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Sulfate Self-Exchange and Amino Acid Transport in Calcium-Loaded Human Erythrocytes

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Summary. To analyze the effects of Ca^{2+}-mediated membrane protein changes on the membrane function, we have studied the SO_4^{2-} self-exchange and amino acid transport in human erythrocytes after loading them with Ca^{2+} with the help of ionophore A23187. The SO_4^{2-} self-exchange is inhibited by 20–30% by loading the erythrocytes with 25 μM to 0.5 mM Ca^{2+}. The extent of this inhibition is almost doubled (50–60%) by increasing the Ca^{2+} loading concentration to 1.5 mM. This additional effect of 1.5 mM Ca^{2+} is not correlated with the Ca^{2+}-induced ATP depletion or membrane protein degradation, but is caused by the transglutaminase-catalyzed membrane protein crosslinking. Like the SO_4^{2-} self-exchange, L-alanine and L-cysteine uptakes are also inhibited in Ca^{2+}-loaded cells. However, no effect is observed on the L-lysine uptake under identical conditions. These results have been interpreted to suggest that the Ca^{2+}-mediated effects on the SO_4^{2-} self-exchange and amino acid transport are caused perhaps by the Ca^{2+}-induced structural rearrangement of the band 3 protein.

Key Words erythrocytes · anion self-exchange · amino acid transport · intracellular calcium · membrane protein rearrangement

Introduction

Anion exchange across the erythrocyte membrane plays a key role in CO_2 transport in vivo and is mediated by the major integral membrane protein, called anion exchange or band 3 protein [4]. Also, this protein has been implicated in amino acid transport across the erythrocyte membrane [21, 23].

Numerous studies have shown that loading of human erythrocytes with Ca^{2+} in the presence of ionophore A23187 leads to the activation of several enzymes including transglutaminase and proteinases, which structurally modify the band 3 protein. While the Ca^{2+}-activated transglutaminase catalyzes irreversible membrane protein crosslinking which involves band 3 protein along with spectrin, ankyrin and 4.1 protein [13, 14], the Ca^{2+}-induced activation of proteinases results in degradation of the membrane proteins which also includes the anion channel protein [1, 12].

To analyze the effects of the above Ca^{2+}-induced membrane structural modifications on the erythrocyte membrane function, we have studied the SO_4^{2-} self-exchange and amino acid transport in Ca^{2+}-loaded human erythrocytes. The Ca^{2+} loading was carried out with the help of ionophore A23187. Results of these studies indicate that the increased intracellular free Ca^{2+} levels strongly influence the SO_4^{2-} self-exchange and L-alanine uptake, but not L-lysine uptake, across the erythrocyte membrane.

Materials and Methods

Ionophore A23187, BSA, Hb standard, DIDS, SDS, HEPES, EGTA, PMSF, N,N-dimethylated casein, crystalline putrescine, Mg^{2+}-ATP, ATP estimation kit, L-alanine, L-cysteine, L-lysine, sodium pyruvate and adenosine were purchased from Sigma Chemical.[1] [^{3}H] putrescine was obtained from Amersham, UK. [^{14}C]-L-alanine, [^{14}C]-L-lysine, $^{45}CaCl_2$ and sodium [^{35}S] sulfate were from Bhabha Atomic Research Centre, Trombay, India. [^{35}S]-L-cysteine was from Amersham, England. Erythrocytes were isolated from fresh normal human blood, and stored at 4°C in buffer A (5 mM Tris, 100 mM KCl, 60 mM NaCl, 10 mM glucose; pH 7.4). A 5 mM stock solution of ionophore was prepared in dimethylsulfoxide, and stored in the dark at −20°C.

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[1] *Abbreviations:* BSA, bovine serum albumin; Hb, hemoglobin; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; HEPES, N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid; EGTA, ethylene glycolbis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine-5′-triphosphate; PMSF, phenylmethylsulfonyl-fluoride; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; Tris, Tris-(hydroxymethyl-) aminomethane; and RBC, red blood cells.

Calcium Loading

Ca^{2+} loading was done essentially as described earlier [6]. A control sample containing EGTA (5 mM) and no Ca^{2+} was always run simultaneously.

Erythrocyte ATP Levels

Red cell ATP levels were determined by an enzymatic test employing a Sigma Diagnostics Kit (procedure no. 366-UV), except that isolated erythrocytes were used in the assay instead of whole blood.

Erythrocyte Transglutaminase Activity

Transglutaminase activity was measured by using N,N-dimethylated casein and [^{3}H] putrescine, essentially according to published procedures [20]. Incubations were carried out at 37°C for 60 min. The effect of cystamine on the red cell transglutaminase activity was determined by including 10 mM cystamine in the incubation mixture.

