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METABOLIC DEPENDENCE OF THE FLUIDITY OF INTACT ERYTHROCYTE MEMBRANE

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SUMMARY: The motion of spin labeled phosphatidylcholine embedded in the lipid bilayer of erythrocyte membrane increased in association with metabolic ATP-depletion. The fluidity change was reversed by subsequent ATP-replenishment. However, levels of cellular 1,2-diacylglycerol, GSH or intracellular Ca^{2+} contributed little to the fluidity change. Temperature dependence of the fluidity indicated the disappearance of inflection points in ATP-depleted cells, thereby suggesting alterations in protein-lipid interactions. The fluidity change was also reproduced with oxidizing agents which cross-link spectrin. We suggest that the lipid phase state of membrane is maintained by protein-lipid interactions which depend on the metabolic state of the cells, particularly ATP levels.

The fluidity change of intact erythrocyte membrane due to metabolic depletion was demonstrated using spin labeled phosphatidylcholine (PC) by the incubation of erythrocytes without glucose. We looked for factors which may contribute to the fluidity change, including cellular ATP, GSH, intracellular Ca^{2+}, membrane lipid fractions and protein-lipid interactions.

Materials and Methods

Incubation procedure: Erythrocytes from freshly collected heparinized (5units/ml) human blood were washed three times with 10mM Tris buffered saline (TBS) (pH 7.4, 310mOsm) to eliminate the buffy coat. The cells were depleted of ATP by incubating washed erythrocytes in 10 vol of TBS at 37°C under gentle shaking. The ATP was replenished by incubating the depleted cells in 20mM phosphate buffered saline (PBS)(pH 7.4, 310mOsm) containing 10mM adenosine and 12mM glucose at 37°C [1]. Repletion of ATP was made feasible by incubating erythrocytes at 37°C for 20h in TBS containing 1mM adenosine and 12mM glucose from the initiation of incubation. Each incubating medium contained 100 units of penicillin/ml and 100 μg of streptomycin/ml. Incubation under anaerobic conditions was performed according to the method detailed by Palek et al. [2]. Hemolysis was less than 2% after each incubation, as determined by the method of Roelofsen and Zwall [3].

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Abbreviations: PC, phosphatidylcholine; TBS, Tris buffered saline; PBS, phosphate buffered saline; DAG, 1,2-diacylglycerol; ESR, electron spin resonance.

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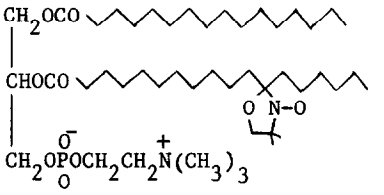
Spin labeling: Erythrocytes were periodically picked up from the incubating suspensions and washed three times with the same incubating medium and then spin labeled by incubating with PC label vesicles (made from each incubating medium, final concentration: 0.25mg/ml) according to the method of Tanaka and Ohnishi [4]. PC label, a gift from Professor S. Ohnishi, was synthesized by the method of Hubbell and McConnell [5]. The chemical formula of the PC spin label is shown above.

PC spin label



Depletion of intracellular Ca^{2+}: Ca^{2+}-depletion was carried out by incubating cells with 5 μM of A23187, a divalent cation ionophore, and 1mM EDTA at 37°C for 30 min, according to the method described by Ferrell and Huestis [6].

Membrane lipid analysis: Membrane lipids were extracted by the method of Rose and Oklander [7] and were chromatographed on a HPTLC plate (silica gel 60 precoated plate, Merck) using two different developing solvents. The one was benzene:ether:ethanol:acetic acid (50:40:2:0.2 by vol) for the separation of 1,2-diacylglycerol (DAG). The other was chloroform:methanol:acetic acid:water (25:15:4:1 by vol) for the phospholipid fractionations. Each lipid was detected by spraying of 10% H_2SO_4 or Dittmer reagent [8], measured by a dual wave length densitometer (Shimadzu, Kyoto, Japan) and quantitated by Chromatopack (Shimadzu, Kyoto, Japan)

Measurement of ATP and GSH: Levels of cellular ATP and GSH were measured using the method of Bücher [9] and that of Beutler et al. [10], respectively.

