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A contribution of calmodulin to cellular deformability of calcium-loaded human erythrocytes

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The effect of intracellular calcium on the deformability of human erythrocytes was studied with a rheoscope, especially in relation to the dynamic structure of membrane cytoskeleton. (1) The appropriate calcium-loading and calcium-depletion were performed to intact erythrocytes with A23187 in potassium buffer. The total calcium content was varied in the range of 0.25 to 3 times as much as normal content, without complete ATP depletion and shape change (the reduction of mean cell volume and the condensation of hemoglobin due to dehydration were avoided). (2) Increasing the intracellular calcium content by about 1.5 times of normal, the deformability was distinctly decreased, while calcium depletion did not affect the deformability. (3) Reduced deformability of the calcium-loaded erythrocytes was restored by the treatment with calmodulin inhibitors, W-7 or trifluoperazine. However, such an effect by calmodulin inhibitors was not detected on normal or calcium-depleted erythrocytes. In conclusion, the interaction between calcium-calmodulin complex and cytoskeletal proteins may affect the membrane stiffness which is regulated through the change of the cytoskeletal structure, and contributes to the deformability of erythrocytes.

Introduction

The regulation of erythrocyte deformability or of membrane stiffness by the intracellular calcium, through the modulation of cytoskeletal dynamic structure, has been frequently discussed [1–3]. In order to measure the effect of calcium quantitatively, it is necessary to establish the relationship between the calcium content and the deformability. However, in addition to the membrane stiffness, the cellular deformability is also influenced by the cell shape and the intracellular viscosity [4]. Actually, it is hard to modify the calcium content arbitrarily without changing these factors. Upon artificial calcium-loading, for example, Gardos [5] in 1958 found K^+ and H_2O efflux and Dreher et al. [6] showed ATP depletion and echinocytosis. Further, we have observed the cell age-dependence of these phenomena [7]: i.e., calcium entry induced by calcium-ionophore takes place preferably to in vivo aged cells, in which ATP utilization exceeds ATP regeneration and dehydration occurs upon calcium accumulation. Although we have shown that the decreased deformability due to calcium accumulation results from the increased intracellular viscosity and the increased membrane stiffness, no direct observation about the changes in cytoskeletal structure has been made except for the decreased membrane fluidity.

The spectrin network in the erythrocyte cytoskeleton is an important factor for cellular deformability and for maintenance of the discocytic

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shape [1–3]. A number of protein-protein interactions contributes to the dynamic structure of spectrin network [8], e.g., actin-band 4.1, ankyrin-band 3, calmodulin and its binding protein, etc. Among them, calmodulin interacts with band 4.1 and/or spectrin and may modify the membrane stiffness, when it binds with calcium [9–11], though there has been some disputes whether or not calmodulin binds to intact spectrin in in vitro studies [12,13]. Therefore, we conducted an in vivo study to detect the effect of calmodulin inhibitors on the deformability of calcium-loaded and calcium-depleted erythrocytes. However, concerning cell shape and volume, (a) the extensive calcium-loading depletes ATP, decreases cell volume and induces echinocytosis [6,7,14–17], on the other hand (b) most of calmodulin inhibitors are known as stomatocytic agents [18]; thus a cautious experiment is required.

This paper deals with the relation between calcium content and deformability and with the effect of calmodulin inhibitors, for human erythrocytes. First of all, the characteristics of calcium-loaded and calcium-depleted erythrocytes was examined in detail, in order to obtain the specimens without changing cell shape and volume and with maintaining certain amount of ATP. Then we demonstrated that (1) the deformability gradually decreased by calcium loading up to 1.5 times (of normal), then the effect saturated (calcium could be accumulated up to 3 times), while the deformability was not affected by calcium-depletion and (2) calmodulin inhibitors restored the decreased deformability of calcium-loaded cells. Additionally, the influence of calcium-loading on energy metabolism is discussed.

Materials and Methods

Erythrocytes. The fresh human erythrocytes were obtained from heparinized venous blood, after centrifugation (3000 rpm, 10 min, at 4°C) and removal of buffy coat. The cells were washed three times with 20 volumes of isotonic Hepes-buffered saline (130 mM KCl, 50 mM Hepes (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid), 5.6 mM D-glucose; pH 7.4 adjusted with KOH; 285 mosM: abbreviated as 'K-buffer'). Instead of KCl and KOH, in some cases, NaCl and NaOH were used ('Na-buffer'). K-buffer was used throughout, unless otherwise noted.

