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PHORBOL ESTER STIMULATES THE PHOSPHORYLATION OF RABBIT ERYTHROCYTE BAND 4.1

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The effect of phorbol esters on membrane phosphorylation was examined in intact rabbit erythrocytes prelabeled with [^{32}P]orthophosphate. Tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate, but not the inactive 4α-phorbol 12,13-didecanoate, specifically stimulated the phosphorylation of two proteins in the erythrocyte membrane. They have apparent molecular weight of 100,000 dalton and 85,000 dalton. When intracellular calcium concentrations were raised by ionomycin, A23187, both the 85,000 dalton polypeptide and the 85K phosphoprotein were degraded. The 85,000 dalton phosphoprotein showed cross-reactivity with antiserum to humam erythrocyte band 4.1. Based on its susceptibility to calcium-activated protease and its immunological property, the 85,000 dalton phosphoprotein was identified to be the band 4.1 of the erythrocyte membrane skeletal network.

Erythrocyte membrane skeletal proteins, eg. spectrin, actin and band 4.1, are known to be phosphorylated by cAMP-dependent and cAMP-independent protein kinases (for review see ref. 1 and 2). It has been suggested that phosphorylation of the membrane skeletal components may be linked to processes such as endocytosis, exocytosis, regulation of cell shape and lateral mobility of membrane proteins. Recent studies show that biologically active phorbol ester induced phosphorylation of platelet proteins. The phosphorylation of these proteins are associated with the release of platelet granules (3-5). There is evidence indicating that phorbol esters directly activate the phospholipid and calcium-dependent protein kinase C (6,7). This enzyme is ubiquitously distributed in various tissues (8,9) and has been implicated in regulation of various cellular responses including exocytosis (1,10). Vinculin, a cytoskeletal protein, was recently reported to be a specific

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Abbreviations used: PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulphate-polyascrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethyl sulfoxide.

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substrate for protein kinase C (11). Since protein kinase C and cAMP-dependent protein kinase can often use the same proteins as substrate (13), it is therefore of interest to examine if phorbol ester also stimulates the phosphorylation of erythrocyte membrane skeletal proteins. We report that addition of phorbol 12-myristate 13-acetate to intact rabbit erythrocytes stimulated the phosphorylation of Mr 100,000 (100K) and Mr 85,000 (85K) proteins in the membrane. Evidence is presented here to demonstrate that the 85K is the band 4.1 of the membrane skeleton.

MATERIALS AND METHODS

Blood was collected in heparinized tubes and centrifuged to sediment the cells. Cells were washed twice with and resuspended in Hank's balanced salt solution containing no phosphate. Buffy coat was carefully removed after each washing to rid of platelets and leucocytes. The cell suspension was then divided into aliquots (1 ml) which were incubated with [^{32}P]orthophosphate for one hour at 37°C in order to label the cellular ATP pool. Cells were either allowed to continue to incubate for ten minutes or were treated for ten minutes with phorbol esters, or A23187. Incubation was carried out with gentle mixing of the cell suspensions. Erythrocyte membranes were obtained by immediate lysis of cells in hypotonic phosphate buffer (pH 7.4) containing 1 mM PMSF and 5 mM EDTA at 4°C to minimize proteolytic breakdown of membrane proteins. Protein concentration was determined by the method of Lowry (14). Membrane phosphoproteins were analyzed by SDS-PAGE (15) and stained with Coomassie blue. After destaining, the gels were dried down onto paper for autoradiography with Kodak XAR-5 film. Autoradiograms were scanned with a LKB Soft Laser Densitometer and the incorporation of ^{32}Pi into protein bands was quantitated by measuring the peak area in the tracings.

Membrane proteins, electrophoresed on 6-12% polyacrylamide gradient gel, were transferred to nitrocellulose paper at 200 mA for 2.5 hour. Electroblots were stained with anti-human band 4.1 antiserum and counterstained with goat anti-rabbit IgG conjugated with horse radish peroxidase (16).