Results

As shown in Fig. 1-A, the spectra in ATP-depleted cells differed from that in ATP-repleted cells, especially in the lower-field peak (see heavy arrow). The overall splitting values in the ATP-depleted cells were smaller than those in ATP-repleted cells (49.7 gauss and 51.1 gauss, respectively). Since the upward protrusion in the lower-field peak (see heavy arrow) is considered to originate from the spin labels in the more fluid portions of the lipid bilayer, we introduced the ratios of signal intensities at the fixed positions, h_{-1} (g=2.016) to h_o (g=2.019), as the parameter of the membrane fluidity change (g values were measured from manganese peak). We also used the overall splitting values to detect the change of membrane fluidity. Increased membrane fluidity is associated with a decreased overall splitting and an increased peak height ratio value. Fig. 2 shows the time course of changes of ATP, GSH and two parameters of membrane fluidity during incubation. Both ATP and GSH levels decreased with time during incubation, as reported by other investigators [1,2]. The overall splitting values decreased and the peak height ratio values increased with time. The fluidity change was

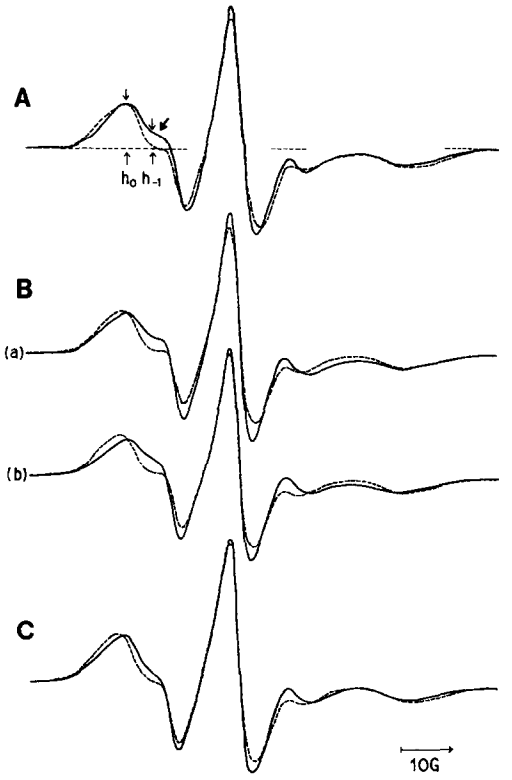


Fig. 1. ESR spectra of erythrocytes spin labeled with PC labels.
1-A ESR spectra of ATP-repleted and ATP-depleted erythrocytes. Fresh cells were incubated for 20h at 37°C with (------) or without (———) 1mM adenosine and 12mM glucose in TBS and then spin labeled. The spectra of ATP-repleted cells were identical to thsoe of fresh cells. Peak heights at the fixed positions (h_{-1} and h_o) were measured and peak height ratio (h_{-1}/h_o) values were used to monitor the change of membrane fluidity (see text). ESR spectra were obtained at 25°C.

1-B ESR spectra of erythrocytes treated with tetrathionate or diamide. Fresh cells were incubated with 20mM tetrathionate (a)(———) or 5mM diamide (b) (———) in medium (90mM KCl, 45mM NaCl, 10mM Na_2HPO_4/NaH_2PO_4, 30mM sucrose, pH 8.0) [11] for 1h at 37°C under gentle shaking. Control cells (------)(a)(b) were obtained by incubating fresh cells with 12mM glucose in the medium. These cells were washed three times with the medium (pH 7.4) containing 12mM glucose and then spin labeled with PC label vesicles made from the washing buffer. ESR spectra were obtained at 25°C.