(i) 'Calcium-loaded' erythrocytes were prepared as follows: calcium-loading was carried out by incubating the washed cells (hematocrit, ca. 20%) with 1.5 μM A23187 (Calbiochem Co., San Diego, CA, USA; dissolved in ethanol/dimethyl sulfoxide (3:1, by vol) mixture), 50 μM $CaCl_2$ and EGTA (0.03–0.10 mM) in K-buffer for 60 min at 37°C. After centrifugation, the cells were resuspended in K-buffer containing 0.5 g/dl human albumin (fatty acid free, fraction V; Miles Lab., IN, U.S.A.) for 5 min at 4°C and washed once with K-buffer, in order to remove ionophore ('first incubation'). Since the erythrocytes prepared by the above procedure were ATP-depleted and contained echinocytes (see Results), the cells were further incubated in K-buffer at hematocrit of approx. 20% for 75 min at 37°C, in order to restore the cell shape and ATP content ('second incubation').

(ii) Partially 'calcium-depleted' erythrocytes were prepared by incubating washed cells (hematocrit, approx. 20%) with 1.5 μM A23187 and 1.0 mM EGTA in K-buffer for 60 min at 37°C. Then the cells were treated with albumin solution as above, and finally washed once with K-buffer.

(iii) 'Control' erythrocytes were also incubated in K-buffer at hematocrit of approx. 20% for 60 min at 37°C. The cell shape and ATP content of control and calcium-depleted cells unaltered, but the 'second incubation' was also made.

The calmodulin inhibitors used were W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride; Lot No. S-003, Seikagaku Kogyo Co., Tokyo, Japan) and TFP (trifluoperazine-2 HCl; Lot No. 54F-0541, Sigma Chem. Co., St. Louis, MO, U.S.A.). The erythrocytes were incubated with the calmodulin inhibitor dissolved in K-buffer (at hematocrit of approx. 10%, for 30 min at 37°C).

Hematological indices were calculated from (i) hematocrit measured with a microhematocrit centrifuge (Kubota Manuf. Co., model KH-120, Tokyo, Japan), (ii) cell number counted with an automatic counter (Toa Medical Electronic Co., model CC-110, Kobe, Japan) and (iii) hemoglobin concentration determined by CN-methemoglobin method.

Cell shape was observed by using a microscope (Olympus Optics, model BH, Tokyo, Japan) or a

scanning electron microscope (SEM; Hitachi Manuf. Co., model S-500, Hitachi, Japan), after fixation in 1% glutaraldehyde then 1% OsO_4 (adjusted to 285 mosM with buffer). For the cells exposed to calmodulin inhibitor, the fixative solution containing the same concentration of inhibitor was used. As an index for the shape alteration, the morphological index of Fujii et al. [19] (based on the classification of Bessis [20]) was adopted: 100–150 cells were counted on scanning electron microscopy photographs, and the transformation to echinocytes and stomatocytes was represented by positive and negative values, respectively.

Determination of intracellular calcium. Prior to calcium determination, the erythrocytes were washed four times with 20 volumes of isotonic NaCl solution (285 mosM; inevitably containing 0.12–0.18 μM calcium) according to O'Rear et al. [21], then packed by centrifugation (final hematocrit of approx. 80%). Then, the total amount of calcium in erythrocytes was determined with a flameless atomic absorption apparatus (Shimadzu Manuf. Co., model 646, Kyoto, Japan) as described previously [7], and expressed as μmol/l packed cells (abbreviated as 'Ca content' or $[Ca]_i$). The concentration of calcium in our laboratory water was 0.13 ± 0.03 μM ($n = 30$) and that of the K-buffer was 0.26 ± 0.07 μM ($n = 5$). The Ca content of control erythrocytes was 16.6 ± 1.4 μmol/l packed cells ($n = 20$). This value agreed with the reported value [21].

Determination of intracellular nucleotides. Nucleotides were determined by the procedure described elsewhere, using a liquid chromatograph (Shimadzu Manuf. Co., model LC-3A, Kyoto, Japan) [22,23]. The energy charge was then calculated: energy charge (EC) = $\{2[ATP] + [ADP]\}/2\{[ATP] + [ADP] + [AMP]\}$.

Electrophoretic analysis of membrane proteins. The crosslinking of membrane proteins was analyzed by 4% polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate, and the gel was scanned after staining with Coomassie brilliant blue R-250, as reported previously [24].

Measurement of cellular deformability. A rheoscope [25], consisted of an inverted microscope and a transparent 0.8° cone-plate viscometer, was employed for observation of erythrocyte deformation due to high shear stress, as described elsewhere [7,24]. The erythrocytes were suspended in 14% Dextran T-40 dissolved in K-buffer (12 cP at 25°C, 285 mosM; in the case of calcium-depleted and the control erythrocytes, 1 mM EGTA was further added; Dextran T-40 was purchased from Pharmacia Fine Chem. Co., Uppsala, Sweden), and in some cases, 20% Dextran T-40 in K-buffer (19 cP at 25°C, 285 mosM) was used. The shear rate of 75–750 s^{-1} was applied for erythrocyte deformation.

