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The Degree of Resistance of Erythrocyte Membrane Cytoskeletal Proteins to Supra-Physiologic Concentrations of Calcium: An In Vitro Study

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Abstract Calcium is a key regulator of cell dynamics. Dysregulation of its cytosolic concentration is implicated in the pathophysiology of several diseases. This study aimed to assess the effects of calcium on the network of membrane cytoskeletal proteins. Erythrocyte membranes were obtained from eight healthy donors and incubated with 250 μM and 1.25 mM calcium solutions. Membrane cytoskeletal proteins were quantified using SDS-PAGE at baseline and after 3 and 5 days of incubation. Supra-physiologic concentrations of calcium (1.25 mM) induced a significant proteolysis in membrane cytoskeletal proteins, compared with magnesium ($p < 0.001$). Actin exhibited the highest sensitivity to calcium-induced proteolysis (6.8 ± 0.3 vs. 5.3 ± 0.6, $p < 0.001$), while spectrin (39.9 ± 1.0 vs. 40.3 ± 2.0, $p = 0.393$) and band-6 (6.3 ± 0.3 vs. 6.8 ± 0.8, $p = 0.191$) were more resistant to proteolysis after incubation with calcium in the range of endoplasmic reticulum concentrations (250 μM). Aggregation of membrane cytoskeletal proteins was determined after centrifugation and was significantly higher after incubation with calcium ions compared with control, EDTA and magnesium solutions ($p < 0.001$). In a supra-physiologic range of 1.25–10 mM of calcium ions, there was a nearly perfect linear relationship between calcium concentration and aggregation of erythrocyte membrane cytoskeletal proteins ($R^2 = 0.971$, $p < 0.001$). Our observation suggests a strong interaction between calcium ions and membrane cytoskeletal network. Cumulative effects of disrupted calcium homeostasis on cytoskeletal proteins need to be further investigated at extended periods of time in disease states.

Keywords Calcium · Membrane cytoskeletal proteins · Aggregation · Erythrocyte

Introduction

During the origin and evolution of life, calcium ion (Ca^{2+}) was given a unique opportunity among all divalent cations

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(Jaiswal 2001). From the beginning of new cell life at fertilization to its termination in the process of programmed cell death, Ca^{2+} plays important roles in a wide variety of cellular functions, from secretion and contraction to metabolism and gene expression (Brini and Carafoli 2009). Recruitment of Ca^{2+} as a dynamic signal was made only possible when compartmentation of intra-cellular space afforded the cell the ability to regulate Ca^{2+} cytoplasmic concentration (Williams 2006). Basal cytosolic concentration of free Ca^{2+} in human eukaryotic cells is about 62–221 nM and may increase to 1 μM or higher by physiologic stimulations (Aiken et al. 1995; Yu and Hinkle 2000; Brini and Carafoli 2009). Free Ca^{2+} concentration in endoplasmic reticulum, the main intracellular calcium store, is much higher (about 500 μM) (Yu and Hinkle 2000). Several Ca^{2+} regulatory mechanisms, including Ca^{2+} channels, pumps and exchangers, are employed to strictly regulate intra-cellular Ca^{2+} concentration (Carafoli 1987). Nevertheless, dysregulation of cellular Ca^{2+} homeostasis is implicated in the pathophysiology of several diseases, including Alzheimer's disease and neuronal ageing (Verkhratsky 2005), cardiac disease (Wehrens et al. 2005), diabetic neuropathy (Verkhratsky 2005), affective disorders (Yamawaki et al. 1998) and inflammatory bowel disease (Schmidt et al. 1995).

The cell skeleton is a tool by which nearly all dynamic processes take place within the cell (Fletcher and Mullins 2010). It is a system of microtubules and microfilaments running all through the cell, particularly just under the plasma membrane. Membrane cytoskeleton is the site in which the complex interaction between cell membrane and cytoskeletal proteins determines cell shape and its intercellular attachments (Bennett 1989; Luna and Hitt 1992). It also plays an important role in signal transduction and cell response to external stimuli (Luna and Hitt 1992). The best-studied example of membrane cytoskeletal complex is that of red blood cells (RBCs). This cortical cytoskeleton is a two-dimensional network of spectrin filaments attached to internal domain of integral membrane proteins through a variety of intermediate protein links (Bennett 1989). The spectrin network plays a crucial role in maintaining normal biconcave shape of erythrocytes and allows these cells to deform and pass through capillaries in the microcirculation.