[^{32}P]orthophosphate was obtained from New England Nuclear. PMA and 4α-PDD were acquired from P.L.-Biochemicals. A23187 was obtained from Sigma. Antiserum to human erythrocyte band 4.1 was generously provided by Dr. Carl Cohen of Tufts University Medical School.

RESULTS AND DISCUSSION

Conventionally, when erythrocyte membrane is subjected to SDS-PAGE, ten to fifteen major polypeptides can be visualized on Coomassie blue stained gels. As shown in Fig. 1, spectrin was the only ^{32}P-labelled protein in the control erythrocyte membrane. This phosphorylation probably resulted from endogenous cAMP-independent and/or cAMP-dependent protein kinases activity (17). Autophosphorylation of spectrin has also been reported (18). Treatment of cells with PMA for ten minutes markedly increased the phosphorylation of two additional polypeptides. They have apparent molecular weight of 100K and 85K as determined by electrophoresis on 8-16% and 6-12% gradient polyarylamide gels. In contrast, the extent of phosphorylation of spectrin, band 3, band 4.2 and actin remained

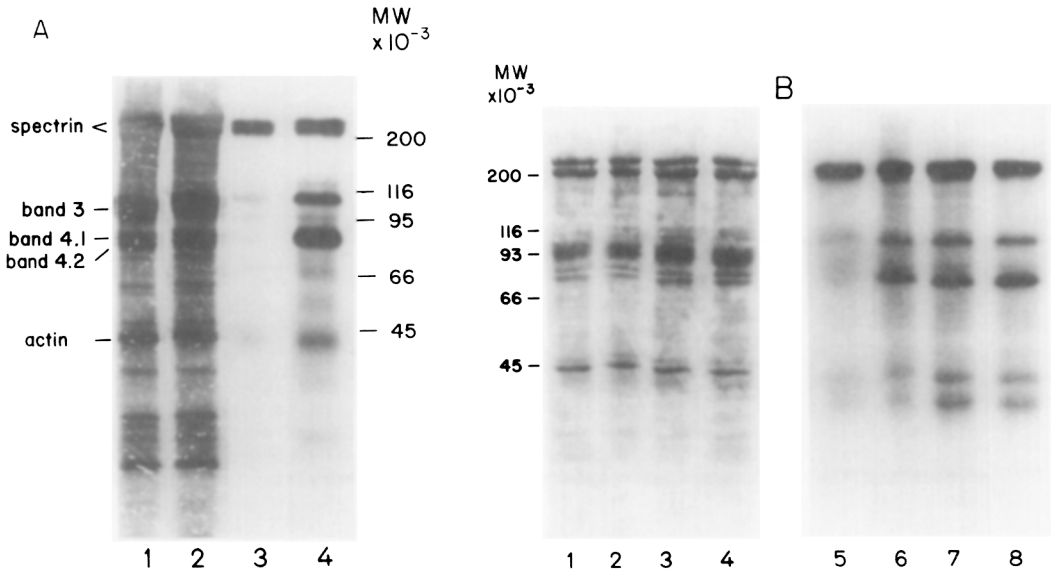

Figure 1: (A). Effect of PMA on rabbit erythrocyte membrane phosphorylation. Cell suspsensions (1 ml) were prelabelled with 60 uCi [^{32}P]orthophosphate at 37°C for 60 min with gentle mixing. Cells were either allowed to continue to incubate for 10 min or were treated for 10 min with 1 uM PMA in DMSO (final concentration, 0.1%) at 37C. Membranes were prepared as decribed in methods. The membrane proteins were analysed by electrophoresis on 8-16% polyacrylamide gradient gel and stained with Coomassie blue. After destaining, the gels were dried and autoradiographed. Lanes 1 and 2, Coomassie blue stained proteins profile (100 ug) of control and PMA treated erythrocyte membranes, respectively. Lanes 3 and 4 correspond to autoradiogram of lanes 1 and 2, respectively. Molecular weight markers were myosin (200,000), β-galactosidase (116,250), phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000).