Erythrocyte ATP Depletion and Repletion

Washed normal red cells were suspended to 10% hematocrit in the ATP depletion medium (50 mM glycylglycine, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, penicillin G (50 IU/ml) and streptomycin sulfate (0.1 mg/ml); pH 7.4), and the mixture was incubated for 18 hr at 37°C under a constant flow of humidified N_2. The pH was adjusted throughout incubation when necessary. The cells were harvested by centrifugation and then washed with isotonic saline (0.9% NaCl, wt/vol). The cells were repleted with ATP by incubating them at 10% hematocrit in the ATP repletion medium (5 mM sodium phosphate, 140 mM NaCl, 5 mM KCl, 5 mM glucose, 5 mM ionosine, 0.5 mM adenine, 5 mM sodium pyruvate; pH 7.4) for 3 hr at 37°C. The cells were harvested, washed with phosphate-buffered saline, and then used for transport measurements. Although this procedure led to over 90% ATP depletion, only about 30% ATP repletion in these ATP-depleted cells could be achieved under our experimental conditions.

Cystamine Pretreatment and ATP Enrichment of Red Cells

Cells were pretreated with 10 mM cystamine for 30 min at 37°C and the cystamine allowed to remain in the medium during Ca^{2+} loading. ATP enrichment of erythrocytes prior to their loading with Ca^{2+} was carried out as reported earlier [2]. About a two- to threefold increase in the intracellular ATP levels was achieved under these conditions (Table 1).

Calcium Loading in ATP-Enriched Cells

Ca^{2+} loading in ATP-enriched cells was carried out essentially as described for the normal cells. The ATP levels after Ca^{2+} loading were around 0.7 μmol/ml packed cells (Table 1). To examine the effect of ATP enrichment on the Ca^{2+} loading efficiency, trace amounts of $^{45}Ca^{2+}$ were also included in the loading medium. The amount of $^{45}Ca^{2+}$ uptake/100 mg Hb in ATP-enriched cells (45.7 $\times$ 10^4 cpm) was similar to that observed with the normal cells (44.4 $\times$ 10^4 cpm), demonstrating that the Ca^{2+} loading efficiency is not affected by the ATP enrichment.

Table 1. ATP levels in normal and ATP-enriched human erythrocytes before and after Ca^{2+} loading (1.5 mM/3 hr)

Cells	ATP levels (μmol/ml packed cells)
Normal control	1.27 ± 0.05
Normal Ca^{2+}loaded	0.12 ± 0.02
ATP-enriched control	2.68 ± 0.50
ATP-enriched Ca^{2+}-loaded	0.72 ± 0.10

Values shown are means of three determinations ± SD.

Total Cell Water Determination

Since Ca^{2+} loading is known to induce cell shrinkage due to K^+-dependent water loss (Gardos effect), the cell water content of Ca^{2+}-loaded cells was also determined. A known amount of packed cells (control and Ca^{2+}-loaded) was dried to constant weight in a vacuum oven at 110°C and the cell water content determined.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis

Membrane protein composition in erythrocytes was determined by SDS-polyacrylamide gel electrophoresis. White ghosts were prepared by the method of Dodge, Mitchell and Hanahan [7]. Packed cells were lysed in 40 volumes of ice-cold 5 mM sodium phosphate buffer (pH 7.8) containing 100 μM EDTA and 30 μM PMSF. One or two additional washings were given to obtain white ghosts. The ghost proteins were immediately denatured with SDS and β-mercaptoethanol, and frozen until electrophoresis was run on the following day. Protein in the denatured ghosts was estimated by the method of Lowry et al. [16]. Electrophoresis was run on 5% acrylamide gels using the method of Fairbanks, Steck and Wallach [8]. Gels were stained with Coomassie blue R250 and scanned on a Shimadzu dual-wavelength TLC scanner, model CS-930.

Sulfate Self-Exchange

SO_4^{2-} self-exchange was measured according to the method of Castranova, Weise and Hoffman [5]. Both influx and efflux rate constants were determined in the same set of experiments.

Influx

Packed red cells were suspended to 10% hematocrit in the SO_4^{2-} flux medium (10 mM HEPES, 50 mM Na_2SO_4, 75 mM KCl and 40 mM sucrose; pH 7.4) and allowed to equilibrate for 3 hr at 37°C. Subsequently, $^{35}SO_4^{2-}$ was added to a final concentration of 10 μCi/ml cell suspension. Aliquots of 0.5 ml were withdrawn in duplicate at the appropriate time periods. Cells were harvested

by centrifugation in an Eppendorf 5415 Centrifuge. The supernatant was discarded and the cells were washed three times with 1.0 ml of ice-cold 10 μM DIDS dissolved in the SO_4^{2-} flux medium. Washings were carried out at 14,000 rpm for 10 sec. Cells were packed and lysed by adding 0.5 ml of 0.5% Triton X-100. The amount of Hb in the lysate was determined after diluting 40 μl of the hemolysate to 100 μl with distilled water, and then adding to it 3.0 ml of Drabkins reagent (1.0 g $NaHCO_3$, 0.2 g $K_3[Fe(CN)_6]$ and 0.05 g KCN in 1.0 liter distilled water). The absorbance was measured at 540 nm, 30 min after adding the reagent in a Bausch and Lomb Spectronic 21 UV/visible spectrophotometer against standard Hb solutions.