The relationship between Ca^{2+} and cytoskeletal proteins is complex and bidirectional. Actin protein of cytoskeleton plays an inhibitory role in the activation of Ca^{2+} entry, making cytoskeletal remodeling a key event in cytosolic Ca^{2+} homeostasis (Rosado and Sage 2000; Rivas et al. 2004). On the other hand, transient rises in cytoplasmic Ca^{2+} concentration activate several proteases and associate with dramatic changes in cell morphology and alterations in microtubule cytoskeleton (Bennett and Weeds 1986; O'Brien et al. 1997). Several observations suggest an important role for Ca^{2+} in modifying erythrocyte cytoskeletal organization and membrane deformity (Lew and Tiffert 2007; Kabaso et al. 2010). Important regulatory effects of Ca^{2+} on cytoskeletal functions have been recognized since 1980s (Fowler and Taylor 1980; Takakuwa and Mohandas 1988). With implication of Ca^{2+} as a major cell stressor, long-term consequences of intracellular Ca^{2+} dysregulation have been subject to intensive research in recent years.

We hypothesized that increased concentration of cytosolic free Ca^{2+} can adversely affect the structure of cell membrane by affecting its cytoskeletal architecture. We examined this hypothesis in a cytosol-free in vitro model of erythrocyte membrane and in supra-physiologic and endoplasmic ranges of Ca^{2+} concentrations.

Materials and Methods

Erythrocyte Membrane Preparation

Peripheral blood samples were obtained by venipuncture from eight healthy blood donors. Blood samples were collected in vacutainer tubes containing lithium heparinate as anticoagulant and were used within 2 h. Whole blood was centrifuged at 3,000 rpm for 10 min. After isolation of white blood cells, platelets and serum plasma, erythrocyte membranes were prepared by osmotic lysis using the method of Dodge et al (1963). Briefly, erythrocytes were washed twice with a buffer containing 5 mM sodium phosphate and 150 mM NaCl, with pH 7.4 at 4 °C. After centrifugation at 40,000 rpm for 10 min, the pellet was resuspended in the same phosphate buffer. Erythrocytes were then lysed by osmotic choc using 1 volume of erythrocyte suspension for 40 volumes of hypotonic solution (5 mM sodium phosphate buffer, pH 7.4). Samples were then centrifuged at 40,000 rpm for 10 min at 4 °C and were washed with phosphate tampon three times and resuspended. Final suspensions containing 4 mg protein/ml were prepared and used for following experiments. Concentration of membrane proteins was determined by membrane suspension in 1 % SDS and absorbance measurement at 280 nm face standard curve.

Relative Percentages of Membrane Cytoskeletal Proteins

The effects of calcium and magnesium on the integrity of membrane cytoskeletal proteins were investigated by measurement of relative percentages of these proteins. Water solutions of $CaCl_2$ (at calcium concentrations of 250 μM and 1.25 mM) and $MgCl_2$ (at magnesium concentration of 8 mM) were added to the suspension of

erythrocyte membrane. Relative percentages of membrane cytoskeletal proteins were measured at baseline, after 3 and 5 days of incubation at 4 °C. Cytoskeletal membrane proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 3.5 % polyacrylamide gel (40 mM tris, 2 mM EDTA, pH 7.4 as electrophoresis tampon). 40 μg of membrane proteins were deposited on gel and protein separation was made at 75 V for 110 min. The gels were stained with Coomassie blue and were fixed with acetic acid and isopropanol. Standard bands consisted of erythrocyte membrane cytoskeletal proteins spectrin, ankyrin, band 3, band 4.1, actin and band 6. Other non-specific bands were analyzed if existed on electrophoresis.