In order to compare the deformability of calcium-loaded erythrocytes with control erythrocytes, the mean cellular hemoglobin concentration of specimens had to be matched in every series of experiment: thus in the measurement of cellular deformability, the control cells were suspended in Dextran T-40 dissolved in appropriately hypotonic K-buffer (265–285 mosM) to increase the cell volume, while the calcium-loaded cells, of which the cell volume was inevitably increased up to 3% during preparation, were suspended in Dextran T-40 in isotonic K-buffer (285 mosM). In these preparations, the mean cellular hemoglobin concentration (MCHC) was 33.9 ± 0.5 g/dl, and the mean cell volume (MCV), 98.6 ± 2.4 μm^3 ($n = 14$).

The erythrocytes deformed to ellipsoidal discocytes, when the shear stress was applied [7,24–27]. On flash microphotographs, the short and long axes of ellipsoidal discocytes were read by a digitizer (Graphtec Co., Mitablet-II, Tokyo, Japan) connected to a microcomputer (Nippon Electronic Co., model PC-9801, Tokyo, Japan), then the ratio of short axis to long axis was calculated. The mean of the ratio (obtained from more than 100 cells) represented the averaged value of deformability; the smaller ratio meant the better deformability.

Measurement of osmolarity and intracellular Na^+ and K^+. The osmolarity of various solutions was calculated from the freezing point, measured with a Halbmikro osmometer (Knauer, type M, F.R.G.). The intracellular concentration of Na^+ and K^+ was determined with an ion meter (Toyo Kagaku Sangyo Co., model PT-3D, Tokyo, Japan) with Na^+- and K^+-electrode, respectively, after hemolysis and appropriate dilution.

Statistical procedure. The statistical significance was judged on the basis of Student's *t*-test.

Results

A. Characteristics of calcium-loaded erythrocytes

The properties of erythrocytes frequently changes by the procedure of calcium-loading with ionophore [6,14–17,28], in particular for in vivo aged cells as demonstrated previously [7]. Therefore, it was necessary to assure the constancy of the determinant factors of deformability (except for calcium content), such as cell shape, intracellular hemoglobin concentration, and energy metabolism.

(1) Intracellular calcium contents and cell volume. When calcium-loading was carried out in K-buffer containing A-23187 and $CaCl_2$, the Ca content in erythrocytes augmented up to 40–50 μmol/l packed cells. The intracellular Ca content gradually increased, decreasing the EGTA concentration in the incubation medium. The cellular dehydration due to calcium loading was prevented in K-buffer (the cell volume slightly increased and the intracellular hemoglobin concentration inversely decreased), as noted in the early report of Gardos [5]. Calcium depletion was performed in K-buffer containing EGTA (without $CaCl_2$), and the Ca content could be decreased to 4–7 μmol/l packed cells.

In this connection, the changes in intracellular Na^+ and K^+ concentration during calcium-loading procedure were within the experimental error: before calcium loading, $[Ca]_i$ = 18.3 μmol/l packed cells, $[Na]_i$ = 5.4 mmol/l packed cells and $[K]_i$ = 125 mmol/l packed cells, while after calcium loading, $[Ca]_i$ = 34.5 μmol/l packed cells, $[Na]_i$ = 5.6 mmol/l packed cells and $[K]_i$ = 124 mmol/l packed cells, respectively.

On the other hand, calcium-loading in Na-buffer inevitably induced considerable dehydration (thus the cell volume decreased remarkably) as a function of the accumulated calcium, as described previously [7].