To determine the cell-associated radioactivity, 400 μl of the above hemolysate was acidified with 400 μl of 10% (wt/vol) TCA, the precipitated protein was removed by centrifugation, and 400 μl of the supernatant was counted for ^{35}S in an LKB Wallac 1217 Rackbeta counter. The amount of radioactivity initially added to the cell suspension was determined by treating 400 μl of the cell suspension with 400 μl of TCA, removing the precipitated protein by centrifugation, and then measuring the radioactivity in the supernatant.

Efflux

Packed red cells were suspended to a 10% hematocrit in the SO_4^{2-} flux medium containing 10 μCi/ml $^{35}SO_4^{2-}$ and incubated at 37°C for 3 hr to ensure maximal SO_4^{2-} equilibration. Cells were quickly washed two times at 4°C with chilled nonradioactive SO_4^{2-} flux medium to remove extraneous radioactivity. Efflux was commenced by adding the above cells to the SO_4^{2-} flux medium at 1% hematocrit. Incubations were carried out at 37°C, and 1.0-ml aliquots were withdrawn for determining the efflux. The cell suspension was immediately centrifuged at 14,000 rpm for 25 sec. Seven hundred microliters of the supernatant was added to 700 μl TCA, and subsequently 700 μl of the supernatant was taken for measuring the radioactivity. The total radioactivity in the medium was determined by acidifying the cell suspension with TCA and measuring ^{35}S in the supernatant as given above.

Data Analysis

The first order rate constant for SO_4^{2-} exchange was calculated using the following equation:

$$\ln(1 - P_t/P_\infty) = -kt \tag{1}$$

where, P_t and P_∞ are the dpm at time 't' and 'infinity' respectively, and 'k' is the first order rate constant. During influx, P_t was obtained from values at time 't' and P_∞ from the 2 or 3-hr values, whichever was the maximum. The dpm were first corrected to dpm/100 mg Hb and this gave the P_t values. The values obtained at 0 min were subtracted from all the values prior to further calculations.

Amino Acid Transport

Packed red cells were suspended to 10% hematocrit in the incubation medium (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 15 mM Tris, 0.1 mM EDTA; pH 7.4) containing the requisite amount of unlabeled and 0.2 μCi/ml cell suspension of the labeled amino acid. The isosmolality of the medium was maintained by adjusting the NaCl concentration, depending on the unlabeled amino acid. The mixtures were incubated at 37°C. Measured aliquots (0.5 ml) were withdrawn at predetermined time intervals. The cells were pelleted in an Eppendorf 5415 centrifuge (1400 rpm, 6 sec) and washed three times with ice-cold washing buffer (10 mM Tris, 106 mM $MgCl_2$; pH 7.4). The cell-associated radioactivity was determined after lysing the cells with 0.5% Triton X-100 (wt/vol) and removing the precipitate by centrifugation.

Analysis of Concentration Dependence Curves

Saturable uptake curves with linear components of unknown magnitude were fitted by a maximum likelihood method (the nonlinear regression program BMDP 3R [10]). This was used to estimate parameters for both hyperbolic and linear components using the following equation:

$$v = \frac{V_{max} S}{(K_m + S)} + K_d \cdot S \tag{2}$$

where V is the initial uptake rate, S is the amino acid concentration, V_{max} is the maximum velocity, K_m is the apparent affinity constant and K_d is the apparent diffusion constant.

Results

Human erythrocytes were loaded with varying amounts of Ca^{2+} using the ionophore A23187. The loading efficiency, as determined by our published method [6], was maximum (80–90%) when the cells were incubated with Ca^{2+} for $\geq$1 hr at 37°C. There was no cell shrinkage due to K^+ and water loss, since we used high K^+ concentration in the incubation medium, and also observed no difference between the cell water contents before and after the Ca^{2+} loading.

Calcium-Induced Changes in Membrane Proteins

Ca^{2+}-induced effects on the membrane proteins were examined by SDS-polyacrylamide gel electrophoreses (*data not shown*). Increased intracellular Ca^{2+} levels invariably resulted in appearance of new protein bands with concomitant decrease in the intensities of polypeptides 2.1, 4.1, spectrin and anion channel protein. Also, a high molecular weight band, which failed to penetrate into the gels, was observed at $\geq$0.5 mM Ca^{2+} loading concentration. Formation of this band was related with the transglutaminase activation, as erythrocyte treatment with the transglutaminase inhibitor cystamine, prior to the Ca^{2+} loading, inhibited not only this enzyme but also the formation of the high molecular weight band. These results are in complete agreement with the earlier studies [13, 14].