Aggregation of Erythrocyte Membrane Cytoskeletal Proteins

The effects of calcium and magnesium on the aggregation of erythrocyte membrane cytoskeletal proteins were investigated at different concentrations. Water solutions of $CaCl_2$ (at calcium concentrations of 1.25, 2.5, 5 and 10 mM), $MgCl_2$ (at magnesium concentrations of 8 and 16 mM) and $CaCl_2$ plus EDTA (5 mM $CaCl_2$ + 5 mM EDTA) were used in these experiments. 30 min after adding each solution to 2 ml of cytoskeletal membrane proteins suspension (with concentration equals to 4 mg/ml) at 25 °C, samples were centrifuged at 1,500 rpm for 3 min. Concentrations of proteins were subsequently measured in supernatant liquid after centrifugation. It was previously shown that the degree of cell ghosts aggregation after rotation of their suspension is proportional to the decrease of the relative membrane protein concentration (the ratio of the protein concentrations determined after and before experiment) in the top layer of the suspension (Deman and Bruyneel 1973). Because basal concentration of membrane cytoskeletal proteins equals the sum of aggregated and remnant membrane proteins concentrations in the supernatant fluid, the relationship between calcium concentration and aggregation rate of RBC membranes can be inferred from the relationship between calcium and supernatant fluid proteins concentrations. Densitometric analysis was carried out in the absorbance mode at 280 nm to determine protein concentration after incubation and centrifugation in supernatant fluid.

Ethics

The study protocol was approved by research ethics committee of Tehran University of Medical Sciences and complied with the Declaration of Helsinki. Written informed consents were obtained from blood donors participating in this study.

Statistical Analysis

Experiments were replicated three times and mean values were recruited for analysis. Statistical Package for Social Sciences (SPSS for windows, version 19; Chicago, IL, USA) and R-package (version 2.15.2, R Core Team (2012), R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/) were used for data analysis. Repeated measurements general linear model was used to investigate changes in concentrations of membrane cytoskeletal proteins during and after incubation. Multivariate general linear model was recruited to investigate differences between incubation solutions. In this model, changes in spectrin, ankyrin, band 3, band 4.1, actin and band 6 were simultaneously introduced as outcome variables and type of solution was considered as independent variable. Post-hoc analysis was made using bonferroni adjustment for multiple comparisons. Calcium effects on the aggregation of membrane cytoskeletal proteins were investigated by using linear and quadratic regression models. Analysis of variance and corrected Akaike's Information Criteria (AICc) were used to compare two regression models and select the better one. Alpha = 0.05 was considered as the level of significancy.

Results

Membrane Cytoskeletal Proteins Lysis

Erythrocyte membranes were incubated with 250 μM calcium, 1.25 mM calcium and 8 mM magnesium solutions for 5 days. Relative percentages of membrane cytoskeletal proteins were measured at baseline, days 3 and 5 after incubation and are presented in Table 1. Changes in concentrations of membrane cytoskeletal proteins during incubation with 250 μM calcium were significant for ankyrin, band 3, band 4.1 and actin, but not for spectrin and band 6. In membranes incubated with 1.25 mM calcium, such changes were significant for all types of membrane cytoskeletal proteins. No significant changes in concentrations of cytoskeletal proteins were observed after incubation with 8 mM magnesium solution. After 3 days of incubation with 1.25 mM calcium and 5 days of incubation with 250 μM calcium, small non-specific bands appeared between band 4.1 and actin and farther than band 6. These bands were not present during incubation with 8 mM magnesium solution (Table 1).

To compare linear changes in concentrations of cytoskeletal proteins among three types of solutions, a multivariate general linear model was used. Concentrations of cytoskeletal proteins were jointly introduced as outcome variables (Table 2). Type of solution was a significant