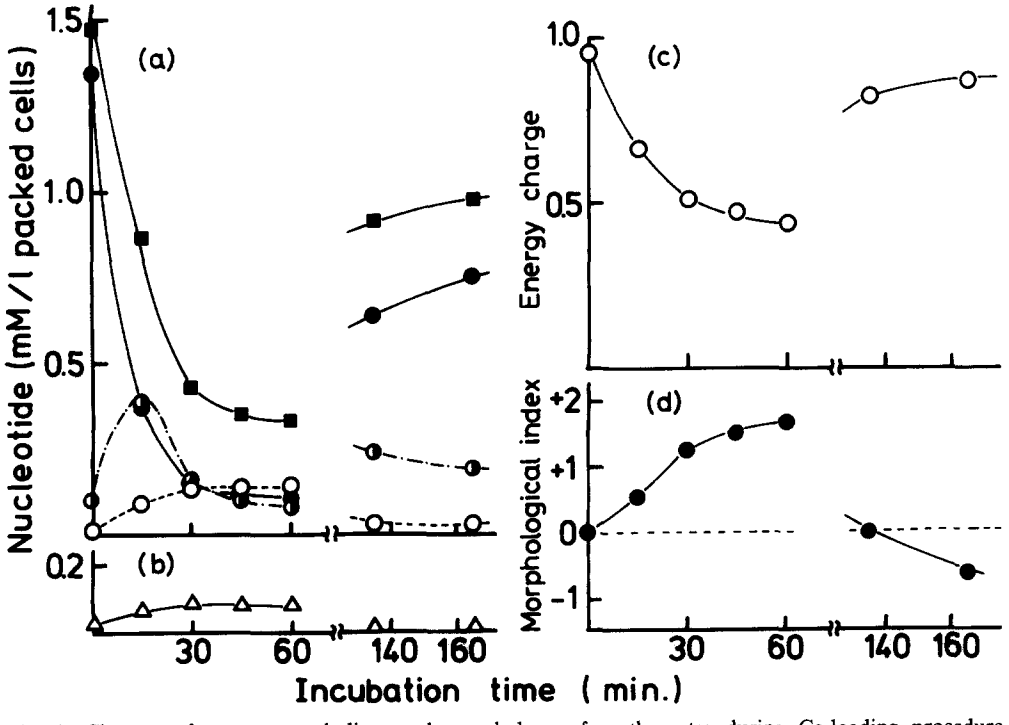

Fig. 1. Changes of energy metabolism and morphology of erythrocytes during Ca-loading procedure and their restoration. Erythrocytes (hematocrit, 20%) were incubated with 1.5 μM A23187, 50 μM $CaCl_2$ and 40 μM EGTA in K-buffer at 37°C for 60 min ('first incubation', t = 0–60 min). After removing ionophore, the cells were further incubated for 75 min to restore ATP content and the shape ('second incubation', t = 60–135 min). Then the restored cells ('calcium-loaded cells'; hematocrit, 10%) were treated with 30 μM W-7 for 30 min (t = 135–165 min). (a) Adenylates: AMP (○), ADP (◑), ATP (●) and total adenylates (■); (b) IMP; (c) energy charge; (d) morphological index: +, to echinocytosis, −, to stomatocytosis.

(2) Energy metabolism. Calcium-depletion did not affect the ATP content. However, as shown by Kirkpatrick et al. [14] and Dreher et al. [6] earlier, the calcium loading with A23187 depleted ATP considerably. In Fig. 1(a)–(c), the time-courses of ATP, ADP, AMP, IMP and energy charge during first incubation in K-buffer and further the regeneration process of ATP in second incubation were shown. After 60 min of calcium-loading procedure, (i) ATP content decreased to approx. 10% of the original level, (ii) ADP slightly decreased after a transient increase (approx. 4 times of the original), (iii) both AMP and IMP increased (to approx. 10 times and 4 times of the original, respectively), and (iv) the energy charge decreased markedly. However, upon further incubation without external calcium and A23187, ATP was regenerated and the energy charge was restored to the normal level (i.e., more than 0.8). The Ca content in erythrocytes was scarcely changed by the second incubation, in spite of regain of ATP.

(3) Cell shape. Calcium-depletion did not affect the cell shape. However, after calcium-loading procedure in K-buffer, the cells transformed to echinocytes, as presented by the morphological index in Fig. 1(d). The transformation in K-buffer

TABLE I

MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF CALCIUM-DEPLETED, CONTROL AND CALCIUM-LOADED ERYTHROCYTES IN K-BUFFER

Values in parenthesis show the results after the 'second incubation' for 75 min, to restore ATP content and the shape. MCV, mean cell volume; MCHC, mean cellular hemoglobin concentration.