Table 2. Effect of the Ca^{2+} loading time on the intensity of the human erythrocyte membrane Band 3 protein

Loading time (hr)	Percent peak area[a]		Band 3/Band 5
	Band 3	Band 5	
0	32.68 ± 2.02	4.46 ± 0.06	7.33
6	29.27 ± 3.99	4.97 ± 0.36	5.89
12	29.48 ± 2.45	6.02 ± 1.20	4.90
18	17.40 ± 0.52	7.81 ± 0.63	2.23

[a] Percent peak areas were taken from the densitometric scans of Coomassie blue-stained SDS/polyacrylamide gel electrophoretograms of red cell ghosts.
Values shown are means of peak areas taken from 3–5 scans ±SD. The extracellular Ca^{2+} concentration used for loading was 1.5 mM.

Table 3. Effect of the ATP enrichment on the Ca^{2+}-induced transglutaminase activation in human erythrocytes

Experiment number	Cells	Ca^{2+} concentration	^3H (dpm)
1	Normal	0	705 ± 61
		0.5	1261 ± 71
		1.5	2096 ± 160
	ATP-enriched	0	995 ± 66
		0.5	892 ± 84
		1.5	823 ± 28
2	Normal	0	1125 ± 61
		0.5	1572 ± 101
		1.5	2605 ± 164
	ATP-enriched	0	1003 ± 76
		0.5	838 ± 113
		1.5	720 ± 88
3	Normal	0	933 ± 45
		0.5	1992 ± 260
		1.5	2798 ± 170
	ATP-enriched	0	958 ± 213
		0.5	1123 ± 68
		1.5	985 ± 65

Transglutaminase was assayed using [^3H] putrescine, as given in Materials and Methods. Values shown are means of four determination ± SD.

Together with the polypeptides 2.1 and 4.1, the band 3 protein was also degraded in the Ca^{2+}-loaded cells. This degradation depended upon the duration of erythrocyte incubation with Ca^{2+} in the presence of ionophore A23187 and was maximum at 18 hr (Table 2). Unlike the normal erythrocytes, Ca^{2+} loading in the ATP-enriched cells did not structurally modify the membrane proteins (Fig. 1). This was quite consistent with our finding that even at 1.5 mM Ca^{2+} concentration, the transglutaminase could not be activated in the lysates of these cells (Table 3).

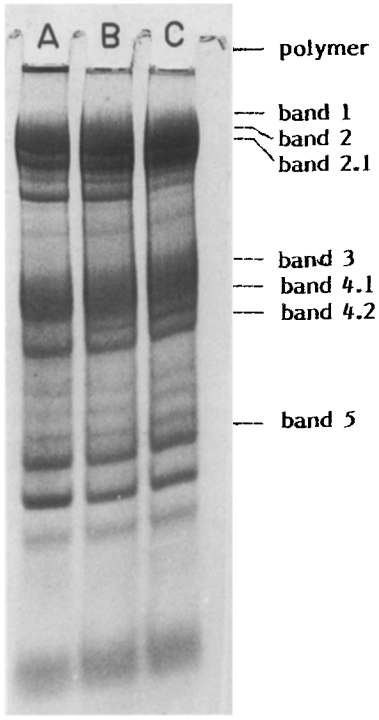

Fig. 1. Effect of ATP enrichment on the Ca^{2+}-induced membrane protein alterations in human erythrocytes. (*A*) Normal cells loaded with 1.5 mM Ca^{2+}. (*B*) ATP-enriched cells loaded with 1.5 mM Ca^{2+}. (*C*) ATP-enriched control cells

Calcium-Induced Effects on Anion Self-Exchange

Anion self-exchange activity in Ca^{2+}-loaded erythrocytes was ascertained by measuring both the influx and efflux of SO_4^{2-} across the membranes of these cells. Results shown in Table 4 indicate that the SO_4^{2-} self-exchange was decreased by 20–30% upon loading the cells with 25 μM Ca^{2+}. The extent of this decrease remained unaltered by increasing the Ca^{2+} loading concentration up to 0.5 mM, but a further decrease of 25–30% in the exchange was observed at 1.5 mM Ca^{2+} loading concentration. This additional decrease observed at 1.5 mM Ca^{2+} did not further increase by increasing the erythrocyte incubation time with Ca^{2+} in the presence of the ionophore (Table 5). It, however, disappeared by treating the cells with cystamine or by enriching them with ATP prior to the Ca^{2+} loading (Table 6).

Calcium-Induced Effects on Amino Acid Transport

L-alanine is transported stereospecifically by the Na^+-dependent ASC system at its physiological concentration [22]. The apparent K_m of this system

Table 4. Effect of enhanced intracellular free Ca^{2+} on the SO_4^{2-} self-exchange across the human erythrocyte membrane

Ca^{2+}-loading concentration (mM)	Cells	Influx		Efflux	
		$k(hr^{-1})$	I (%)	$k(hr^{-1})$	I (%)
0.025	Control	2.662 ± 0.272	22.6	1.795 ± 0.232	22.8
	Ca^{2+}-loaded	2.061 ± 0.333		1.385 ± 0.175	
0.25	Control	3.267 ± 0.252	27.2	2.142 ± 0.224	23.2
	Ca^{2+}-loaded	2.377 ± 0.226		1.644 ± 0.135	
0.50	Control	2.965 ± 0.186	18.2	2.482 ± 0.233	24.2
	Ca^{2+}-loaded	2.426 ± 0.113		1.881 ± 0.302	
1.5	Control	2.718 ± 0.561	56.8	1.777 ± 0.314	57.3
	Ca^{2+}-loaded	1.175 ± 0.079		0.759 ± 0.047	

Values shown are means of 3–5 determinations ±SD $k(hr^{-1})$, first order rate constant; I, inhibition. The Ca^{2+} loading was carried out for 3 hr.

