents the plasma levels of calcium and may thus play a similar role in modulating the fluidity of plasma proteins at body temperature.

The temperature transition of calcium-loaded RBCs took place in the same temperature range in which a sudden viscosity drop of bulk hemoglobin solutions was observed. A necessary condition for the transition might be the small spacing between the hemoglobin molecules. We propose that the temperature transitions observed are attributable to

a gel-sol transition and a partial release of the hemoglobin-bound water. Calcium modifies the rheological behavior of RBCs as well as the viscosity of hemoglobin suspension. However, the transition temperature itself is almost unaffected by calcium, indicating two different modes of action, the chemical action of calcium and the physical action of temperature.

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