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Alteration in protein kinase C activity and subcellular distribution in sickle erythrocytes

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In agreement with previous data, membrane protein phosphorylation was found to be altered in intact sickle cells (SS) relative to intact normal erythrocytes (AA). Similar changes were observed in their isolated membranes. The involvement of protein kinase C (PKC) in this process was investigated. The membrane PKC content in SS cells, measured by [^{3}H]phorbol ester binding, was about 6-times higher than in AA cells. In addition, the activity of the enzyme, measured by histone phosphorylation was also found to be increased in SS cell membranes but decreased in their cytosol compared to the activity in AA cell membranes and cytosol. The increase in membrane PKC activity was observed mostly in the light fraction of SS cells, fractionated by density gradient, whereas the decrease in cytosolic activity was only observed in the dense fraction. PKC activity, measured in cells from the blood of reticulocyte-rich patients, exhibited an increase in both membranes and cytosol, thus explaining some of the effects observed in the SS cell light fraction, which is enriched in reticulocytes. The increase in PKC activity in the membranes of SS cells is partly explained by their young age but the loss of PKC activity in their cytosol, particularly in that of the dense fraction, seems to be specific to SS erythrocytes. The relative decrease in membrane PKC activity between the dense and the light fractions of SS cells might be related to oxidative inactivation of the enzyme.

Introduction

In sickle cells the phosphorylation of membrane proteins is abnormal [1–4]. Spectrin phosphorylation is reduced, whereas phosphorylation in band 3 and in the region of bands 4.5–4.9 is increased. Although an increase in the activities of cAMP-dependent and -independent protein kinases has been demonstrated in these abnormal cells, this alteration has been ascribed to their young age [5]. PKC, which is present in human erythrocytes [6–8], is another candidate potentially responsible for the high level of protein phosphorylation in sickle cells [9]. This phospholipid-dependent enzyme is physiologically activated by Ca^{2+} and diacylglycerol [10,11]. Tumor-promoting phorbol esters, such as PMA, bind to PKC, causing its redistribution from cytosol to the membrane, and activate it by virtue of their structural analogy to diacylglycerol [12,13].

In the present study, evidence is provided that an increase in the activity and content of membrane PKC is the cause of the increase in membrane protein phosphorylation of SS cells. This alteration may be attributed to the presence of reticulocytes. In addition, a decrease in cytosolic PKC activity, characteristic of the dense fraction, may be ascribed to a specific abnormality in sickle cells.

Abbreviations: ISC, irreversibly sickled cells; AA cell, intact normal erythrocyte; SS cell, intact sickle cell; PMA, 4β-phorbol 12β-myristate 13α-acetate; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C, calcium-activated and phospholipid-dependent protein kinase; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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Materials and Methods

[γ-^{32}P]ATP and [20(n)-^{3}H]PDBu were obtained from Amersham (France), [^{32}P]P_i from International CIS (Division France). PDBu, PMA, phosphatidylserine, diolein, histone type III-S, Tris, Hepes, EGTA and ATP were all purchased from Sigma. All other chemicals were of analytical grade.

Blood collection. Blood samples were obtained from patients with homozygous hemoglobin S and from patients with comparable degrees of reticulocytosis due to hemorrhage or hereditary spherocytosis without splenectomy. Two classes of patient were excluded: (1) patients who were treated by transfusion during their crisis or during the preceding 3 months, and (2) patients with Sβ-thalassemia. Normal controls were healthy adult volunteers. Blood was collected by venipuncture using heparin as anticoagulant and centrifuged ($1000 \times g$, 10 min) at 4°C. White cells and platelets were removed and the cells were washed three times in buffer A (154 mM NaCl/5 mM KCl/10 mM Hepes/10 mM glucose/1 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4)). Cells were resuspended in buffer A, stored at 4°C and used within 2 days.

Cell fractionation in density gradients. Fresh red cells were washed and resuspended (hematocrit 30%) in buffered saline (BSKG) containing 5 mM KCl, 10 mM glucose, 10 mM sodium phosphate buffer (pH 7.4) and sufficient NaCl to increase the osmolarity to 291 mosM. These preparations were fractionated by the method of Corash et al. [14] using discontinuous Stractan density gradients [15]. Stractan solutions (densities at 20°C from 1.060 to 1.150) were layered on ultra-clear centrifuge tubes. 3 ml of blood suspension were layered on top of the gradient. The tubes were centrifuged at 4°C, in a L8-55 Beckman ultracentrifuge (SW 41 rotor, $110000 \times g$, 1 h). The top fraction from the gradient (density 1.060–1.068), which contained white cells and part of reticulocytes, was discarded. The erythrocyte bands obtained after centrifugation were washed twice in BSKG and once in buffer A. The red cells were stored as described above.

