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INCREASED MEMBRANE-ASSOCIATED PHORBOL-12,13 DIBUTYRATE (PDBu) RECEPTOR FUNCTION IN SICKLE RED CELLS

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Summary: A four-fold increase in the binding of ^{3}H-PDBu by red cell membrane ghosts isolated from sickle red cells compared to that from normal controls is presented. Phosphorylation studies with γ-^{32}P-ATP indicate a similar (two to three-fold) increase in the radiolabelling of the acid-precipitable membrane proteins in sickle red cells. When red cells were loaded with Ca^{2+} using Ionophore A23187, both normal and sickle red cells enhanced their phosphorylation and sickle red cells to a greater extent than normal red cells. Polyacrylamide slab gel electrophoretic separation of the phosphoproteins and autoradiography also reveal phosphorylation, predominantly of protein bands 3, 4.1 and 4.9 which are known in the red cells as specific substrates for the PDBu receptor, protein kinase C. These results indicate that membrane association of protein kinase C in sickle red cells is increased, possibly as a consequence of the pathological change in their ability to accumulate intracellular calcium.

The biological receptor for the tumor-promoting phorbol esters like PMA or PDBu has been identified as the Ca^{2+}-dependent phospholipid sensitive enzyme protein kinase C (1,2). Phorbol esters bind to this enzyme in a one to one ratio causing a redistribution of the enzyme from cytosol to the membrane and activate it by virtue of their structural analogy to DG, the physiological activator of the enzyme, produced transiently in the cell membranes during the turnover of phosphoinositides (3-6). Phorbol esters have therefore been used often to demonstrate the presence of protein kinase C in various cell types and recently in red blood cells (7,8). Raising intracellular Ca^{2+} levels by treatment of intact red cells with Ca^{2+} and Ionophore A23187 which in turn

Abbreviations: DG, diacylglycerol; PS, phosphoatidylserine; ^{3}H-PDBu, phorbol-12,13 dibutyrate; PMA, phorbol 12-myristate-13-acetate; PBS, 1 volume 0.1 M phosphate buffer, pH 7.4 and 9 volumes of 0.85% sodium chloride; PMSF, phenyl methyl sulfonyl fluoride; Normal membranes, membranes isolated from normal red cells; SS membranes, membranes isolated from sickle red cells; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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brings about enzyme translocation and protein phosphorylation, has also been employed to demonstrate the presence of protein kinase C in red cells (8). However, such studies in the red cell may not reflect any variation in the extent of endogenous membrane association of protein kinase C consequent to changes in intracellular Ca^{2+} levels in pathological states. In sickle cell anemia, red cells display markedly increased permeability to Ca^{2+} leading to abnormal accumulation of $[Ca^{2+}]_i$ often to as high as eight times the mean normal levels of 25 μmoles per liter packed cells (9,10). In addition, Ca^{2+}-induced changes in vitro in the metabolism of membrane phosphoinositides as well as their increased breakdown in vivo have also been noticed by earlier workers (11,12). Since protein kinase C is dependent on cellular Ca^{2+} and membrane phospholipids for its translocation to the membrane and eventual activation, we examined if the red cell distribution of protein kinase C is altered in this disease state. In this report, we present evidence that such a translocation of cytoplasmic protein kinase C to the membrane is indeed occurring in sickle red cells.

MATERIALS AND METHODS

Preparation of red cells and isolation of membranes: Fresh blood specimens collected in EDTA tubes were centrifuged in the cold, white cells and platelets removed thoroughly and red cells washed three times in PBS and packed. Membranes were prepared according to Dodge et al. (13) by hypotonic lysis of the packed cells in a 20-fold excess of Tris-HCl buffer 10 mM, pH 7.5 containing 1 mM EDTA and 0.1 mM PMSF and centrifugation at 18000 x g for 30 min. The membranes were washed five times in Tris buffer, frozen in methanol-dry ice mixture, thawed and a uniform suspension made by several passes through the fine end of a Pasteur pipette. Protein concentration was estimated (14) and adjusted to 2 mg/ml using bovine serum albumin as the standard and used immediately.

Binding studies with ^3H PDBu: The procedure of Wolf et al. (15) was followed with some modifications. The assay mixture (200 μl) contained 10 mM Tris-HCl buffer, pH 7.5, 1 mM dithiothreitol, 200 μM Ca^{2+}, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 0.1 uCi ^3H PDBu (15.8 Ci/mmole, New England Nuclear) with unlabelled PDBu to range concentrations from 20-200 nM and 100 μg membrane protein. After incubation at 30°C for 10 min, the contents were diluted 20-fold, membrane-bound PDBu separated from the unbound by filtration through Whatman GF/C glass filters, washed thoroughly and radioactivity counted in a Beckman LS 230 liquid scintillation counter. Radioactivity that remained bound to the membrane incubated for 10 min in the presence of 20 ug ml^{-1} unlabelled PDBu was taken to be non-specific binding (16) and all values were corrected accordingly.