1-C ESR spectra of ATP-repleted erythrocytes spin labeled before or after incubation. Fresh cells were incubated for 20h at 37°C in TBS containing 1mM adenosine and 12mM glucose. The one was spin labeled after incubation (------), the other was spin labeled before incubation (———). The ESR spectra of the latter resembled those of ATP-depleted cells spin labeled before or after incubation in TBS (Fig. 1-A). ESR spectra were obtained at 25°C.

then reversed by subsequent ATP-replenishment, by incubating cells with adenosine, glucose and phosphate for 4h. The presence of adenosine and glucose from the beginning of incubation completely prevented the change in cellular ATP and GSH and membrane fluidity up to at least 30h. Maintaining GSH by anaerobic

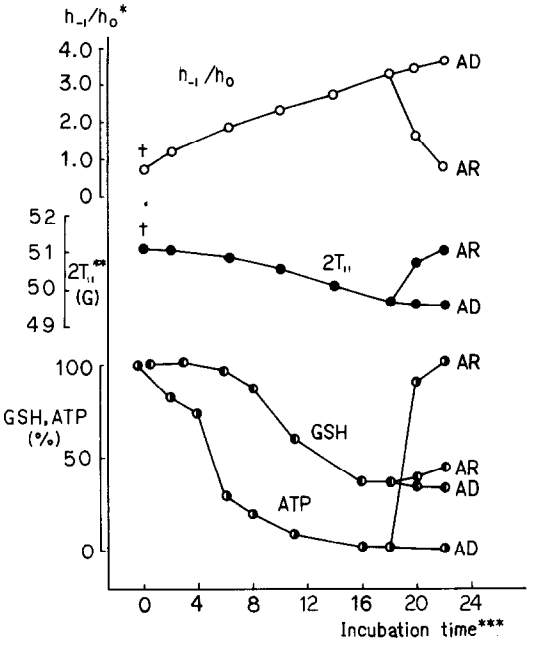


Fig. 2. Time course of changes in erythrocyte membrane fluidity, ATP and GSH accompanied by metabolic depletion. Fresh cells incubated in TBS at 37°C were periodically picked up and their membrane fluidity (peak height ratio* and overall splitting** values), cellular ATP and GSH were measured for up to 22h. After incubation for 18h, the cells were re-incubated for up to 4h under two different conditions. One was incubation in TBS (AD) and the other was incubation in PBS containing 10mM adenosine and 12mM glucose for ATP-replenishment (AR). ***Incubation time included 2h for spin labeling. †Fresh cells were spin labeled under the presence of 1mM adenosine and 12mM glucose. ESR spectra were obtained at 25°C.

ATP-depletion produced identical changes to those observed with aerobic incubation in which GSH was depleted (Table 1). The presence of EDTA in the medium did not prevent the fluidity change in ATP-depleted cells. A23187 plus EDTA, a treatment for depleting intracellular Ca^{2+}, did not reverse the fluidity change. There was an increase in cellular DAG levels along with ATP-depletion, as has been reported by others [12]. ATP-replenishment, which led to reversion of the fluidity change failed to decrease DAG levels, as reported by others [13](Table 2). Fig. 3 shows the temperature dependence of the overall splitting values in both ATP-depleted and ATP-repleted cells incubated for 20h. There were two inflection points (18°C and 33°C) in ATP-repleted cells, as noted by Tanaka and Ohnishi in fresh cells [4]; however, the inflection points were nor clearly visible in ATP-depleted cells. Table 3 and Fig. 1-B show that the increased fluidity was

Table 1.