Intact cell phosphorylation. After a preincubation at 37°C in buffer A with 2 mM adenine and 10 mM inosine, the cells were incubated for 2 h in the presence of $[^{32}P]P_i$ (28 MBq/ml of cells) to label intracellular ATP pools. When required, PMA, dissolved in Me_2SO, was added at the beginning of the second hour of incubation. After the cells had been washed twice with 154 mM NaCl/1.5 mM Hepes/1 mM EGTA (pH 7.4), they were hemolyzed at 4°C in 10 mM Tris-HCl/1 mM EDTA (pH 7.4). Membranes were washed twice by centrifugation ($30000 \times g$, 10 min) at 4°C and were prepared for electrophoresis by solubilization with SDS buffer.

Assay of PKC activity. Membranes were prepared by hypotonic hemolysis of packed cells in a lysis buffer containing 10 mM Tris-HCl, 0.1 mM EDTA and 0.01 mM PMSF (pH 7.4) [6]. After centrifugation at 30000 $\times g$ for 10 min, the supernatant was removed; the membranes were washed twice in hemolysis buffer and resuspended to a final concentration of 2–3 mg of protein/ml of lysis buffer. Cytosol samples were prepared by hypotonic hemolysis of cells in 20 vols of ice-cold 5 mM sodium phosphate (pH 7.0)/0.1 mM EGTA [7]. The supernatants were diluted by a 5-fold addition of the above buffer to a protein concentration of 3–3.5 mg/ml.

Protein kinase C activity was measured by phosphorylation of histone as described by Kikkawa et al. [16]. Cytosol fractions were incubated in a reaction mixture (final vol 100 μl) containing (final concentrations) cytosol (100–150 μg of protein), 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 200 μg/ml histone (Sigma type III-S), 10 μM [γ-^{32}P]ATP (spec. act. 400–700 cpm/pmol) and either 50–100 μg/ml phosphatidylserine, 12–25 μg/ml diolein and 1 mM $CaCl_2$ or 1 mM EGTA. Diolein and phosphatidylserine in chloroform/methanol (2 : 1, v/v) were dried under a stream of N_2, suspended in H_2O by vortexing and sonicated for 15 min at 4°C. The reaction initiated by the addition of ATP was performed at 30°C for 7 min (linear conditions). The reaction was stopped by transferring 35 μl aliquots onto Whatman ET 31 paper squares (2×2 cm) followed by several immersions in 25% trichloroacetic acid baths [17]. Histone-bound ^{32}P was counted by liquid scintillation. Phosphorylation of membranes (100–150 μg of protein) was performed in a 100 μl mixture comprising 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 3 mM $MgCl_2$, 27 μM [γ-^{32}P]ATP (spec. act. 300–400 cpm/pmol) with or without 200 μg/ml histone. The incubation was done at 30°C and the reaction was initiated by the addition of MgATP and stopped after 5 min (linear conditions) by addition of SDS buffer. Histone phosphorylation was assessed after electrophoresis as described.

Binding studies with [^{3}H]PDBu. Cells were preincubated for 15 min at 37°C in buffer A with or without 500 nM PDBu, and washed twice in buffer A. Then membranes were prepared as described. The binding of [^{3}H]PDBu to PKC was measured by the method of Wolf et al. [18]. The reaction mixture (200 μl) contained 10 mM Tris-HCl (pH 7.4), 1 mM DTT, 3 mM $MgCl_2$, 30 μ /ml BSA, 0.5 mM $CaCl_2$, 50 nM of [^{3}H]PDBu (740 GBq/mmol) and 100–150 μg of membrane proteins, without or with 25 μM of unlabelled PDBu (non-specific binding). The reaction mixture was incubated at 24°C for 45 min. Bound [^{3}H]PDBu was separated from free ligand by filtering through Whatman GF/C glass fibre filter and washed twice with 10 mM Tris-HCl buffer (pH 7.4). The radioactivity bound to the filters was determined by liquid scintillation.