In vitro phosphorylation of erythrocyte ghosts: Phosphorylation was studied using ^{32}P-ATP in the presence and in the absence of both PS and DG under condi-

tions employed for assay of protein kinase C (17). The increment in ^{32}P incorporation brought about by the lipids was taken to be a measure of protein kinase C-mediated phosphorylation. The reaction mixture contained 10 mM Tris-HCl, pH 7.5; 5 mM magnesium chloride; 0.2 mM ATP containing 1 uCi ^{32}P-ATP ($2.5-3.5 \times 10^4$ cpm/ mole); 0.01 mM calcium chloride, 7.5 µg PS; 1 ug diolein and 100 ug membrane protein in a final volume of 200 µl. Membranes were preincubated with the sonicated lipids for 5 min at 30°C, reaction started by the addition of ATP, continued for 4 min and stopped by plunging the tubes into methanol-dry ice followed by addition of ice-cold TCA. Proteins were collected by filtration using millipore filters (0.45 µm) previously soaked in a solution containing 5 mM sodium phosphate and 1 mM ATP, washed with 5 x 3 ml volumes of cold 5% TCA, dried for 30 min at 80°C and radioactivity counted.

SDS-PAGE analysis of phosphorylated polypeptides: Both normal and SS membranes were phosphorylated for 10 min as described and the reaction arrested by addition of 200 ul of SDS sample buffer and left at room temperature for 30 min. Solubilized membrane proteins (25 ug/lane) were separated on 10% polyacrylamide slab gels and stained with 0.1% Coomassie blue R-250 according to Laemmli (17) and the different bands identified according to Steck (18). For autoradiography, the Coomassie blue-stained gels were dried under partial vacuum at 80°C on Whatman No. 3 MM filter paper, exposed to Kodak X-Omat AR-5 film for 72 hrs with a Dupont cronex intensifying screen and photographed.

Ca^{2+}-loading of intact red cells: Red cells from fresh blood collected in EDTA tubes were washed in PBS and resuspended in the same medium (hematocrit 25%) and incubated in the presence and in the absence of both Ionophore A23187 (5 µM) and Ca^{++} (1 mM) for 30 min at 37°C. At the end of incubation, the ionophore was removed by centrifugation and cells were quickly washed once with cold PBS and membranes prepared and PK-C activity in the isolated membranes were estimated as described.

RESULTS

Results of phorbol ester binding studies are shown in Fig. 1. In both normal and SS membranes, the binding reaction increased in direct proportion with the increase in the concentration of PDBu in the medium and reached a plateau at 150 nM PDBu concentration. At this concentration of PDBu SS membranes bound about four times more ^3H-PDBu than normal red cell membranes indicating increased membrane association of protein kinase C in sickle cells.

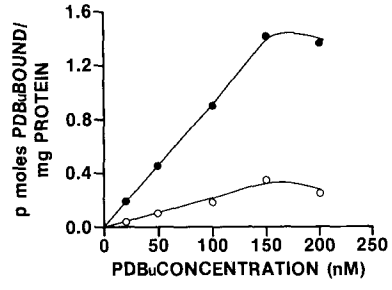

Fig. 1. Binding of ^3H-PDBu to red cell membrane ghosts. Experimental details are given in the text. Normal, o—o; SS, ●—●. Results presented here are mean of duplicate assays of two each of normal and SS samples.

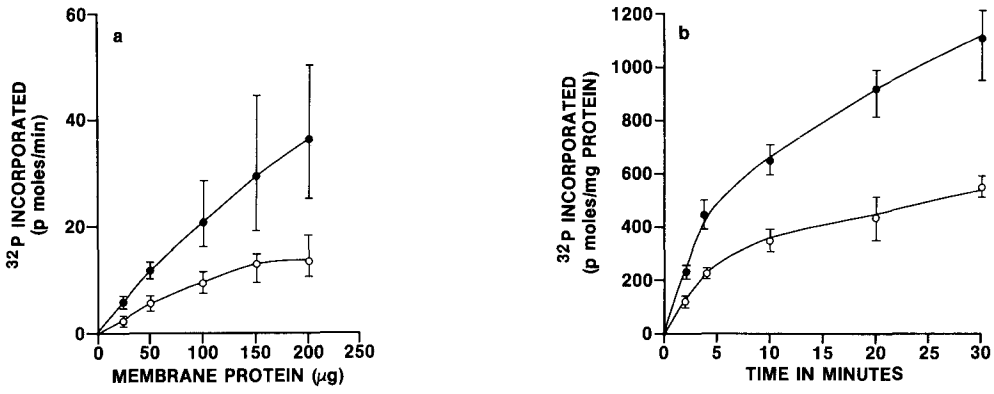

Fig. 2. Effect of membrane protein concentration (1a) and incubation time (1b) on red cell membrane 'protein kinase C' activity. Experimental details as described in the text. Normal membranes, o——o; SS membranes, ●——●.