Changes in erythrocyte ATP, GSH and membrane fluidity under different incubating conditions

Treatment of erythrocytes	ATP(%)	GSH(%)	$2T_{II}$	h_{-1}/h_o
Fresh*	100.0	100.0	51.1	0.00
ATP-repleted (incubated for 20h)	100.0	100.0	51.1	0.00
ATP-depleted (incubated for 20h)				
aerobic	5.6	37.9	49.7	0.32
anaerobic	6.0	82.2	49.7	0.30
with 1mM EDTA	5.1	30.0	49.0	0.32
ATP-replenished**	102.7	42.3	51.1	0.08
ATP-depleted/A23187+1mM EDTA***	0.0	2.2	48.6	0.46

*Spin labeled with PC label vesicles made from TBS containing 1mM adenosine and 12mM glucose. **ATP-depleted cells incubated for 18h were re-incubated in PBS containing 10mM adenosine and 12mM glucose for 4h (including 2h labeling time) at 37°C. ***ATP-depleted cells incubated for 18h were re-incubated in TBS containing 1mM EDTA and 5 μM A23187 for 1h and then spin labeled with PC label vesicles made from TBS. ESR spectra were obtained at 25°C. $2T_{II}$: overall splitting value. h_{-1}/h_o: peak height ratio value.

reproduced in fresh cells treated with tetrathionate or diamide. In one experiment in which erythrocytes were first spin labeled and then incubated for 20h, the spectra showed no difference between ATP-repleted and ATP-depleted cells and were identical with those of ATP-depleted cells spin labeled after incubation for 20h (Fig. 1-C and 1-A).

Table 2.

Membrane lipids composition of fresh, ATP-depleted and ATP-replenished erythrocytes

Samples (n=4)	C/PL (mol ratio)	PE (mol%)	PS (mol%)	PC (mol%)	SM (mol%)	lyso-PC (mol%)	DAG/C (density ratio)
Fresh	1.01±0.01	30.48±3.13	13.05±1.36	28.54±2.42	27.13±3.90	1.04±0.19	2.66±1.21
ATP-depleted*	1.01±0.03	31.32±1.60	14.70±0.99	27.56±2.75	25.59±1.77	0.88±0.16	5.88±2.81
ATP-replenished**	1.06±0.04	30.80±1.78	15.29±1.63	27.23±1.25	25.74±4.11	0.93±0.45	6.67±3.94

*Fresh cells were incubated for 20h at 37°C in TBS.
**ATP-depleted cells were re-incubated for 4h in PBS containing 10mM adenosine and 12mM glucose at 37°C. C/PL: free cholesterol to phospholipid mol ratio, PE: phosphatidylethanolamine, PS: phosphatidylserine, PC: phosphatidylcholine, SM: sphingomyelin, lyso-PC: lysophosphatidylcholine, DAG/C: 1,2-diacylglycerol to free cholesterol density ratio.

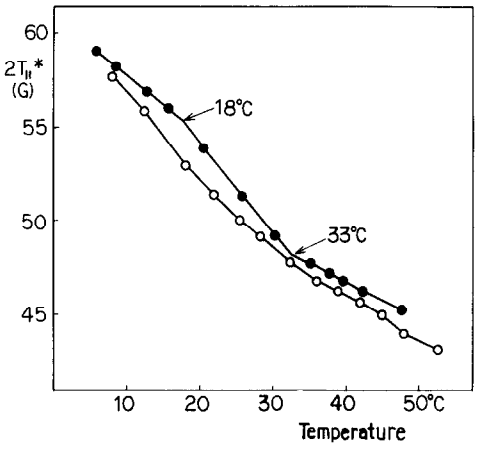


Fig. 3. Temperature dependence of the erythrocyte membrane fluidity. —o—o—o— ATP-depleted cells which were spin labeled after incubation in TBS for 20h at 37°C. —●—●—● ATP-repleted cells which were spin labeled after incubation in TBS containing 1mM adenosine and 12mM glucose for 20h at 37°C. The curve of the latter is identical to that of fresh cells. $2T_{II}$: overall splitting value.

Discussion

We found that erythrocyte membrane fluidity changed in association with metabolic ATP-depletion. The factors which affect the fluidity of biological membrane include lipid-lipid interaction, lipid-protein interaction and interactions of membrane with certain drugs and cations [4,14,15]. There is considerable evidence that ATP-depletion gives rise to increased cellular DAG [12], cross-linking spectrin to oligomers $>10^6$-dalton complexes [2] and accumulation of intracellular

Table 3.