Table 5. Effect of the Ca^{2+} loading time on the SO_4^{2-} self-exchange across the human erythrocyte membrane

Loading time (hr)	Cells	Influx		Efflux	
		$k(hr^{-1})$	I (%)	$k(hr^{-1})$	I (%)
6	Control	1.905 ± 0.236	52.9	1.275 ± 0.164	52.7
	Ca^{2+}-loaded	0.897 ± 0.096		0.603 ± 0.134	
12	Control	2.067 ± 0.238	53.4	1.530 ± 0.224	59.3
	Ca^{2+}-loaded	0.963 ± 0.177		0.623 ± 0.041	
18	Control	2.275 ± 0.260	64.6	1.539 ± 0.079	50.5
	Ca^{2+}-loaded	0.806 ± 0.087		0.761 ± 0.104	

The extracellular Ca^{2+} concentration used for loading was 1.5 mM. Values shown are means of 3–4 determinations ±SD $k(hr^{-1})$, first order rate constant; I, inhibition.

corresponds to the normal levels of L-alanine in the blood plasma. Figure 2 shows the effects of two different Ca^{2+} loading concentrations, 5 μM and 1.5 mM, on the L-alanine uptake at the physiological L-alanine concentration (0.2 mM). The L-alanine uptake was inhibited by 20–30% upon loading the cells with 5 μM Ca^{2+}. This inhibition was further increased (35–45%) by increasing the Ca^{2+} loading concentration to 1.5 mM, and remained unaffected even upon treating the cells with cystamine prior to the Ca^{2+} loading (Fig. 3).

To determine the specific effects of Ca^{2+} loading on the L-alanine uptake, we have measured also the concentration-dependent uptake of L-alanine in erythrocytes (Fig. 4), and calculated the various kinetic constants. Although the uptake remained unaffected by loading the cells with Ca^{2+}, the maximum velocity was reduced to about one-third (Table 7). Also, the apparent diffusion constant for the nonsaturable uptake was decreased by about 25%. From these results, it would appear that the efficiency of the ASC system to transport amino acids is decreased by increasing the intracellular Ca^{2+}. To further examine the validity of this conclusion, we have measured the uptake of another amino acid (L-cysteine) which is known to be transported in the human erythrocytes through the ASC

Table 6. Effect of the cystamine treatment and ATP enrichment, prior to Ca^{2+} loading (1.5 mM/3 hr), on the SO_4^{2-} self-exchange across the human erythrocyte membrane

Sample	Influx		Efflux	
	$k(hr^{-1})$	I (%)	$k(hr^{-1})$	I (%)
A	3.161 ± 0.233	30.0	2.505 ± 0.195	24.6
B	2.213 ± 0.166		1.889 ± 0.116	
C	5.401 ± 0.282	21.6	4.259 ± 0.366	33.2
D	4.233 ± 0.342		2.844 ± 0.238	

Values shown are means of 3–4 determinations ±SD $k(hr^{-1})$, first order rate constant; I, inhibition; A, cystamine-treated cells; B, cystamine-pretreated Ca^{2+}-loaded cells; C, ATP-enriched cells; D, ATP-enriched Ca^{2+}-loaded cells.

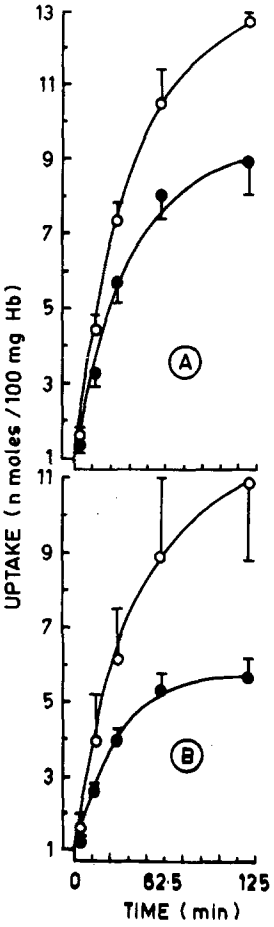

Fig. 2. Effect of Ca^{2+} loading on the L-alanine (0.2 mM) uptake in human red cells. (*A*) 5 μM Ca^{2+}-loaded cells. (*B*) 1.5 mM Ca^{2+}-loaded cells. Open circles, control cells; filled circles, Ca^{2+}-loaded cells. Values are means of four determinations ±SD