Gel electrophoresis. The polypeptide constituents of the membranes were separated by SDS-7.5% polyacrylamide gel electrophoresis [19]. The total polypeptide content of the membranes was demonstrated by staining the gels with Coomassie brilliant blue R250 [20]. The dried gels were analyzed by autoradiography and the radioactivity of appropriate bands was measured by Cerenkov counting [21].

Other procedures. The protein concentration was

measured by the method of Lowry et al. [22] using BSA as standard. Morphologic evaluation of the cells was done under a phase-contrast microscope after fixation in glutaraldehyde solution. Cells whose length was greater than twice their width and which possessed one or more pointed extremities under oxygenated conditions were considered to be ISC. The percentage of ISC in the different patients was 20.7 ± 4.8 ($n = 12$). Reticulocytes were counted in stained smears of washed cells.

Results

Phosphorylation in intact cells and in isolated membranes

Incorporation of ^{32}P into membrane proteins was first measured in unfractionated AA and SS cells. An increase in phosphorylation of band 3, protein 4.1 and protein 4.9 was observed in SS cells together with a decrease in phosphorylation of spectrin + ankyrin (Table I). Protein phosphorylation was also measured in isolated membranes in the presence of [γ-^{32}P]ATP. ^{32}P incorporation was found to be increased in band 3, protein 4.1 and protein 4.9 of the SS cell membranes compared with AA cell membranes (Table I). Therefore, the endogenous protein phosphorylation remains more elevated even in isolated membranes from SS cells, suggesting the presence of an additional membrane-bound protein kinase activity.

AA and SS cells were fractionated according to their density on Stractan gradients. The light fractions (A_1 and S_1) comprised those cells of densities between 1.068 and 1.078 and contained about 5–7 and 60% of reticulocytes, respectively, for A_1 and S_1. The dense fraction A_2 ($d = 1.082$–1.096) contained the oldest AA cells; S_2 ($d = 1.082$–1.096) was discarded. S_3 ($d > 1.105$) contained about 70% of ISC. After fractionation, cell membranes were prepared and phosphorylated (Table I). In both AA and SS cell membranes, band 3 and protein 4.1 and 4.9 phosphorylation was significantly higher in the light fractions (A_1 and S_1) than in the dense fractions (A_2 and S_3). This difference was not seen in the case of spectrin + ankyrin.

PKC content and activity in membranes from fresh or phorbol ester-pretreated cells

PKC content and activity were measured in membranes by [^{3}H]PDBu binding or histone phosphorylation, respectively. As shown in Table II, the binding of the [^{3}H]phorbol ester in SS membranes was 6-times higher than that in AA membranes. Histone phosphorylation was also found to be increased in SS cell membranes (Table III). However, with this second method, which measures the enzymatic activity, the difference between AA and SS membranes, although statistically significant, was smaller than that obtained

TABLE I

Phosphorylation of membrane proteins in intact cells (A), in isolated membranes from unfractionated cells (B) or in isolated membranes from fractionated cells (C)

(A) Cells were incubated for 2 h in the presence of [^{32}P]P_i. Cells were washed and membranes were prepared by hemolysis. Membrane proteins were separated by SDS-PAGE electrophoresis and the radioactivity in each protein was measured by Cerenkov counting of the corresponding portion of the gels. Results are means ± S.E. of three to five independent experiments carried out in duplicates. (B) Membranes were incubated in the presence of [γ-^{32}P]ATP for 5 min. Protein radioactivity was measured as described in (A). Results are means ± S.E. of eight independent experiments carried out in duplicates. (C) A_t and S_t: unfractionated cells; A_1 and S_1: $d = 1.068$–1.078; A_2: $d = 1.082$–1.096; S_3: $d > 1.105$. AA and SS cells were fractionated by centrifugation on Stractan gradients. Membranes were prepared by hemolysis and incubated with [γ-^{32}P]ATP for 5 min. Protein phosphorylation was measured as described in (A). Results are means ± S.E. of five independent experiments carried out in duplicate.