(B). Effect of inactive phorbol ester and varying concentration of PMA on erythrocyte membrane phosphorylation. Prelabeling with [^{32}P]orthophosphate, treatment with phorbol ester and preparation of membrane were as described previously. Membrane proteins (75 ug) were analyzed on a 6-12% polyacrylamide gradient gel. Lane 1, membrane of erythrocytes treated with 1 uM 4 -PDD in DMSO (final concentration, 0.1%). Lanes 2, 3 and 4, membrane of cells treated with 0.1 uM, 0.2 uM and 1.0 uM PMA, respectively. Lanes 5 to 8 correspond to autoradiograms of lanes 1 to 4, respectively.

unchanged. Biologically inactive phorbol ester, 4α-PDD, which does not bind to the phorbol receptor or activate protein kinase C, failed to enhance phosphorylation of the 100K and 85K (Fig. 1B, lane 5). Thus, the effect of PMA on erythrocyte membrane phosphorylation was highly specific. The PMA stimulated phosphorylation is concentration-dependent with significant phosphorylation apparent at 0.1 uM PMA (Fig. 1B, lane 6). As PMA concentrations were raised to 0.2 uM and 1 uM, phosphorylation of the 85K was increased by approximately 50% and 100%, respectively. A less consistent increase was observed in the 100K while the overall increase in spectrin never exceeded 7% in all experiments. As the PMA levels were

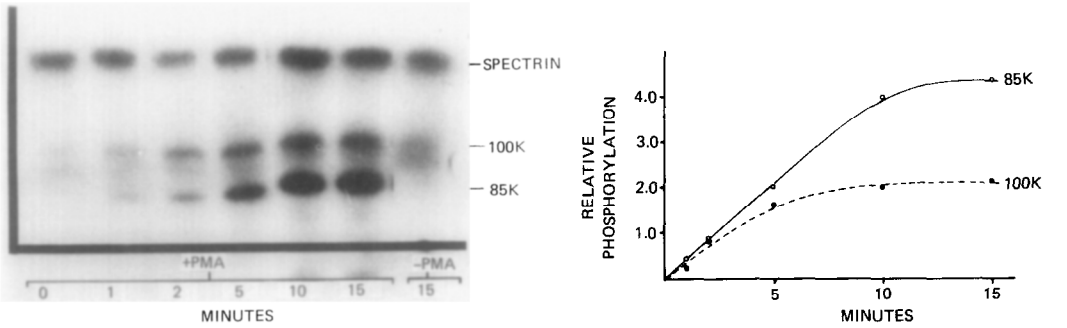

Figure 2. Time course of 100K and 85K phosphorylation induced by PMA addition to rabbit erythrocytes. PMA (1 uM) was added to erythrocytes prelabeled with [^{32}P]orthophosphate. Incubation was carried out at 37°C and terminated at various times. Membrane samples were analysed by SDS-PAGE and autoradiography. The incorporation of ^{32}Pi into 100K and 85K was quantitated as described in methods and the results are expressed as relative phosphorylation in the lower panel.

raised, there was also a small increase of labeled polypeptides between 45K and 50K (Fig. 1B) which may possibly be breakdown products. Figure 2 shows a time course of PMA-induced phosphorylation of intact erythrocytes. The increase in phosphorylation of both the 100K and 85K was linear for ten minutes, at which time the phosphorylation reached a maximum level. As expected, phosphorylation of spectrin remained essentially unchanged for fifteen minutes.

In all these experiments, DMSO was used as the solvent for PMA with the final concentration of DMSO at 0.1%. Since 4α-PDD in 0.1% DMSO did not enhance the 100K and 85K phosphorylation, it is unlikely that the effect could be attributed to DMSO. Recent work has demonstrated that protein kinase C copurified with phorbol ester receptor and that the enzyme is the receptor for phorbol (7,19,20). Therefore, it seems reasonable to assume that the PMA-induced phosphorylation observed here is mediated by protein kinase C.