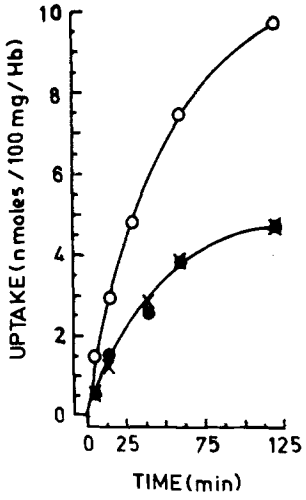

Fig. 3. L-alanine uptake in the erythrocytes that were loaded with 1.5 mM Ca^{2+} after the cystamine treatment. Open circles, control cells, filled circles, Ca^{2+}-loaded cells; crosses, cells loaded with Ca^{2+} after the cystamine treatment

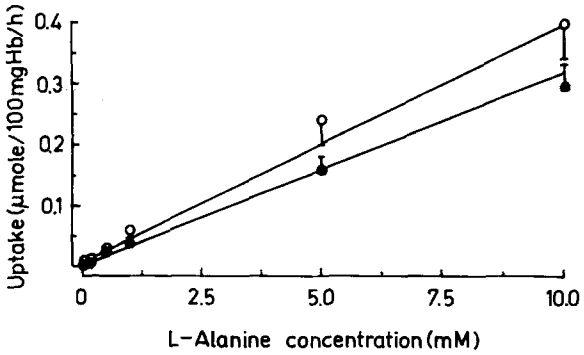

Fig. 4. Effect of Ca^{2+} loading on the concentration-dependent uptake of L-alanine in human red cells. Open circles, control cells; filled circles, 1.5 mM Ca^{2+}-loaded cells. Values are means of four determinations ±SD

Table 7. Kinetic constants for L-alanine and L-lysine uptakes in Ca^{2+}-loaded (1.5 mM/3 hr) human erythrocytes

Amino acid	Cells	K_m (mM)	V_{max} (nmol/100 mg Hb/hr)	K_d (nmol/100 mg Hb/hr/mM)
L-alanine	Control	0.26	30.4	36.8
	Ca^{2+}-loaded	0.25	10.5	27.0
L-lysine	Control	0.11	65.2	75.7
	Ca^{2+}-loaded	0.13	60.0	72.3

Uptake measurements and data analysis were performed as given in Materials and Methods.

system [22]. Figure 5 shows that the L-cysteine uptake was considerably reduced by loading the erythrocytes with 1.5 mM Ca^{2+}. A reduction of 44–55% in the uptake was observed at 20 μM external L-cysteine concentration, which is close to the K_m for uptake of this amino acid by the ASC system [22].

The effect of the increased levels of cellular Ca^{2+} on the amino acid transport through the Ly^+ system [22] was analyzed by measuring the time-dependent L-lysine uptake in 1.5 mM Ca^{2+}-loaded human erythrocytes. Figure 6 shows that the L-lysine uptake was not affected by loading the cells with Ca^{2+}. This is further confirmed by our measurements of the L-lysine uptake at the varying external concentrations (Fig. 7). Neither the saturable nor the nonsaturable uptake of L-lysine seems to be significantly influenced by the Ca^{2+} loading (Table 7).

Besides the Ca^{2+}-loaded cells, we have measured the L-alanine uptake also in the ATP-depleted cells as well as in the cells that were enriched with ATP prior to their loading with Ca^{2+}. The time-dependent (0–120 min) uptake of L-alanine in ATP-depleted cells was measured at 0.2 mM external

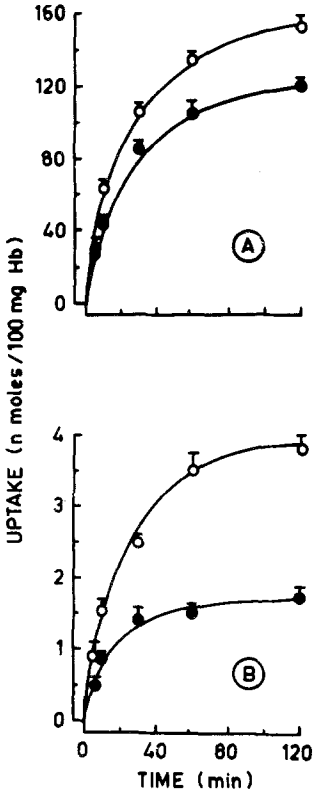

Fig. 5. Effect of Ca^{2+} loading on the L-cysteine uptake in human erythrocytes at 2.0 mM (*A*) and 20 μM (*B*) external amino acid concentrations. Open circles, control cells; filled circles, 1.5 mM Ca^{2+}-loaded cells. Values are means of four determinations ±SD