Cells	Spectrin + ankyrin	Band 3	Band 4.1	Band 4.9
A: units, ^{32}P cpm/μg protein				
AA	7.1 ± 1.1	4.3 ± 0.8	1.4 ± 0.2	1.3 ± 0.2
SS	4.9 ± 0.6 [a]	6.1 ± 0.6 [a]	2.5 ± 0.4 [a]	2.7 ± 0.2 [a]
B: units, pmol P_i/mg protein per 5 min				
AA	58.4 ± 4.6	51.7 ± 5.5	8.0 ± 1.0	11.2 ± 1.7
SS	55.5 ± 4.8	72.9 ± 7.9 [a]	20.3 ± 3.0 [a]	30.7 ± 3.3 [a]
C: units, %				
A_t	100	100	100	100
A_1	114.0 ± 14.0	121.2 ± 10.5	127.2 ± 8.1	117.0 ± 5.6
A_2	95.3 ± 18.7	85.0 ± 6.8 [b]	97.0 ± 16.0 [b]	90.5 ± 9.7 [b]
S_t	100	100	100	100
S_1	85.3 ± 4.4	98.2 ± 6.1	114.8 ± 11.1	140.0 ± 18.9
S_3	95.5 ± 18.4	66.0 ± 6.3 [c]	64.0 ± 6.6 [c]	63.0 ± 6.8 [c]

[a] Significantly different from control ($P < 0.02$).
[b] Significantly different from A_1 ($P < 0.05$).
[c] Significantly different from S_1 ($P < 0.01$).

TABLE II

Binding of [^{3}H]PDBu to AA and SS membranes

AA and SS cells were preincubated either in control medium or in the presence of 500 nM PDBu. Membranes were prepared by hemolysis and were incubated with 50 nM [^{3}H]PDBu. Nonspecific binding was estimated in the presence of 25 μM PDBu. Results are means ± S.E. of three independent experiments or of two experiments.

Cells	fmol PDBu bound/mg protein	
	control	PDBu-pretreated
AA	42 ± 13	254 ± 15
SS	264 ± 95	527

with the ^{3}H-binding method. This may indicate that only a fraction of the membrane-associated PKC was activated and exhibited phosphotransferase activity. Interestingly, histone phosphorylation, measured on membranes from density-fractionated cells, exhibited striking differences according to the fractions (Table III). In both types of cell, this activity was higher in the membranes from the light fraction (A_1 and S_1) than in the membranes from the corresponding dense fraction (A_2 and S_2). Compared to the corresponding AA cell membranes, histone phosphorylation was increased in both dense and light SS fractions but this increase was much

TABLE III

Distribution of PKC activity in unfractionated and density-fractionated AA and SS cells

A_1 and S_1: $d = 1.068–1.078$; A_2: $d = 1.082–1.096$; S_2: $d > 1.105$. Cells were fractionated by centrifugation on Stractan gradients. Fraction S_1 contained about 40–60% reticulocytes and fraction S_2 about 50–70% ISC. Cytosol and membranes were prepared by hemolysis and PKC activity was measured from histone phosphorylation. Both membrane and cytosol PKC activities are expressed in a common unit (pmol P_i/min per ml of cells). This was calculated from the original data expressed in pmol P_i/min per mg of membrane or cytosolic protein using the following conversion factors: 1 mg of membrane protein per 189 μl of cells and 1 mg of cytosol protein per 3.55 μl of cells [6]. Cytosolic protein is mostly hemoglobin but is generally used as a reference value for the expression of cytosolic PKC activity [6,7]. Values are means ± S.E. of n experiments (number in parenthesis).

	pmol P_i/min per ml cell	
	AA	SS
Membrane		
Unfractionated	34.1 ± 4.1 (9)	47.2 ± 5.0 (9) [a]
Light (A_1, S_1)	33.7 ± 3.5 (5)	52.7 ± 5.2 (6) [a]
Dense (A_2, S_2)	21.4 ± 1.9 (5) [b]	29.1 ± 3.6 (6) [a,b]
Cytosol		
Unfractionated	303.7 ± 30.9 (10)	219.6 ± 28.1 (10) [a]
Light (A_1, S_1)	303.7 ± 33.7 (7)	303.7 ± 39.3 (6)
Dense (A_2, S_2)	236.5 ± 39.4 (7)	146.6 ± 25.4 (6) [a,b]

[a] Significantly different from the corresponding AA fraction ($P < 0.05$).

[b] Significantly different from the corresponding A_1 or S_1 fraction ($P < 0.05$).