	Ca depletion		Ca loading		
	control	depleted	control	loaded	
				(i)	(ii)
Incubated with					
A23187 (μM)	0	1.5	0	1.5	1.5
$CaCl_2$ (μM)	0	0	0	50	50
EGTA (μM)	1000	1000	0	46	40
After incubation for 60 min at 37 °C ('first incubation')					
Ca content					
(μmol/l packed cells) [a]	16.1	4.0	18.6	18.9	39.4
(amol/cell)	1.50	0.37	1.72	1.81	3.81
MCV (μm^3)	93.0	93.3	92.4	96.1	96.8
MCHC (g/dl)	33.9	34.0	35.1	33.8	33.5
Morphological index [b]	0	0	0	+1.6	+1.6
	(0)	(0)	(0)	(0)	(0)
Nucleotides (mmol/l packed cells)					
[ATP]	1.24	1.09	1.23	0.14	0.11
	(1.21)	(1.10)	(1.36)		(0.68)
[ADP]	0.18	0.22	0.19	0.12	0.09
	(0.17)	(0.22)	(0.18)		(0.26)
[AMP]	0.02	0.03	0.02	0.14	0.15
	(0.02)	(0.03)	(0.02)		(0.04)
Total adenylates	1.44	1.34	1.44	0.40	0.35
	(1.41)	(1.35)	(1.56)		(0.98)
[IMP]	0.01	0.01	0.02	0.07	0.08
	(0.01)	(0.01)	(0.01)		(0.01)
Energy charge of adenylates	0.92	0.89	0.92	0.50	0.44
	(0.92)	(0.90)	(0.93)		(0.83)

[a] The average and standard deviation for control erythrocytes: 16.6 ± 1.4 ($n = 20$).

[b] According to Fujii et al. [19] based on the Bassis' classification [20]. + shows echinocytosis.

was quite similar to that in Na-buffer, though less distinct. However, although ATP could not be completely regained by the second incubation with glucose, the cell shape was perfectly restored to discocyte, and the cellular deformability at this level of ATP was not impaired, as demonstrated previously [23,29]. The phenomena may be probably due to the complete restoration of energy charge.

(4) Membrane proteins. A crosslinking of membrane proteins was not detected in calcium-loaded erythrocytes prepared in the present experiment by polyacrylamide gel electrophoresis.

(5) Cell age dependence. Since the effects of calmodulin, e.g., to activate $(Ca^{2+} + Mg^{2+})$-ATPase, decline with cell age [30], any calcium-loading procedure showing the distinct age-dependency is inadequate for the present study. After calcium loading in K-buffer, the erythrocytes were fractionated by using the density gradient centrifugation with Percoll [7,23,31]. The cells in the light and heavy fractions (upper and lower fractions separated in isotonic K-buffer containing 75.6% Percoll, respectively) differed in the mean cellular hemoglobin concentration and calcium concentration, but contained a similar amount of adenylates and showed the same activity of glucose-6-phosphate dehydrogenase (which also declines with cell age [7,32–34]). This result is contrasted to the previous calcium-loading in Na-buffer, in where calcium accumulation and dehydration occurred in preference to the aged cells [7]. In the present calcium-loading procedure, therefore, the cell age-dependent calcium accumulation was not observed (vide infra).

The characteristics of calcium-depleted, control and calcium-loaded erythrocytes, prepared for the study of calmodulin inhibitor, are summarized in Table I, as the representative data.

B. Decreased deformability of calcium-loaded cells

The erythrocytes were deformed to ellipsoidal discocytes under uniform shear force (at shear rates of 150–750 s^{-1} in 12 cP dextran solution, i.e., under shear stress of 18–90 dyn/cm^2) as shown in Fig. 2. The deformability quantified by the ratio of short axis to long axis was summarized as a function of the intracellular Ca content (Fig. 3). The calcium-depletion unaffected the deformability, but as calcium accumulation proceeded, the deformability decreased (i.e., the ratio increased): there seemed to be a gradual transition from more deformable (low Ca content) to less deformable (high Ca content) states, at around 20–30 μmol/l packed cells of total intracellular calcium.

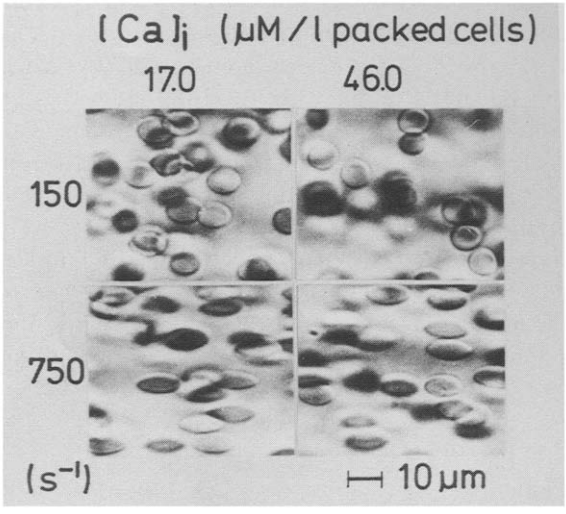

Fig. 2. Deformation of erythrocytes under uniform shear stress observed by high shear rheoscopy. The deformation of (a) control cells ($[Ca]_i = 17.0$ μmol/l packed cells) and of (b) calcium-loaded cells ($[Ca]_i = 46.0$ μmol/l packed cells) was observed in 14% Dextran T-40 in K-buffer at 25°C under the shear rates of 150 s^{-1} (upper) and 750 s^{-1} (lower).

