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CALCIUM AND IONOPHORE A23187 INDUCE THE SICKLE CELL MEMBRANE PHOSPHORYLATION PATTERN IN NORMAL ERYTHROCYTES

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Pre-treatment of normal erythrocytes with micromolar Ca^{2+} and ionophore A23187 induces abnormal phosphorylation of membrane polypeptides, as determined by labeling with exogenous $^{32}P_i$. The Ca^{2+}-induced effects, which include increased incorporation of ^{32}P into acid-stable linkages and increased labeling in the Band 3 and 4.5–4.9 regions of SDS gels, are similar to those seen in untreated sickle erythrocytes. Part of the abnormal phosphorylation of sickle cells may be caused by their elevated intracellular Ca^{2+} levels.

Introduction

The phosphorylation of membrane polypeptides in intact sickle cells is abnormal, in that the total amount of phosphorylation and the distribution of ^{32}P from exogenous $^{32}P_i$ is altered [1]. There are a number of abnormalities in sickle erythrocytes that are secondary to the polymerization of hemoglobin S, including membrane permeability changes, altered intracellular cation concentrations and lipid peroxidation. We have examined the effect of some of these parameters on membrane phosphorylation, and wish to report that some of the altered ^{32}P labeling can be caused by exposure of the intracellular contents to elevated Ca^{2+} levels.

Methods

The general procedure for intact cell phosphorylation has been described [1]. Cells from normal donors were filtered through cellulose to remove white cells [2], and washed in 118 mM NaCl/5 mM KCl/2 mM $MgCl_2$/35 mM Tris-HCl/20 μg/ml PMSF/1 mM EGTA/10 mM glucose (pH 7.4). They were resuspended at a hematocrit of 40 and 1 mM Na $^{32}P_i$ (approx. 0.5 Ci/mmol) was added. During incubation at 30°C, 1.0 ml of the cell suspension was removed and the cells were washed three times briefly in 10 ml ice-cold phosphorylation buffer to remove external residual [^{32}P]P_i. The sedimented cells were hemolysed in 1.5 ml of ice-cold buffer comprising 5 mM Tris-HCl, 1 mM EDTA/7 mM NaCl/1 mM PMSF (pH 7.4). The hemolysate was centrifuged at 4°C and 1.0 ml clear supernatant solution was removed for ATP determination. Ghosts were washed several times in ice-cold hemolysis buffer until white. The polypeptides were separated on 4–12% gradient acrylamide gels in SDS using the buffers of Fairbanks et al. [3]. The gels were stained with Coomassie blue, and ^{32}P located by autoradiography or by Cerenkov counting of gel slices. Autoradiograms were quantitated using a Zieneh integrating densitometer.

Ca^{2+} loading

Washed cells at hematocrit 20 were exposed to 4 μM ionophore A23187 (Calbiochem) and varying amounts of Ca^{2+} for 1 h at 30°C in 5 mM

sodium phosphate/75 mM KCl/75 mM NaCl/10 mM glucose (pH 7.4) which was passed through Chelex resin (Biorad) to remove Ca^{2+}. This buffer prevents volume loss through the Ca^{2+}-activated K^+ efflux [4]. They were then washed three times in 10 vol. phosphorylation buffer to remove external Ca^{2+} and ionophore A23187. Ca^{2+} was determined by atomic absorbance on protein-free extracts of EDTA washed cells [4] prepared with redistilled trichloroacetic acid [5].

ATP depletion

Cells were passed through cellulose and washed in 5 mM sodium phosphate/140 mM NaCl/1 mM EDTA (pH 7.4). They were resuspended to 20% hematocrit and then incubated at 37°C in the same buffer. At different times, aliquots were removed, washed in phosphorylation buffer and then labeled as described.

Determination of ATP concentration and specific activity

An aliquot of the red cell suspension (4°C) was made 4% in perchloric acid with ice-cold 71% perchloric acid. The precipitated protein was removed by centrifugation. A measured volume of the protein-free extract, usually 200 μl, had 1 μl of 0.5% Methyl orange indicator added and sufficient 3 M K_2CO_3 to neutralize the perchloric acid. The white precipitate of sodium perchlorate was removed by centrifugation and 20 μl of supernatant was injected into a Whatman Partisil 10-SAX column on a Varian high-pressure liquid chromatograph in 0.45 M KH_2PO_4, pH 3.60. ATP emerged in about 6 min. The concentration of ATP was determined by automatic peak integration, and radioactivity by collecting the entire ATP peak for Cerenkov counting. A calibration curve of the amount of ATP (nmol) versus peak integration units was linear in the range 1–50 nmol ATP.