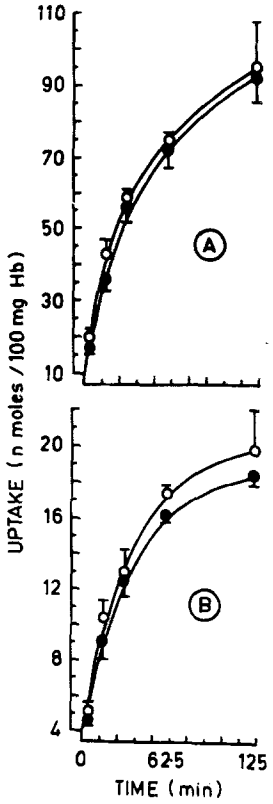

Fig. 6. Effect of Ca^{2+} loading on the L-lysine uptake in human erythrocytes at 0.2 mM (*A*) and 20 mM (*B*) external amino acid concentrations. Open circles, control cells; filled circles, 1.5 mM Ca^{2+}-loaded cells. Values are means of four determinations ±SD

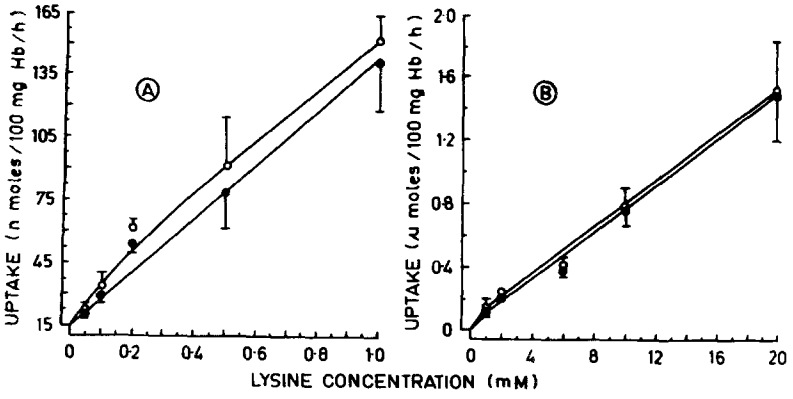

Fig. 7. Effect of Ca^{2+} loading on the concentration-dependent uptake of L-lysine in human erythrocytes. Open circles, control cells; filled circles, 1.5 mM Ca^{2+}-loaded cells. Values are means of four determinations ±SD

concentration. Results of these measurements revealed that the L-alanine uptake is inhibited by 20–35% by depleting the cellular ATP. However, this inhibition could not be reversed by ATP repletion at least up to 30%.

The L-alanine uptake in ATP-enriched erythrocytes, before and after 1.5 mM Ca^{2+} loading, was measured at 0.2 mM external concentration. Figure 8 shows that the L-alanine uptake in the ATP-enriched cells was 1.5–1.8 times greater than that observed above in the normal erythrocytes (*see* Fig. 2). However, this uptake, unlike the normal erythrocytes, was not reduced by loading the ATP-enriched cells with 1.5 mM Ca^{2+} (Fig. 8).

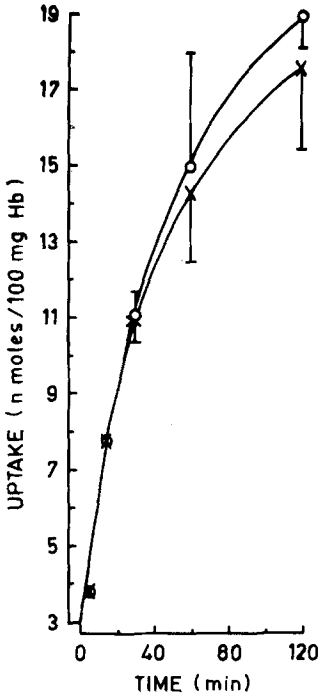

Fig. 8. L-alanine uptake in ATP-enriched 1.5 mM Ca^{2+}-loaded human erythrocytes. Open circles, control cells; crosses, Ca^{2+}-loaded cells. ATP-enriched cells incubated (3 hr, 37°C) with 5 mM EGTA and 10 μM ionophore in the absence of Ca^{2+} were used as controls. Values are means of three determinations ±SD

Discussion

This study shows that both the SO_4^{2-} self-exchange and L-alanine uptake are inhibited by increasing the intracellular Ca^{2+} concentration in human erythrocytes. These effects of Ca^{2+} are accompanied by changes in the structure of erythrocyte membrane proteins, which could be inhibited by treating the cells with the transglutaminase inhibitor cystamine or enriching them with ATP, prior to their loading with Ca^{2+}. However, unlike the L-alanine uptake, L-lysine uptake is not affected in Ca^{2+}-loaded erythrocytes, as compared to the normal cells.

Only 20–30% SO_4^{2-} self-exchange was inhibited by loading 25 μM to 0.5 mM Ca^{2+}, but this inhibition was almost doubled (50–60%) by increasing the Ca^{2+} loading concentration to 1.5 mM (Table 4), which is quite consistent with the earlier study which showed similar effects of Ca^{2+} on the Cl^- self-exchange [17]. As Ca^{2+} loading in erythrocytes with the help of ionophore A23187 besides leading to an increased concentration of the intracellular free Ca^{2+}, also leads to the structural modifications of membrane proteins as well as the ATP depletion, it may be visualized that the observed effects of Ca^{2+} on the SO_4^{2-} self-exchange or L-alanine uptake could have been mediated by one or more of these factors.