It should be noted that the frequency distribution of deformed cells vs. the short axis/long axis ratio was Gaussian in all cases, thus calcium-loading procedure in K-buffer decreased the deformability of whole cell population. This is contrasted to our previous report [7], which demonstrated that calcium accumulation in Na-buffer took place in cell age-dependent manner: i.e., the aged cell population was highly dehydrated due to calcium accumulation and became undeformable, whereas the younger cell population resisted to calcium loading (due to higher activity of Ca^{2+} extrusion) and maintained the same deformability as the intact cells.

C. Effect of calmodulin inhibitors on the deformability

Many calmodulin inhibitors are known to induce the stomatocytosis [18,35]. Actually, W-7 and

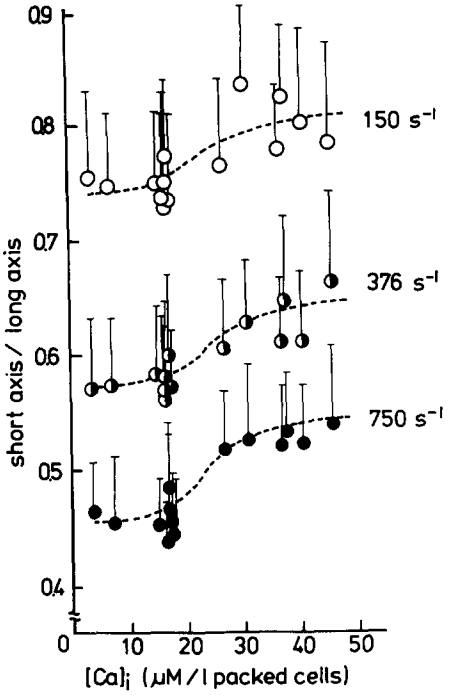

Fig. 3. Changes of erythrocyte deformability as a function of intracellular Ca content. The deformability was measured in 14% Dextran T-40 in K-buffer at 25°C under the shear rates of 150 (○), 376 (◑) and 750 (●) s^{-1} (the shear stress, 18, 45 and 90 dyn/cm^2, respectively) and represented by the mean of short axis/long axis ratio of more than 100 deformed cells with the standard deviation (expressed by half bar). The result of five different series of experiment are summarized. Mean cell volume was 98.6±2.4 (mean±S.D.) μm^3, and mean cellular hemoglobin concentration, 33.9±0.5 (mean±S.D.) g/dl (n = 14).

TFP, like chlorpromazine [26,27], induced stomatocytosis then spherostomatocytosis, as increasing their concentration in the incubation medium. As shown in Fig. 4, the effect of TFP on the transformation was stronger than that of W-7. Interestingly, calcium-loaded cells resisted against the stomatocytic transformation by both W-7 and TFP (especially at high concentration), as compared with control and calcium-depleted cells.

To study the effect of these calmodulin inhibitors on the deformability, the incubation medium containing 30 μM W-7 or 7 μM TFP was used at hematocrit of approx. 10%. In the measurement of deformability, the inhibitor was also added to dextran solution in the same concentration. At these concentrations, a slight stomatocytosis was

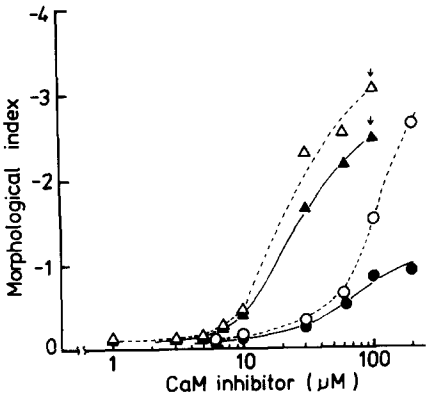

Fig. 4. Stomatocytic transformation of erythrocytes by calmodulin (CaM) inhibitors. ○, control and W-7-treated cells ($[Ca]_i$ = 18.6 μmol/l packed cells); △, control and trifluoperazine treated cells ($[Ca]_i$ = 17.0 μmol/l packed cells); ●, calcium-loaded and W-7-treated cells ($[Ca]_i$ = 39.4 μmol/l packed cells); ▲, calcium-loaded and trifluoperazine-treated cells ($[Ca]_i$ = 46.0 μmol/l packed cells). Hemolysis by trifluoperazine began at the concentration shown with the arrow.