Results

In attempts to determine the cause of altered ^{32}P-labeling of membrane proteins in sickle erythrocytes, normal red cells were subjected to a number of procedures which duplicate some of the secondary membrane abnormalities of the sickle cell. These included: lipid peroxidation, performed as described by Chiu and Lubin [6] and cellular dehydration [7] by incubation in hypertonic NaCl. Neither of these procedures modified intact cell

TABLE I

EFFECT OF Ca^{2+}-A23187 PRETREATMENT ON $^{32}P_i$ INCORPORATION

Normal erythrocytes were exposed to 4 μM ionophore A23187 and the indicated amounts of Ca^{2+} for 1 h at 37°C in 5 mM sodium phosphate (pH 7.4)/75 mM KCl/75 mM NaCl/10 mM glucose. They were then washed three times in phosphorylation buffer, and incubated with sodium [^{32}P]phosphate for 4 h. The isolated membranes were separated on SDS gels, autoradiographed, and the film density determined in arbitrary units on a Zieneh scanner. The areas chosen for integration are shown in Fig. 1. Equal amounts of radioactivity were electrophoresed for each sample. The average and standard deviation of the relative polypeptide phosphorylation for normal and sickle erythrocytes after 4 h of incubation are shown for comparison. Units for ATP concentration are μmol/ml of packed cells. Specific activities (SA) are given as percent of control, since the values were dependent on the amount of ^{32}P added. For the Ca^{2+} experiments, $n = 5$ except for the ATP determination, where $n = 4$.

$[Ca^{2+}]$ (μM)	cpm/ghosts (percent of control)	Band 3 cpm/ Band 2 cpm	Band 4.5–4.9 cpm/ Band 2 cpm	ATP before phosphorylation	After phosphorylation	
					ATP	SA (ATP)
0	100	0.58±0.11	0.39±0.14	1.40±0.14	1.41±0.27	100
1	313±25	0.74±0.08	0.53±0.15	1.26±0.13	1.43±0.29	120±40
5	129±19	0.84±0.09	0.66±0.21	1.20±0.05	1.45±0.32	123±38
10	129±27	0.94±0.21	0.67±0.21	0.94±0.05	1.37±0.29	111±28
15	156±14	0.89±0.10	0.61±0.16	0.71±0.06	1.08±0.30	158±43
50	124±20	0.96±0.20	0.69±0.22	0.59±0.09	0.95±0.09	98±53
AA rbc ($n = 8$)		0.57±0.13	0.35±0.11			
SS rbc ($n = 4$)		0.95±0.05	0.57±0.10			