Table 1 Relative concentrations of erythrocyte membrane cytoskeletal proteins

		Spectrin	Ankyrin	Band 3	Band 4.1	Actin	Band 6	Bands was considered as >6	Bands between 4.1 and actin
$CaCl_2$	Baseline	39.9 ± 1.0	5.1 ± 0.5	33.7 ± 1.0	8 ± 0.2	6.8 ± 0.3	6.3 ± 0.3	–	–
250 μM	3 days	40.3 ± 1.2	5.2 ± 0.3	33.7 ± 0.3	7.7 ± 0.2	6.3 ± 0.2	6.8 ± 0.8	–	–
	5 days	40.3 ± 2.01	4.5 ± 0.5	31 ± 2	7.7 ± 0.3	5.3 ± 0.6	6.8 ± 0.8	2.7 ± 0.6	1.5 ± 0.9
	Significance	$p = 0.393$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.005$	$p = 0.191$		
$CaCl_2$	Baseline	39.9 ± 0.2	4.7 ± 0.7	34 ± 2.3	8.3 ± 0.8	6.7 ± 0.6	6.3 ± 0.5	–	–
1.25 mM	3 days	34.9 ± 0.6	3.7 ± 0.3	30.7 ± 0.3	7.7 ± 0.8	6.0 ± 0.1	12.2 ± 0.1	4.7 ± 0.3	–
	5 days	22.5 ± 2.5	3.2 ± 0.3	21.8 ± 1.5	6.3 ± 0.6	3.3 ± 0.3	13.3 ± 1.5	23 ± 0.3	6.7 ± 0.1
	Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$		
$MgCl_2$	Baseline	40.1 ± 0.8	5.3 ± 0.5	34 ± 0.3	7.9 ± 1.0	6.5 ± 0.9	6.2 ± 0.2	–	–
8 mM	3 days	39.7 ± 0.7	5.4 ± 0.5	34.4 ± 0.6	7.8 ± 0.1	6.3 ± 0.3	6.3 ± 0.3	–	–
	5 days	39.8 ± 1.1	5.7 ± 0.3	34 ± 0.3	7.6 ± 0.8	6.3 ± 0.7	6.5 ± 0.7	–	–
	Significance	$p = 0.645$	$p = 0.098$	$p = 0.835$	$p = 0.705$	$p = 0.060$	$p = 0.539$		

Data is presented as mean ± standard error of mean. All values are presented in percent (%) of total concentration of membrane cytoskeletal protein. Repeated measurements general linear model was used for statistical analysis

Table 2 Statistical analysis for linear changes in relative percentages of RBC membrane cytoskeletal proteins during incubation

	Significance	R^2	Significance Ca 250 μM/Mg 8 mM	Significance Ca 1.25 mM/Mg 8 mM	Significance Ca 250 μM/Ca 1.25 mM
Spectrin	$p < 0.001$	0.946	$p = 0.604$	$p < 0.001$	$p < 0.001$
Ankyrin	$p < 0.001$	0.614	$p < 0.005$	$p < 0.001$	$p = 0.217$
Band 3	$p < 0.001$	0.877	$p < 0.05$	$p < 0.001$	$p < 0.001$
Band 4.1	$p < 0.001$	0.538	$p = 0.99$	$p < 0.001$	$p < 0.005$
Actin	$p < 0.001$	0.843	$p < 0.001$	$p < 0.001$	$p < 0.001$
Band 6	$p < 0.001$	0.915	$p = 0.99$	$p < 0.001$	$p < 0.001$

p values are obtained from multivariate general linear model with type of incubation solution as independent variable and cytoskeletal proteins concentrations as outcome variables. R^2 expresses the percent of total variance for each protein concentration that is explained by independent variable. Post-hoc analysis to compare each two solutions was made by bonferroni adjustment

independent predictor of each type of cytoskeletal proteins, controlling for its effect on other cytoskeletal proteins ($p < 0.001$). Alterations in concentrations of ankyrin, band 3 and actin were significant for 250 μM calcium, compared with 8 mM magnesium solution. Differences between 1.25 mM calcium and 8 mM magnesium were significant for all cytoskeletal proteins. The same differences were observed between 250 μM and 1.25 mM calcium, except for ankyrin (Table 2).

Aggregation of Erythrocyte Membrane

RBC membranes were incubated with 1.25, 2.5, 5 and 10 calcium solutions. Three control solutions were used (5 mM calcium + 5 mM EDTA, 8 mM magnesium and 16 mM magnesium). By increasing calcium concentration in incubatory solutions, the concentration of erythrocyte membrane proteins in supernatant fluid generally decreased, suggesting enhanced aggregation of these proteins by Ca^{2+} ions (Table 3). When 5 mM EDTA was added to 5 mM calcium solution, this effect was reversed (*p* value = 0.16 compared with baseline, *p* value <0.001 compared with 5 mM calcium solution). The amount of aggregation of erythrocyte cytoskeletal proteins after incubation with 8 mM magnesium was significantly lower than baseline values (*p* value <0.01), but higher than 5 mM calcium solution (*p* value <0.001).