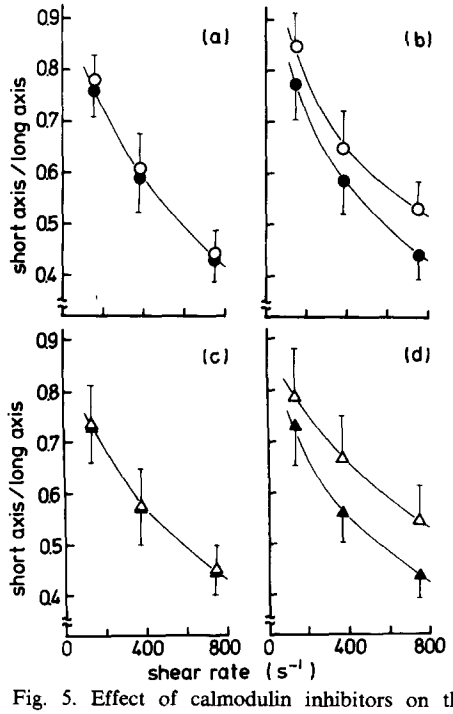

Fig. 5. Effect of calmodulin inhibitors on the erythrocyte deformability. (Upper) Effect of W-7: (a) control cells ($[Ca]_i$ = 18.6 μmol/l packed cells, $[Hb]_i$ = 33.2 g/dl); (b) calcium-loaded cells ($[Ca]_i$ = 39.4 μmol/l packed cells, $[Hb]_i$ = 33.5 g/dl). ○, W-7-untreated cells; ●, 30 μM W-7-treated cells. (Lower) Effect of trifluoperazine (TFP): (c) control cells ($[Ca]_i$ = 17.0 μmol/l packed cells, $[Hb]_i$ = 35.6 g/dl); (d) calcium-loaded cells ($[Ca]_i$ = 46.0 μmol/l packed cells, $[Hb]_i$ = 35.4 g/dl). △, TFP-untreated cells; ▲, 7 μM TFP-treated cells. Conditions for the measurement of deformability as in Fig. 3.

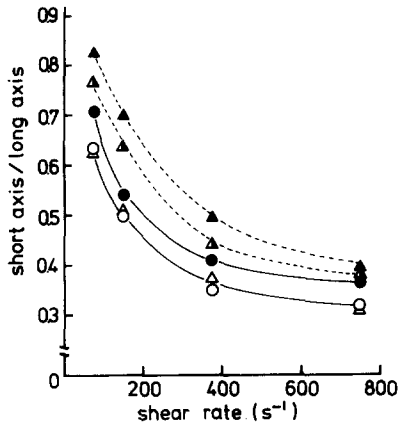

Fig. 6. Comparison of the deformability between calcium-loaded and diamide-treated erythrocytes. Measured in the medium containing 20% Dextran at various shear rates. (1) Present experiment; control cells (○, $[Ca]_i = 17.0$ μmol/l packed cells) and calcium-loaded cells (●, $[Ca]_i = 46.0$ μmol/l packed cells). (2) Previous experiment (Fig. 4(A) of Ref. 24); cells treated with 0 mM (△), 0.4 mM (▲) and 0.8 mM (▲) diamide (the degree of spectrin-crosslinking determined by electrophoresis, 0, 4 and 9%, respectively, which are represented by % loss of band 2).

observed in all kinds of preparations in the same degree, but the cells were deformed to ellipsoidal discocytes under uniform shear stress. It should be noted that the intracellular Ca content was not altered by incubation with calmodulin inhibitors.

The effects of W-7 and TFP on the erythrocyte deformability are shown in Fig. 5. The decreased deformability of calcium-loaded cells was clearly restored by the calmodulin inhibitors, but the effect on the deformability of control and calcium-depleted cells was not detected. In these cases again, the frequency distribution of deformed cells vs. the short axis/long axis ratio was Gaussian, thus all population was uniformly affected by the calmodulin inhibitors and no cell-age dependence was noticeable.

Further, the cells, when treated with high concentration of inhibitors, transformed to spherostomatocytes and deformed under shear stress to the ellipsoid (rugby ball-like shape), as observed in the case of high concentration of chlorpromazine [27]. In such situation, the comparison of deformability between the ellipsoidal disk and ellipsoid on the basis of the short axis/long axis ratio was meaningless.