Fig. 2 gives the extent of protein kinase C-mediated membrane protein phosphorylation in normal and SS cases using different concentrations of membrane proteins and reaction time. Under the conditions of assay, the reaction showed linear rates of activity up to 5 min and 150 μg membrane protein. The enhanced radiolabelling of sickle red cell membranes was distinctly seen at every protein concentration tested (Fig. 2a) or incubation time (Fig. 2b). For routine assays conducted in duplicate 100 μg membrane protein and a reaction time of 4 min were chosen. Expressed as pmoles Pi incorporated per mg membrane protein per minute, the 'protein kinase C

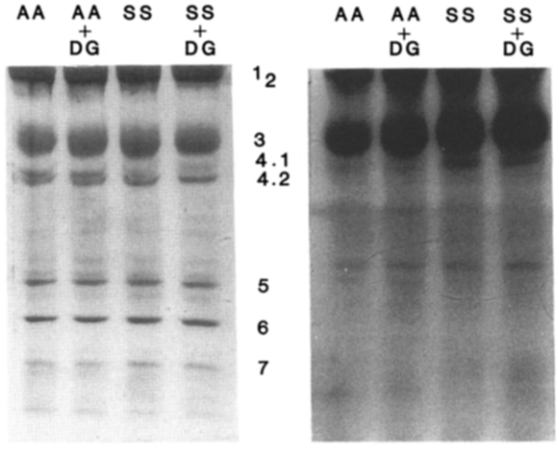

Fig. 3. Separation of phosphoproteins by SDS-PAGE. First four lanes on the left represent the Coomassie blue stained gel of the separated normal (AA) and SS membrane proteins. The last four lanes on the right represent the autoradiogram. The protein bands are numbered according to Steck (19).

Table 1. Influence of Ca^{++} introduced by Ionophore A23187 into normal and sickle red cells on membrane PK-C activity

Source	Protein kinase C activity pmoles Pi/min/mg protein		
	(-) Ionophore	(+) Ionophore	% Activation
Normal	38.8	46.0	18.6
	66.2	72.8	10.0
	83.4	101.6	21.0
	90.4	102.2	13.2
Sickle Cell Anemia	104.0	153.6	47.7
	133.6	205.6	53.9
	157.8	205.8	30.4
	203.8	331.6	62.7

activity' in 8 normal controls was 58.7 ± 18.3 and in 8 SS cases, 135.2 ± 25.7, thus parallelling the results obtained with ^{3}H-PDBu binding.

Fig. 3 gives the Coomassie blue-stained gel and the autoradiogram of membrane polypeptides separated by SDS-PAGE. Phosphorylation of bands 3, 4.1, and 4.9 in both normal and SS cases were noticed confirming the involvement of protein kinase C in the phosphorylation. Comparison of the normal and SS cases also showed a definite increase in the phosphorylation of all these three bands in SS cases.

When red cells were loaded with Ca^{2+} using Ionophore A23187, both normal and sickle red cells exhibited increased membrane-associated protein kinase C activity over the pre-ionophore treatment values. But sickle red cells responded to ionophore treatment to a greater extent (two-fold increase) than normal red cells (Table 1). In the case of normal, the membrane associated enzyme activity even after Ca^{2+}-loading did not reach the 'basal' levels observed in sickle red cells (i.e. before ionophore treatment; Table 1).