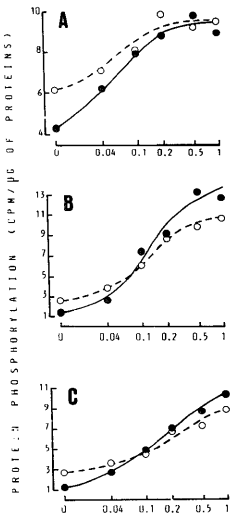

Fig. 1. Effect of PMA on membrane protein phosphorylation in AA (●) and SS cells (○). After 1 h preincubation of the cells with [^{32}P]P_i, PMA (in Me_2SO) or Me_2SO alone (control) was added for a further 1 h incubation. Cells were washed, hemolysed and membrane protein phosphorylation was measured after SDS-PAGE electrophoresis. Values are means of two experiments or means ± S.E. of three or four experiments carried out in duplicate. (A) band 3; (B) protein 4.1; (C) protein 4.9.

pronounced in the light fractions. Membrane PKC content and activity were also measured after pretreatment with a phorbol ester known to induce the translocation and the activation of PKC from the cytosol to the membrane [6–8]. [^{3}H]PDBu binding to membranes was increased after a preincubation of the cells with (unlabelled) PDBu (Table II). This pretreatment induced a similar increase in the binding of the [^{3}H]phorbol ester in both types of cell (212 and 263 fmol/mg of protein). Membrane protein phosphorylation in intact cells was measured after pretreatment with different doses of PMA. An increase in ^{32}P incorporation into band 3, proteins 4.1 and 4.9 was observed in the presence of PMA in both AA and SS cells (Fig. 1). However, the stimulation of phosphorylation induced by PMA in the three proteins was less in SS cells than in AA cells.

TABLE IV

Protein phosphorylation in isolated membranes from unfractionated and density-fractionated AA cells from normal donor and reticulocyte-rich patients

A_t and R_t = unfractionated cells from AA and reticulocyte-rich blood, respectively; A_1 and R_1 = light fraction ($d = 1.068–1.078$): A_2 and R_2: dense fraction ($d = 1.082–1.096$). Protein phosphorylation was measured in isolated membranes as described in the legend to Table I. Results are the means of two separate experiments carried out with the blood from two normal donors and two reticulocyte-rich patients (containing 10.5 and 12% reticulocytes). One patient who was reticulocyte-rich had hereditary spherocytosis (HS). A defect in the phosphorylation of spectrin in HS erythrocytes has been reported in some studies but not in others (see Ref. 23). Absolute values of phosphorylation in cells from this patient were increased 3–4-fold in protein 3, 4.1 and 4.9 and 1.5-fold in spectrin + ankyrin when compared to AA cells from a normal donor. The same increases in membrane protein phosphorylation were found with the other reticulocyte-rich patient and were thus attributed to the high reticulocyte count.

	pmol P_i/mg protein per 4 min			
	Spectrin + ankyrin	band 3	band 4.1	band 4.9
A_t	33.6	62.5	12.4	18.4
A_1	43.1	94.2	15.4	21.5
A_2	41.8	60.1	9.4	15.3
R_t	45.9	256.3	40.9	50.9
R_1	44.2	469.4	65.9	78.8
R_2	31.5	192.2	33.1	40.2

TABLE V

Distribution of PKC activity in unfractionated and density-fractionated AA cells from normal donors and reticulocyte-rich patients

Results are the means of two separate experiments carried out with the cells from two normal donors and from two reticulocyte-rich patients (the same as those used in the experiments of Table IV) unfractionated or density-fractionated (A_1, A_2, R_1 and R_2). PKC activity was measured as described in the legend of Table III. The absolute values of membrane and cytosolic PKC activity in AA cells were markedly lower than those in the experiments shown in Table III. This may be due to some day-to-day variations (difference in the concentration of phosphatidylserine and diolein or in the specific radioactivity of [γ-^{32}P]ATP). The comparison between AA normal and AA reticulocyte-rich cells remained valid since both were measured on the same day.

	pmol P_i/min per ml cell	
	normal	reticulocyte-rich
Membrane		
Unfractionated	26.0	33.0
Light (A_1, R_1)	27.5	50.0
Dense (A_2, R_2)	13.3	22.9
Cytosol		
Unfractionated	163.4	222.5
Light (A_1, R_1)	194.4	281.7
Dense (A_2, R_2)	160.6	112.7

These results indicate that although the amount of PKC translocated was of similar magnitude, the enzyme was less active in SS than in AA cell membranes.