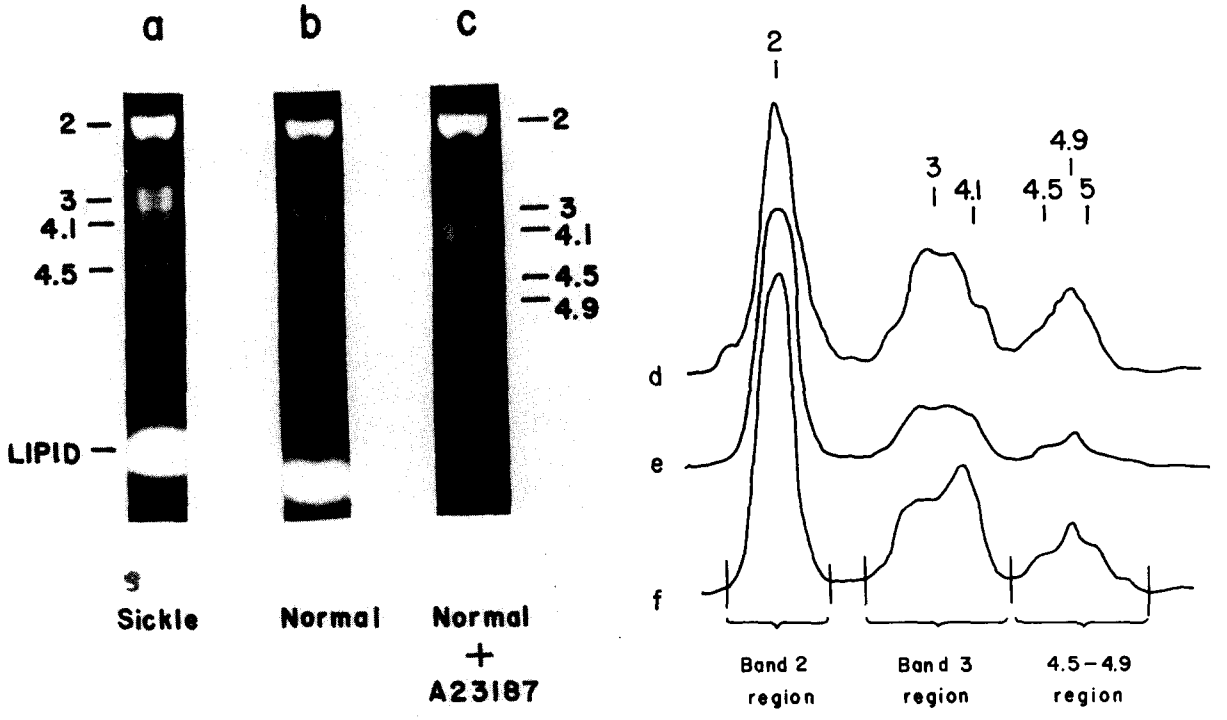

Fig. 1. Autoradiograms of membrane polypeptides labeled with ^{32}P during a 4 h incubation of intact cells with $^{32}P_i$. (a–c) autoradiograms of (a) sickle cells; (b) normal cells; (c) normal cells pretreated with 4 μM ionophore A23187 and 5 μM Ca^{2+} for 1 h at 37°C. (d–f) densitometric scans of the autoradiograms: (d) sickle cells; (e) normal cells; (f) Ca^{2+}-treated normal cells. The areas that were integrated to obtain the ratio given in Table I are indicated.

phosphorylation. However, when normal cells were treated with Ca^{2+} and ionophore A23187 for 1 h before phosphorylation to mimic the elevated intracellular Ca^{2+} of sickle cells [8], the subsequent ^{32}P labeling resembled that found in sickle erythrocytes. In particular, the cpm/ghost was elevated, and there was a relative increase in the percentage of radioactive label in the region of Bands 3 and 4.5–4.9 (Table I). The peak areas that were integrated are indicated in Fig. 1f. The criterion of relative labeling in these regions of the gel has been used earlier by Hosey and Tao [9] to characterize ^{32}P distribution in washed membranes incubated with [γ-^{32}P]ATP. The appearance of a sickle cell-like pattern of phosphorylation in normal cells was also apparent in autoradiograms (Fig. 1).

Ca^{2+} concentration

Immediately after a 1 h incubation with Ca^{2+} and 4 μM ionophore A23187, the cells were washed in phosphorylation buffer to remove the ionophore and medium Ca^{2+}. An aliquot was washed in Chelex-treated saline and assayed for Ca^{2+}. The intracellular Ca^{2+} levels were consistently found to be normal (5–10 nmol/ml packed red cells). This result was consistent with earlier findings [4,9] that erythrocytes can extrude Ca^{2+} via the Ca^{2+}-ATPase as rapidly as Ca^{2+} enters, as long as the ionophore concentration used is sufficiently low. In these experiments, the ionophore/cell ratio was 20 μmol/l packed cells, well below the ratio needed to raise steady-state Ca^{2+} concentrations [4].

ATP depletion

Although no cellular accumulation of Ca^{2+} occurred in these erythrocytes, there was loss of ATP during the Ca^{2+}-A23187 treatment (Table I, column 5). This decline in ATP was largely restored

TABLE II

EFFECT OF ATP DEPLETION ON $^{32}P_i$ INCORPORATION

Washed red cells (hematocrit 20) were incubated in 5 mM sodium phosphate (pH 7.4)/140 mM NaCl/1 mM EDTA at 37°C. At the indicated times, an aliquot was removed for ATP determinations. At the same time, 2.0 ml were removed, washed three times in phosphorylation buffer, resuspended in final volume of 2 ml, and incubated a further 4 h at 37°C with 4 μmol of sodium [^{32}P]phosphate. Membranes were then prepared and electrophoresed as described. The cpm in membranes and in gel slices is shown. The data are means of two experiments.