Changes in erythrocyte membrane fluidity by cross-linking of spectrin with tetrathionate or diamide

Incubation	$2T_{II}$	h_{-1}/h_o
Medium* containing		
12mM glucose	51.1	0.00
none	50.4	0.29
20mM tetrathionate	49.4	0.47
5mM diamide	48.5	0.53

Fresh cells were incubated for 1h in medium* (90mM KCl, 45mM NaCl, 10mM Na_2HPO_4/NaH_2PO_4 and 30mM sucrose, pH 8.0) [11] with or without 20mM tetrathionate or 5mM diamide at 37°C under gentle shaking. These cells were washed three times with the medium (pH 7.4) containing 12mM glucose and then spin labeled with PC label vesicles made from the washing buffer. ESR spectra were obtained at 25°C. $2T_{II}$: overall splitting value. h_{-1}/h_o: peak height ratio value.

Ca^{2+} [1]. Increased levels of cellular DAG contribute little to the fluidity change in ATP-depleted cells, according to the result of ATP-replenishment; i.e. reversed membrane fluidity and non-reversed DAG levels [12]. Intracellular calcium is one of the important factors regulating membrane fluidity, since phase separation can be induced by packing of acidic phospholipids [15]. However, intracellular accumulation of Ca^{2+} also was not a principal factors cintributing to the increased fluidity caused by ATP-depletion, since intracellular depletion of Ca^{2+} by ionophore plus EDTA neither prevented nor restored the membrane fluidity change. Disappearance of inflection points in temperature dependence of the fluidity manifested structural changes in ATP-depleted cell membrane, and suggested alterations in protein-lipid interactions. Although the precise cause of inflection points is unclear, Bieri and Wallach [16] suggested from a paramagnetic quenching analysis the appearance of a new protein-lipid binding at 15°C and its disappearance above 40°C. Therefore, our results suggest that the alterations in protein-lipid interactions are associated with ATP-depletion and these may cause changes in the phase state of the membrane lipid bilayer. This idea is supported by the observations that treatment of cells with oxidizing agents, tetrathionate or diamide, which selectively cross-link spectrin at the concentrations used in this study [11], increased motion of the spin labeled PC. It is biologically important that erythrocyte membrane fluidity is controlled by a modification of membrane proteins which involves cross-linking and dephosphorylation of spectrin in ATP-depleted cells. In the intact erythrocyte membrane, the lipids distribute asymmetrically in double leaflets [3], thereby inducing heterogeneity in the fluidity; a more rigid outer layer and a more fluid inner layer [4]. Spin labeled PC was shown to be incorporated into the outer layer and to be more rigid than spin labeled phosphatidyl-ethanolamine and -serine embedded in the inner layer of intact erythrocytes [4]. The increased fluidity in pre-spin labeled ATP-repleted cells (incubated for 20h) is of interest, since the increased motion of the label (Fig. 1-C) may be due to its re-orientation into the more fluid inner layer by "flip-flop" which has been shown to take about 10h in ATP-repleted erythrocytes [17]. In ATP-depleted cells, there is the possibility that spin labeled PC was incorpo-

rated symmetrically by facilitated "flip-flop" during 2h labeling and as a result there was an increase in its motion. Another and more interesting possibility is the disruption of lipid asymmetry due to ATP-depletion [18], a state whereby induced homogenization in the fluidity increases the motion of the PC label in ATP-depleted cells. There is evidence that lipid asymmetry and heterogeneity of the fluidity are maintained by spectrin-lipid interactions [4,10]. Williamson et al. [19] reported the involvement of spectrin in the maintenance of erythrocyte lipid phase state asymmetry by means of uptake of merocyanine 540 and fluorescence depolarization studies.

The lipid phase-state (fluidity) of the erythrocyte membrane seems to be maintained by protein-lipid interactions which depend on the metabolic state of the cells, particularly cellular ATP levels.

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