PKC activity in cytosol

Cytosolic PKC activity, determined by histone phosphorylation, was found to be decreased by about 30% in unfractionated SS cells when compared to AA cells (Table III). This decrease was not observed for the light fraction of SS cells (S_1) when compared to the same fraction of AA cells (A_1). It was observed only when one compared the dense fraction of SS cells (S_3) to the dense fraction of AA cells (A_2). Histone phosphorylation was slightly decreased in the dense fraction from AA cells (A_2) compared to the light one (A_1), but there was a 2-fold decrease between the dense fraction of SS cells (S_3) and the light one (S_1). In three experiments (data not shown), cytosolic PKC activity measured in four fractions of SS cell of increasing densities (1.068–1.078, 1.078–1.096, 1.096–1.115, 1.115–1.178) was found to be progressively diminished (1.11, 0.89, 0.44 and 0.37 pmol P_i/mg protein per min).

PKC activity in reticulocyte-rich AA subjects

Because blood from sickle-cell anemic patients contains a high percentage of reticulocytes (7–28% in this study, instead of 0.5–1% in normal donors), we have investigated whether the changes in membrane protein phosphorylation and in PKC activity found in SS cells could be ascribed to the presence of these young cells. These parameters were measured in red cells from two reticulocyte-rich AA subjects (containing 10.5 and 12% of reticulocytes, respectively). Phosphorylation of membrane proteins was increased 3–4-fold (band 3, proteins 4.1 and 4.9 but not spectrin) (Table IV). This increase was greater in the light fraction (R_1, $d = 1.068–1.078$) particularly enriched in reticulocytes (about 50% in this fraction) (Table IV). Membrane and cytosolic PKC activity was also especially increased in R_1 when compared to A_1, whereas in R_2 an increase in membrane PKC activity and a small decrease in cytosolic activity were observed when compared to A_2 (Table V).

Discussion

An increase in [^{3}H]PDBu binding to SS cell membranes compared with the binding in AA cell membranes has been reported recently [9]. We have confirmed this data by using this method as well as by measuring PKC activity by histone phosphorylation. The former method gives an estimation of the amount of enzyme and the latter measures the phosphotransferase activity. This second method was also applied to determine cytosolic PKC activity.

The values of PKC activity of the membrane and of the cytosol of AA cells (6.45 ± 0.78 and 1.08 ± 0.11

pmol P_i/min per mg protein, respectively, see the legend to Table III) were about twice as high as those reported by Palfrey and Waseem [6] (2.79 and 0.579 pmol P_i/min per mg protein). This difference may be caused by the use of histone III-S as a substrate in our work instead of histone H_1 in theirs. Indeed, Cohen and Foley [7] have obtained a cytosolic PKC activity of 2.5 ± 0.2 pmol P_i/min per mg protein using histone III-S. In agreement with previous studies [24,25], Ca^{2+}, diolein and phosphatidylserine were required for full activation of PKC using histone III-S as a substrate, but only when the enzyme activity was measured in the cytosol [6]. The membrane components are likely to fulfill the role of the lipid cofactors and the enzyme must be in a Ca^{2+}-insensitive form as already suggested [6].

In unfractionated cells, the PKC activity of SS membranes was higher than that of AA membranes but PKC activity of SS cytosol was lower than that of AA cytosol (Table III). However, in SS membranes compared to AA membranes, the amount of enzyme increased more (Table II) than its activity (Table III). Pretreatment with a phorbol ester induced the translocation of about the same amount of enzyme in both AA and SS cells (Table II) but a lower stimulation of the phosphotransferase activity (Fig. 1). This could indicate that the enzyme in SS membranes is in a partially inactivated state perhaps due to an effect of the lipid environment or to oxidative inactivation as discussed below.

From the data obtained in density-fractionated SS cells it may be concluded that the high level of membrane PKC activity found in the total SS cell population is a consequence of their high content in reticulocytes. Firstly, in S_1 ($d = 1.068–1.078$, 40–60% of reticulocytes) membrane PKC activity was higher than in A_1 ($d = 1.068–1.078$, 5–7% of reticulocytes) and also higher than in S_3 ($d > 1.105$, almost devoid of reticulocytes). Secondly, in cells from reticulocyte-rich patients (10–12% of reticulocytes), this activity was higher than in normal cells (0.5–1% of reticulocytes) and this increase was particularly obvious in R_1 ($d = 1.068–1.078$, 50% of reticulocytes). Fairbanks et al. [5] have also reported elevated protein kinase activities (cAMP-dependent and -independent) in SS cells and in cells from other patients with reticulocytosis. Other enzyme activities (glucose-6-phosphate dehydrogenase and pyruvate kinase) are known to be age-dependent in normal erythrocytes and to correlate with the reticulocyte percentage [14].