Time (h)	[ATP] (mmol/l)	SA [ATP] (cpm/nmol)	cpm/10^8 ghosts				Radioactivity ratio of bands:	
			membrane	Band 2	Band 3	Band 4.5–4.9	3/2	4/2
0	1.41	4.33	12700	427	245	200	0.57	0.47
3	1.32	4.52	9300	–	–	–	–	–
6	1.29	–	–	–	–	–	–	–
9	0.99	3.62	11800	423	187	127	0.44	0.30
15	0.61	5.93	10300	–	–	–	–	–
18	0.55	6.61	9400	500	190	148	0.38	0.30

during the $^{32}P_i$ labeling incubation (Table I, column 6). In order to rule out the possible involvement of ATP concentration per se on membrane phosphorylation, the effect of ATP depletion brought about by cell starvation was assessed (Table II). In these experiments, ATP declined to one-third of the normal level during the incubation without glucose. Nevertheless, during the subsequent labeling period with $^{32}P_i$, the specific activity of ATP was similar in all samples, i.e., the rate of labeling of the ATP pool is independent of the ATP pool size. Moreover, the extent of ^{32}P incorporation into the membrane was unaffected by the intracellular concentration of ATP (column 3) and the labeling of individual bands sliced from the SDS-electrophoretograms was also unchanged. These results suggest that the altered phosphorylation seen in Ca^{2+}-A23187-treated erythrocytes is not a consequence of ATP depletion.

Discussion

The functional abnormalities in the sickle cell secondary to Hb S polymerization include abnormal membrane permeability to cations even when the cells are oxygenated [11], and the generation of a class of deformed and rigid erythrocytes called irreversibly sickled cells. The relation between these functional defects and the known biochemical abnormalities such as the presence of oxidized lipids [6], elevated intracellular Ca [8], or membrane-bound Hb S is not at present completely understood. We have noted an abnormality in the ^{32}P-labeling of sickle cell membrane polypeptides [1], and the present work indicated that much of this abnormal phosphorylation could be reproduced in normal cells by pre-treatment with Ca and ionophore A23187. Incubation of intact cells with $^{32}P_i$ measures both turnover of esterified phosphate and net phosphorylation. The relative contribution of these processes to the total ^{32}P incorporation cannot be assessed, since both kinases and phosphatases are active in the intact erythrocyte. Measurement of net phosphorylation could be done by isolation and analysis of individual polypeptides. This has been done in limited cases [12,13] but is technically formidable. Therefore, the relative increase in ^{32}P in the Band 3 and Band 4.5 regions seen on both sickle cells and Ca^{2+}-treated normal cells may be a manifestation of increased turnover or net labeling of these polypeptides. It should also be pointed out that these regions contain more than one polypeptide, and identity of the labeled species has not been established.

In related work, an effect of intracellular Ca^{2+} on erythrocyte membrane phosphorylation was reported by Nelson and Huestis [14]. The experimental procedures are not directly comparable to ours, in that Nelson and Huestis used red blood

cells depleted of intracellular ATP, and Ca^{2+}-A23187 and $^{32}P_i$ were present simultaneously rather than sequentially. Nelson and Huestis [14] reported a 20–40% increase in spectrin phosphorylation in the presence of A23187 and Ca^{2+}. The autoradiograms of Nelson and Huestis also show a marked increase in ^{32}P incorporation into the Band 3 and Band 4.5 regions in the presence of Ca^{2+}, although the investigators did not discuss this finding. Their results are therefore not inconsistent with those reported here. Recently, Nelson and Huestis have also demonstrated a calmodulin-dependent kinase in erythrocytes [15], suggesting a mechanism for intracellular Ca^{2+} effects. It is of some interest that the Ca^{2+}-mediated effects on the membrane did not require elevated Ca^{2+} levels during the labeling period. At no time did the steady-state intracellular Ca^{2+} rise significantly above normal levels, as determined by atomic absorption. Nevertheless, Ca^{2+} did gain access to the inner surface of the membrane, since the Gardos effect [16] was activated, as evidenced by marked K^+ loss that occurred when erythrocytes were incubated in K^+-free media (data not shown). This result implies that exposing the inner surface of the erythrocyte membrane to low levels of Ca^{2+} can result in alterations that persist even after Ca^{2+} has been removed by the Ca^{2+}-ATPase. This may be relevant to the question of membrane damage in sickle erythrocytes, since there is a transient increase in Ca^{2+} permeability during a sickling episode [8]. Damage sustained during this period might persist even after reoxygenation and removal of Ca^{2+}, beginning the process of membrane deterioration.

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