The apparent molecular weight of the 85K phosphoprotein suggested that it may be the band 4.1 of the erythrocyte membrane skeleton. Band 4.1 is highly susceptible to calcium-dependent proteolysis (21). We found that treatment of erythrocytes for ten minutes with the calcium ionophore A23187 in the presence of 1.2 mM external calcium resulted in the disappearance of the Coomassie blue stained 85K polypeptide (Fig. 3A, lane 3). When the cells were treated with PMA together with A23187 in high external calcium, both the 85K polypeptide and the 85K

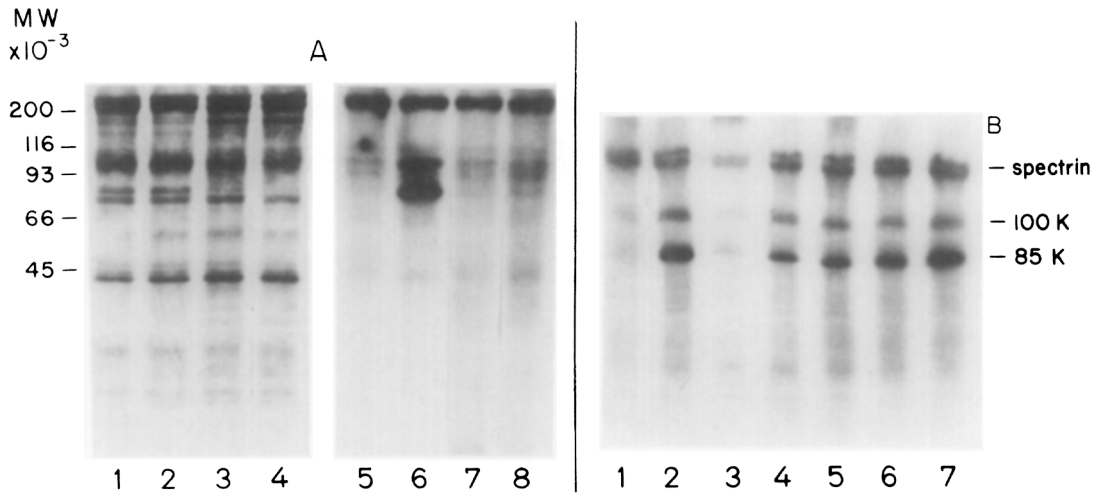

Figure 3: (A). Effect of A23187 and high external calcium on the 85K phosphoprotein. Experimental conditions were as described in methods. Coomassie blue staining (lanes 1-4) and autoradiograms (lanes 5-8) of erythrocyte membrane proteins (75 ug) on a 6-12% polyacrylamide gradient gel are shown here. Lanes 1 and 5, control erythrocytes treated with 1 uM 4α-PDD; Lanes 2 and 6, erythrocytes treated with 1 uM PMA; lanes 3 and 7, erythrocytes treated with 20 uM A23187; lanes 4 and 8, erythrocytes treated with 1.0 uM PMA and 20 uM A23187.

(B). Effect of various external calcium concentration on the phosphorylation of the 85K polypeptide. Erythrocytes were preincubated with $^{32}P_i$ and treated with 20 uM A23187 and 1 uM PMA for 10 min at 37°C in the presence of various external calcium concentrations. Radiolabeled membrane proteins were analyzed on 6-12% polyacrylamide gradient gel. The autoradiogram of the dried gel is shown here. Lane 1, control erythrocytes treated with 1 uM 4α-PDD; lane 2, cells treated with 1 uM PMA; lanes 3 to 7, cells treated with 20 uM A23187 and 1 uM PMA in the presence of 1.2 mM, 0.6 mM, 0.1 mM, 0.05 mM and 0.02 mM external calcium, respectively.

phosphoprotein were absence (Fig. 3A, lanes 4 and 8). The 100K phosphoprotein was diminished by treatment of the cells with A23187. Degradation of spectrin also occured, albeit to a lesser extent. In a separate series of experiments, A23187 was added along with PMA to ^{32}P-labeled cells in various concentration of external calcium. As shown in figure 3B, the 85K phosphoprotein was absence in cells treated with A23187 and 1.2 mM external calcium but was presence in cells incubated in 0.02 mM external calcium. The other calcium concentrations appeared to give intermediate degrees of phosphorylation, indicating significant degradation at or above 0.05 mM external calcium. These results clearly demonstrate that the 85K phosphoprotein is highly sensitive to calcium-activated protease in a calcium-concentration dependent manner.