To investigate the dose–response pattern of the relationship between supra-physiologic calcium concentrations and cytoskeletal proteins aggregation, first-order and quadratic regression analyses were recruited. In the first-order model, calcium concentration was a significant predictor of cytoskeletal proteins aggregation [beta = −0.225, 95 % CI (−0.244, −0.206), *p* value <0.001, $R^2 = 0.971$]. In the

Table 3 Concentrations of membrane cytoskeletal proteins after incubation with calcium and magnesium solutions

	Baseline	$CaCl_2$ 1.25 mM	$CaCl_2$ 2.5 mM	$CaCl_2$ 5 mM	$CaCl_2$ 10 mM	$CaCl_2$ 5 mM + EDTA	$MgCl_2$ 8 mM	$MgCl_2$ 16 mM
Protein concentration (mg/ml)	2.2 ± 0.1	1.97 ± 0.15	1.93 ± 0.15	1.12 ± 0.1	0.04 ± 0.05	2.02 ± 0.33	2.04 ± 0.09	2.03 ± 0.09
p value	–	0.002	<0.001	<0.001	<0.001	0.16	<0.01	<0.01

Data is presented as mean ± standard deviation. *p* values are obtained from mean comparison of each group with baseline values

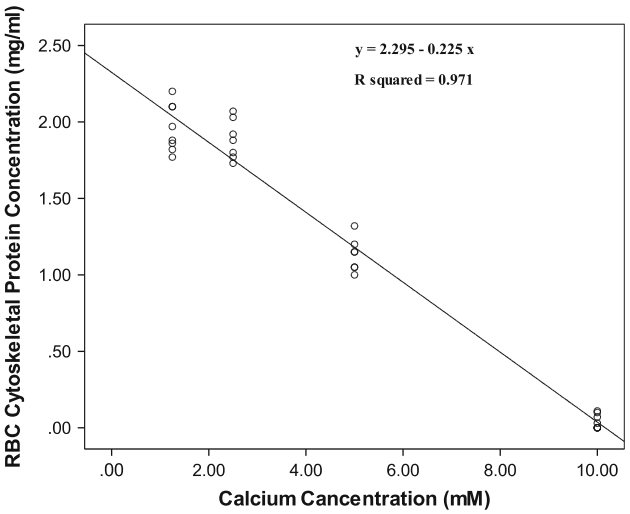

Fig. 1 Linear relationship between concentrations of erythrocyte membrane cytoskeletal proteins and calcium after incubation and centrifugation

quadratic model, the first-order term (calcium concentration) was still a significant predictor [beta = −0.219, 95 % CI (−0.327, −0.112), *p* value <0.001] of protein aggregation, while the second-order term (the second power of calcium concentration) was not [beta = −0.0005, 95 % CI (−0.009, 0.008), (*p* value = 0.908)]. Analysis of variance was used to decide which model is superior in terms of providing better approximation of the underlying relationship. Quadratic term did not significantly improved the predictive power of the model [test statistics = 0.0140, $F(1,2) = 98.50$, *p* value = 0.99]. This was compatible with corrected Akaike's Information Criteria (AICc for first-order model = −10.52, AICc for quadratic model = −8.04, information ratio = 3.45), suggesting that the first-order model was 3.45 times more likely to be correct than quadratic one. Linear relationship between concentrations of calcium and RBC cytoskeletal protein after incubation is presented in Fig. 1.

Discussion

In this study, we used two models to investigate Ca^{2+} effects on membrane cytoskeletal structure. By measuring the concentration of its constituent proteins, we demonstrated that 5-day exposure to supra-physiologic concentrations of Ca^{2+}, even in a cytosol-free medium, leads to enhanced degradation of erythrocyte membrane cytoskeletal proteins, possibly by activating Ca^{2+}-dependent proteases. Effect of Ca^{2+} on membrane cytoskeleton was also manifested by accelerated aggregation of erythrocyte membranes after incubation with increasing concentrations of Ca^{2+}, a phenomenon that was reversed after adding EDTA to the medium. One important finding was the almost perfect linear relationship (with negative slope) between supra-physiologic concentrations of Ca^{2+} and membrane cytoskeletal proteins in supernatant fluid after incubation ($R^2 = 0.971$). This implies a direct linear relationship between above-normal concentrations of Ca^{2+} and aggregation rate of erythrocyte membranes.