In contrast, it is clear that the decrease in cytosolic PKC activity in SS cells was specific to these cells and not related to their high content of reticulocytes (since in these latter there was an increase in the cytosolic PKC activity). Only in S_1, where the percentage of reticulocytes is high, the cytosolic PKC activity was not changed relatively to that in A_1. In S_3, a fraction almost devoid of reticulocytes and rich in ISC, this decrease was particularly obvious. In conclusion, in unfractionated SS cells, membrane PKC activity will be increased and cytosolic activity decreased to different extents depending on the percentage of reticulocytes and ISC.

SS cells are known to have a high calcium content and a stimulation of calcium uptake upon deoxygenation and sickling [26,27]. However, the cytosolic concentration of ionized calcium ($[Ca^{2+}]_i$) is not different from that in AA cells [28], because this extra calcium is sequestrated into endocytic inside-out vesicles [29,30]. Activation of the Ca^{2+}-dependent translocation of PKC from the cytosol to the membrane seemed a straightforward explanation of the high level of membrane-associated PKC in SS cells. Such an explanation has been proposed by Ramachandran et al. [9]. However, the membrane PKC activity in the S_3 fraction enriched in ISC, which have the highest calcium content [26,27], was lower than that in S_1. From these data, it seems unlikely that the high calcium content of SS cells plays any role in the alteration of PKC distribution observed in SS cells.

In SS cells, the antioxidant defences are impaired and they generate spontaneously twice the normal amounts of activated oxygen species [31]. Their membranes contain endogenous products of lipid peroxidation [32,33] and exhibit abnormal intramolecular modification of protein thiols [34]. Oxidative damage correlates with the proportion of ISC [33]. The PKC activity in intact cells, particularly the membrane-bound form, is highly susceptible to oxidative inactivation upon exposure to hydroxyl radicals [35]. Oxidative inactivation of membrane-associated PKC could constitute a potential mechanism to account for the loss of PKC activity in the membranes of the densest SS cells and also of the densest AA cells (A_2), which were reported to exhibit significant peroxidative damage to membrane lipids when compared to the lightest AA cells [36]. However, this process does not account for the high level of membrane PKC in SS cells when compared to AA cells.

The increase in membrane PKC activity is likely to be responsible for the increase in membrane protein phosphorylation observed before [1–5] and confirmed in this study. Firstly, the membrane proteins which showed an enhanced phosphorylation were those described as the substrates of PKC when this latter was activated by a phorbol ester such as PMA in AA cells [6–8]. These are proteins 4.1 and 4.9 and two peptides of 97 and 103 kDa comigrating with band 3. These two peptides, recently identified as an α/β heterodimer, were calmodulin-binding proteins and were named adducin [37,38]. Secondly, there was a correlation between the extent to which protein phosphorylation was enhanced in a membrane fraction and the increase in PKC activity in the same membrane fraction (compare

S_1 and S_2 or A_1 and R_1). The abnormal oxidative status of protein 4.1 in SS cells [39] and perhaps of other proteins, substrates of PKC, may be also a factor contributing to the alteration of their phosphorylation. As we have reported previously, a simulation of the phosphorylation of adducin, protein 4.1 or 4.9, induced by PKC activation, results in an increased phosphorylation of polyphosphoinositides in AA cells [40]. This increase in protein phosphorylation observed in SS cells could also explain the increased phosphorylation of polyphosphoinositides that we have observed in SS cells [41].

One possible consequence of the abnormal amount of membrane-associated PKC is an activation of the Ca^{2+} pump as recently reported [42]. Such an activation could be beneficial to ensure a rapid extrusion of the excess cytosolic Ca^{2+} during the $[Ca^{2+}]_i$ transients experienced by SS cells during deoxygenation.

Of interest is the observation that several of the changes encountered in SS cells are not unique for this hemolytic disorder. Evidence for alteration in cytoskeleton, membrane lipid peroxidation, increased total cell calcium and elevated protein kinase activities have been described in thalassemic erythrocytes and some are found in unstable hemoglobinopathies, such as hemoglobin Köln [43]. Some of these changes have been ascribed to the young age of the cell population, while others were clearly associated to the presence of unstable hemoglobin molecules. This seems also to apply in SS cells for membrane and cytosolic PKC activities, respectively.

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