To further ascertain whether the 85K phosphoprotein was band 4.1, membrane proteins separated by SDS-PAGE were transferred electrophoretically to

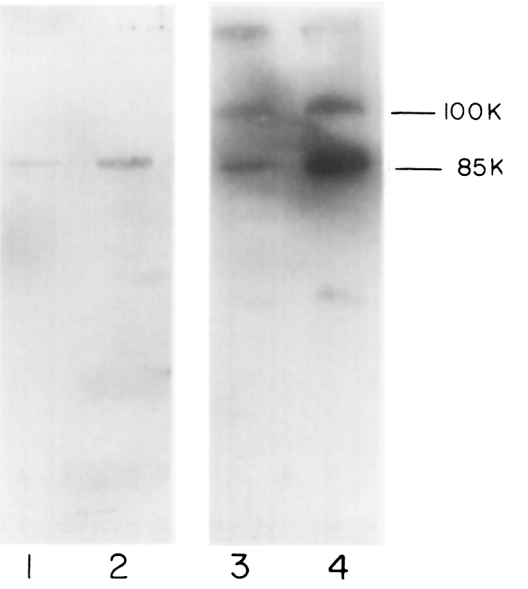

Figure 4: Immunological characterization of the 85K phosphoprotein. Electroblotting of erythrocyte membrane proteins and immunostaining with antiserum to band 4.1 were as described in methods. Lane 1, erythrocytes treated with 1 uM PMA and 20 uM A23187 for 10 min in the presence of 1.2 mM external calcium; lane 2, erythrocytes treated with 1 uM PMA for 10 min; Lanes 3 and 4, autoradiograms correspond to lanes 1 and 2, respectively.

nitrocellulose paper and the cross-reactivity with antiserum to band 4.1 was examined. The data showed that treatment of cells with A23187 and PMA in 1.2 mM external calcium resulted in a substantial loss of band 4.1 immune-reactive material (Fig. 4, lane 1). Autoradiography of the immunoblot revealed a parallel loss of the 85K phosphoprotein which lined up exactly with band 4.1 (Fig. 4, lane 3). Therefore, we conclude that the 85K phosphoprotein observed here is in fact band 4.1. At this moment, the identity of the 100K which was also phosphorylated after addition of PMA is not known. It is well established that erythrocyte membrane possesses binding sites for growth hormone, insulin, prostaglandins and acetylcholine (2). Interestingly, it has recently been reported that the beta subunit of the insulin receptor was phosphorylated in lymphocytes after treatment with phorbol esters (22). The beta subunit is approximately 100,000 dalton in molecular weight. Another possible candidate of the 100K phosphoprotein is the catalytic subunit of the Na,K-ATPase.

The functional significance of the phorbol-induced phosphorylation of band 4.1 remainly to be established. In many cells, including the erythrocytes, cytoskeleton and membrane-associated skeletal proteins play a major role in regulating cell shape and the mobility of integral membrane proteins. In addition, phosphorylation of erythrocyte membrane skeletal proteins has been implicated in endocytosis and exocytosis. Recently, it has been shown that vinculin, a cytoskeletal protein, was phosphorylated by protein kinase C in vitro (11). Our finding is the first report on phorbol-induced phosphorylation of membrane skeleton component in intact cells. It is possible that changes in band 4.1 phosphorylation mediated by protein kinase C alter the actin-band 4.1-spectrin ternary complex which is essential to the stability and shape of erythrocytes. The presence of band 4.1 in neural tissue (23), platelets (24) and fibroblasts (25) suggests that band 4.1 could be a ubiquitous substrate for protein kinase C. In view of the present findings, it is interesting to speculate that protein kinase C may mediate the alterations of band 4.1 phosphorylation, which could possibly be a general mechanism controlling interactions of cytoskeletal elements with the plasma membrane.

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