Erythrocyte aggregation rate increases in a wide range of pathologic states, from infections (Goldin et al. 2007) and malignancy (von Tempelhoff et al. 2000) to inflammation (Almog et al. 2005) and atherosclerosis (Fusman et al. 2002; Sharshun et al. 2003). Erythrocyte Sedimentation Rate (ESR) is a familiar laboratory test based on this feature of RBCs. Sensitivity of erythrocyte membranes to their environmental stressors makes ESR a sensitive candidate for screening purposes, as its value changes in a variety of diseases, particularly inflammatory ones. We used this feature in our experiments to quantify calcium effects on membrane cytoskeletal integrity. Based on previous reports, we expected that adding calcium to the medium of RBC cell membranes results in an increased rate of membrane aggregation (Liu et al. 2005; Mostafavi et al. 2013). Adding Mg^{2+} ions to the sample of erythrocyte membranes also increased their aggregation rate (*p* value = 0.01). Considering higher concentrations of Mg^{2+} compared to Ca^{2+} in these experiments (8–16 mM Mg^{2+} vs. 1.25–10 mM Ca^{2+}), aggregative effects of Ca^{2+} take on more importance, because the observed effect could not be simply attributed to positive charges of Ca^{2+} ions. We aimed to determine the nature of Ca^{2+} regulatory effects on membrane cytoskeletal proteins. The perfect linear relationship between supra-physiologic Ca^{2+} concentration and cytoskeletal proteins aggregation sheds light on the underlying relationship between these two intracellular players. However, the intracellular concentration

of free Ca^{2+} is extremely low in non-stimulated states (in the range of nano-mol) (Yu and Hinkle 2000; Brini and Carafoli 2009). Whether this linear in-vitro relationship can be extrapolated to lower concentrations in vivo states is an important consideration. On the other hand, there are some possibilities that the observed effects may be far more important as both Ca^{2+} and cytoskeletal proteins interplay in intracellular processes. Not within 3 min of centrifugation or 3 days of follow up but on longer periods of time and in a cumulative manner, even mild interaction between Ca^{2+} and membrane cytoskeletal proteins may be a major determinant of cell dynamics.

After adding EDTA to 5 mM Ca^{2+} solution, the standard error of the aggregation rate increased from 0.10 to 0.33. This suggests that RBC membrane cytoskeletal proteins from different blood donors had dissimilar sensitivities to Ca^{2+}-induced aggregative effects. In other words, this implies a subtle inter-individual difference in healthy population. It will be intriguing to repeat these experiments on other cell lines and diseases in which a significant role has been suggested for Ca^{2+} in their pathophysiology.

Different cytoskeletal proteins did not show similar responses to Ca^{2+}-induced proteolysis. Actin had the highest sensitivity among membrane cytoskeletal proteins. Even in endoplasmic-range concentrations of Ca^{2+} (250 μM), significant decrease in actin concentration was evident at day 3 after incubation, as revealed by SDS-PAGE. This takes more importance when considering the relatively lower location of actin band on polyacrylamide gels after electrophoresis, where proteolytic products of larger proteins may misleadingly overfill protein bands.

Several limitations of this study merit consideration. Membrane cytoskeleton is a critical point in cell dynamics and is under regulation of multiple elements. We observed a linear relation between calcium concentration and membrane cytoskeletal protein aggregation in vitro conditions with constant parameters. Generalizing these findings to in vivo conditions without further investigations would be oversimplification of cell biology. This situation gets more complicated when other thermodynamic and biologic parameters are included. Membrane cytoskeleton is under regulatory effects of multiple elements. Interactive effect of calcium and other regulatory elements of membrane cytoskeleton is out of scope of this study. The balance between cumulative damages, caused by various kinds of cell stressors, and anti-stress mechanisms, such as protein refolding chaperones, is also an area of intensive research. This is especially of great importance when considering the very low concentration of cytosolic free Ca^{2+} and high turnover of cytoskeletal proteins. We used a model of constantly elevated Ca^{2+} concentration, whereas transient rises in intracellular Ca^{2+} concentration would be a better simulation of in vivo conditions. In addition, the results may prove to be far different in intact RBCs, compared with cytosol-free medium of RBC membranes. One important consideration in methodology is that theoretically, proteolytic products of larger proteins may have overlapped with specific bands of cytoskeletal proteins. This effect would become more prominent for proteins located in lower parts of SDS gel after electrophoresis and leads to falsely higher estimation of the concentration of these proteins. However, this problem does not seem to have confounded our findings, as such small non-specific bands were only observed after band 4.1 and primarily, after 5 days of incubation.

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