DISCUSSION

Previous work on red cell protein kinase C have indicated that the enzyme is almost totally confined to the cytoplasm and very little remains associated with the membrane (7,8). In view of the low phorbol ester binding activity, Wolf et al. have suggested that inside-out vesicles prepared from human

erythrocytes can be used as a reconstitution system in which intracellular translocation of protein kinase C can be studied (15). These authors have demonstrated a sharp increase in the binding of purified protein kinase C to these vesicles at Ca^{2+} concentrations ranging from 100 nM to 500 nM and predicted that physiological increases of intracellular Ca^{2+} could cause a translocation of protein kinase C from a soluble to a membrane-bound compartment. In sickle cell anemia, the red cells seem to have acquired the ability to sporadically increase their cellular Ca^{2+} content during sickling episodes resulting in a higher steady-state $[Ca^{2+}]_i$ levels. We investigated if this would result in greater mobilization of cytoplasmic enzyme to the membrane by employing tritium-labelled PDBu, a well known specific binder for protein kinase C (1,5,6). Contrary to earlier reports suggesting no appreciable receptors for phorbol esters in intact normal red cells (16,20), we were able to demonstrate, using isolated membrane ghosts, a minimal ^{3}H-PDBu binding in normal cases which was enhanced about four-fold in SS red cells (Fig. 1). This finding supports a recent suggestion by Kramer et al. (21) that intact human red cells do contain specific receptors for ^{3}H-PBDu and in addition, establishes that a pathological rise in red cell Ca^{2+} level as seen in sickle cell anaemia actually leads to an increase in the membrane association of protein kinase C.

To confirm if increased membrane association of the enzyme actually leads to an increase in the phosphorylation of membrane proteins in sickle cell cases, we assayed 'protein kinase C-like activity' as a difference in ^{32}P incorporation into acid-precipitable membrane proteins with and without the addition of both PS and DG. The two-fold increase in the ^{32}P-labelling of endogenous membrane proteins observed in SS cases compared to normal controls compliments the four-fold increase in the binding of ^{3}H-PDBu by SS membranes.

It should be noted that even in the absence of protein kinase C activation by DG, there was considerable phosphorylation especially of protein band 3 in both normal and SS membranes (Fig. 3). This phosphorylation is again significantly increased in SS membranes. It is pertinent to recall

that there are other protein kinases that are active in red cell membranes such as a cyclic AMP-dependent protin kinase (22), a cyclic-AMP independent kinase (23) and Ca^{2+}-activated protein kinase that is different from protein kinase C (8). The relative activities of these kinases in SS red cells are not known except for the reported rise in the activity of the cyclic AMP-independent enzyme (24). What is reported here is the increment over this 'basal' activity brought about by addition of DG and referred to as protein kinase C activity since no other protein kinase in red cells is activated by DG.

The role of intracellular Ca^{2+} in mobilizing the cytoplasmic protein kinase C to the membrane needs no emphasis (1,2,25). Recently, Ganang et al. (26) who studied the mechanism of activation of protein kinase C by DG observed that in the presence of high cellular Ca^{2+}, the enzyme forms a membrane-bound "enzyme-PS-Ca^{2+}" complex which by itself is inactive but a three-point attachment of DG (or PMA?) to this complex is necessary to cause activation in a highly specific fashion. It is likely that high cellular Ca^{2+} in sickle red cells mobilizes the cytoplasmic enzyme to the membrane and the inactive "enzyme-PS-Ca^{2+}" complex bound to the membrane gets activated on addition of DG. While a chronic elevation of cellular Ca^{2+} per se need not result in a greater amount of membrane bound protein kinase C, the sudden surge in Ca^{2+}-influx during sickling process, comparable to the agonist-induced Ca^{2+}-influx in other cellular systems (1,2) can be considered to trigger the membrane association process. "Sickling" is known to cause a progressive failure of the Ca-pump and marked reduction in Ca:Ca exchange (27). It should be noted that the net Ca^{2+} gain during sickling is "preferentially localized in the membrane" (10).

Raising the intracellular Ca^{2+} levels in normal red cells using Ionophore A23187 did increase the enzyme activity over the pre-treatment values (Table 1) but not enough to bring these values up to the levels seen in SS red cells. Sickle red cells, on the other hand, responded to a greater extent. It is possible 1) both normal and sickle cells may have the same amount of total enzyme but a third factor, probably related to the membrane binding of the enzyme,

may be operative in SS red cells, even in the presence of identical cellular Ca^{2+}; 2) SS membranes may have more substrate availability for phosphorylation; 3) the actual intracellular Ca^{2+} attained after ionophore treatment in sickle cells may even be higher than in normal red cells.

Red cell membrane protein bands 3, 4.1 and 4.9 have been identified as specific substrates for protein kinase C by earlier workers using PMA-induced membrane phosphorylation in intact red cells (7,8). The phospohorylation pattern (Fig. 3) that we have seen with isolated membranes matches very well with these studies. However, further studies are necessary for the identification of specific membrane polypeptides that are phosphorylated and the extent of their phosphorylation and then only the effect of such phosphorylation on membrane function, if any, can be predicted. The present observations only indicate that there is an increased membrane-association of protein kinase C in sickle red cells.

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