The increased inhibition of anion exchange at 1.5 mM Ca^{2+} was not directly due to the ATP depletion, since we observed only 20–30% inhibition in cystamine-pretreated 1.5 mM Ca^{2+}-loaded cells (Table 6). Also, the ATP-enriched Ca^{2+}-loaded cells had cellular ATP levels which were much higher than in the normal Ca^{2+}-loaded erythrocytes (Table 1), but even then the extent of anion exchange inhibition in these cells was comparable to that observed with the cystamine-pretreated Ca^{2+}-loaded cells (Table 6). It would therefore seem that the increased inhibition of anion exchange at 1.5 mM Ca^{2+} is caused by the Ca^{2+}-induced membrane protein alterations rather than the cellular ATP depletion.

Ca^{2+}-induced membrane protein modifications besides including transglutaminase-catalyzed membrane protein crosslinking also include membrane protein degradation by Ca^{2+}-dependent proteinases [1, 12–14]. However, the increased anion exchange inhibition observed at 1.5 mM Ca^{2+} cannot be attributed to the membrane protein degradation, as the inhibition (Table 5) did not vary with band 3 degradation in Ca^{2+}-loaded erythrocytes (Table 2). Since Ca^{2+}-dependent proteinases are of cytosolic origin and, therefore, should cleave only the cytoplasmic domain of the band 3 protein, our results are consistent with the earlier study which showed that the cytoplasmic portion of anion exchange protein is not required for its activity [9].

It appears that the increased inhibition of SO_4^{2-} self-exchange at 1.5 mM Ca^{2+} could primarily result from the increased transglutaminase-catalyzed membrane protein crosslinking at this Ca^{2+} concentration. This is quite evident from our findings that cystamine pretreatment, which inhibited both the Ca^{2+}-induced transglutaminase activation and membrane protein polymer formation (*data not shown*), also reduced the degree of anion exchange inhibition to 20–30% (Table 6), in contrast to 50–60% inhibition in untreated cells (Table 4). Further, the anion exchange activity in ATP-enriched Ca^{2+}-loaded cells was inhibited by only 20–30% (Table 6), which was consistent with the observed lack of Ca^{2+}-induced transglutaminase activation (Table 3) and membrane protein polymer formation in these cells (Fig. 1).

The anion exchange inhibition of 20–30% observed at low Ca^{2+} concentrations (25 μM to 0.5 mM) cannot be attributed to the Ca^{2+}-induced activation of erythrocyte transglutaminase, as this inhibition persisted even after pretreating the cells with

cystamine (Table 6). Also, these Ca^{2+} concentrations did not significantly activate transglutaminase in the erythrocyte lysates (*data not shown*). It may, therefore, be inferred that the SO_4^{2-} exchange inhibition caused by low Ca^{2+} levels either represents the direct effects of Ca^{2+} on the anion exchange [15] or could arise from the cellular ATP depletion [3].

Like the SO_4^{2-} self-exchange, L-alanine uptake was also decreased in the Ca^{2+}-loaded erythrocytes, as compared to the normal cells (Fig. 2). However, this decrease was not influenced by treating the cells with cystamine prior to the Ca^{2+} loading (Fig. 3), indicating that the observed inhibition of the amino acid uptake is not due to the Ca^{2+}-induced transglutaminase activation. To examine whether this inhibition is caused by the ATP depletion during Ca^{2+} loading, we measured the L-alanine uptake in ATP-depleted cells. As the L-alanine uptake in these cells was similar to that observed with the Ca^{2+}-loaded erythrocytes, we infer that this uptake in Ca^{2+}-loaded cells is inhibited perhaps due to the ATP depletion.

ATP depletion in erythrocytes besides leading to the decreased intracellular ATP pool, also results in the membrane protein rearrangement [11, 18, 19]. It may, therefore, be envisaged that the reduced activity of the ASC system in Ca^{2+}-loaded cells could result from the membrane protein rearrangement, rather than the decreased cellular ATP content. To analyze this problem, we measured the L-alanine uptake in erythrocytes that were enriched with ATP prior to the Ca^{2+} loading, for this treatment was shown to render the membrane proteins insensitive to the Ca^{2+}-induced structural modifications (Fig. 1, Table 3). As L-alanine uptake in these cells was similar to that in the control erythrocytes (Fig. 8), we conclude that membrane protein rearrangement is the main cause for the observed effects of Ca^{2+} on amino acid uptake through ASC system.

Transglutaminase-catalyzed membrane protein crosslinking involves several erythrocyte membrane proteins including band 3 protein [13, 14]. Also, depletion of the erythrocyte ATP is known to cause rearranagement of membrane proteins which also includes band 3 protein [11, 18, 19]. It is, therefore, tempting to speculate that the structural rearrangement of band 3 protein is perhaps responsible for the observed effects of Ca^{2+} on both the SO_4^{2-} self-exchange (at least partially) and L-alanine uptake in Ca^{2+}-loaded erythrocytes.

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