Discussion

The cellular deformability depends on cell shape, intracellular viscosity and membrane stiffness [2,4,36]. In order to determine the effect of intracellular calcium on membrane stiffness and on cellular deformability, we have to compare the erythrocyte specimens varying only calcium content but maintaining normal discocytic shape and intracellular viscosity. The present procedure of calcium loading and calcium depletion, with A23187 in K-buffer, could successfully vary the total Ca content in the range from 0.25 to 3 times the normal level, without modifying cell shape and mean cellular hemoglobin concentration. In the process of calcium loading, ATP utilization greatly exceeds ATP production, the deamination of adenine moiety proceeded, as pointed out by Lew and Garcia-Sancho [28], and the echinocytosis proceeded (Fig. 1). Further, the inevitable loss of magnesium may occur during calcium loading [37]. During second incubation in glucose-containing buffer, ATP level increased, IMP level decreased and the energy charge was restored. However, ATP level did not regain the original level, presumably due to the breakdown and/or loss of adenosine. In spite of ATP loss of some 30% after second incubation, the calcium loaded erythrocytes could restore their shape, and the deformability was not affected by such a loss of ATP as demonstrated by Feo and Mohandas [29] and by us [23].

As shown in Fig. 3, the cellular deformability gradually decreased as increasing Ca content; it appeared two state transition from more deformable (less calcium) to less deformable (more calcium) states at around 20–30 μmol/l packed cells of total Ca, although the standard deviation of deformability indices was large (because it reflected the deviation of individual cells in cell volume, cell age and so on).

The quantitative comparison of such calcium effect on cellular deformability (Fig. 3) with the effect of covalent spectrin-crosslinking is noteworthy. In Fig. 6, the plots of the ratio of short axis to long axis vs. shear rate for control and calcium-loaded cells are shown, together with the previous data for diamide-treated cells (Fig. 4(A) of Ref. 24; the cytoskeletal proteins are cross-

linked by disulfide bond). The result reveals that (i) both calcium-loading and diamide-treatment decrease the deformability in a similar fashion and (ii) the degree of decreased deformability of calcium-loaded cells (2.6 times the normal Ca content) corresponds to that for diamide-induced spectrin crosslinking of about 2% (expressed as percent loss of monomeric band 2 peak on polyacrylamide gel electrophoresis in sodium dodecyl sulfate [24]). In addition, the irreversible crosslinking of membrane proteins due to Ca^{2+}-activated transglutaminase [38] was not detected for the present calcium-loaded cells. Therefore, this evidence suggests that the increase of cellular calcium content induces the changes in the dynamic structure of spectrin network.

The control mechanism of cytoskeletal dynamic structure is still unknown. In the in vitro study, Sobue et al. [9,10] have first shown that calmodulin complexed with calcium interacts with spectrin. Though there are some disputes whether or not calmodulin binds with native spectrin, the recent in vitro studies have confirmed the binding of Ca-calmodulin complex to intact spectrin [39–41]. Furthermore, Husain et al. [11] have shown that Ca-calmodulin interacts with band 4.1 protein more strongly than spectrin. If these reversible interactions occur inside of intact erythrocytes, the effect of calcium on the deformability (Fig. 3) may be explained: i.e., calcium accumulation leads to an increase of intracellular free Ca^{2+} concentration, and free Ca^{2+} binds with calmodulin, then the organization of the spectrin network is modified in such a way that the membrane stiffness is increased. However, at the moment, we are unable to estimate the free Ca concentration and the binding isotherms among Ca, calmodulin and cytoskeletal proteins. Further, calmodulin-binding protein [42] and/or calmodulin-dependent spectrin kinase [43] may contribute to the phenomena.

It is known that the calmodulin inhibitors preferentially bind with the Ca-calmodulin complex rather than with the calmodulin itself, and that they reduce the effect of Ca-calmodulin complex on the activities of the Ca^{2+}-pump, phosphodiesterase, etc. [44–48]. The present experiment with calmodulin inhibitors, W-7 and TFP, is summarized as follows: (i) for control and calcium-depleted cells, these inhibitors induce the stomatocytosis effectively but unchange the deformability, and (ii) for calcium-loaded cells, they induce the shape change less effectively but perfectly restore the decreased deformability. If the inhibitors reduce the interaction between Ca-calmodulin and cytoskeletal proteins (band 4.1 and spectrin) in intact erythrocytes, as in vitro studies [11,39–41], then the deformability of calcium-loaded cells is restored. On the other hand, in control and calcium-depleted cells, most of the calmodulin may be free of calcium [10,11,40], thus it may interact neither with band 4.1 nor with spectrin.

In conclusion, two phenomena are presented: (1) the erythrocyte deformability is affected by the calcium content and (2) the calmodulin inhibitors can release such an effect of calcium. These may be explained on the basis of the interaction between Ca-calmodulin and cytoskeleton. Although there may be other mechanisms related to the phenomena, the contribution of Ca-calmodulin seems to have a key role for controlling the cellular deformability.

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