Protein Kinase C in the Human Erythrocyte

TRANSLATION TO THE PLASMA MEMBRANE AND PHOSPHORYLATION OF BANDS 4.1 AND 4.9 AND OTHER MEMBRANE PROTEINS*

(Received for publication, June 6, 1985)

H. Clive Palfrey and Ahmad Waseem
From the Departments of Pharmacological and Physiological Sciences and of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Protein kinase C (PK-C) was demonstrated in human erythrocytes using the exogenous substrate histone H1. The enzyme was dependent on the simultaneous presence of micromolar Ca\(^{2+}\) and phosphatidylserine. The Ca\(^{2+}\) requirement was reduced in the presence of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA). Activity was normally recovered in cytoplasmic extracts, but treatment of intact cells with TPA (EC\(_{50}\) = 40 nm) prior to lysis caused a rapid translocation of activity from the cytoplasm to the membrane. Following translocation, PK-C activity in the isolated membrane was independent of Ca\(^{2+}\) and phosphatidylserine and could not be removed by manipulating ionic strength. Ghosts from TPA-treated cells showed a marked increase in the phosphorylation of five proteins (termed PK-C-1-5) of M, 120,000, 110,000, 80,000, 78,000, and 49,000. Addition of purified bovine brain PK-C to ghosts from untreated cells resulted in the phosphorylation of the same five proteins. PK-C-3/4 corresponded to Band 4.1 and PK-C-5 to Band 4.9. Both proteins were isolated from ghosts and shown to be substrates for PK-C in vitro. PK-C-1 and -2 appear to be minor peripheral membrane proteins as both were released from the membrane by alkaline solutions. Incubation of \(^{32}\)P-prelabeled erythrocytes with TPA (EC\(_{50}\) = 40 nm) also resulted in a dose-dependent phosphorylation of PK-C-1-3. These results suggest that PK-C may play an important role in erythrocyte membrane function.

*This work was supported by National Institutes of Health Grant GM-35546 (to H. C. P.) and American Cancer Society Grant BC-95 and National Institutes of Health HL-30121 (to T. L. Steck). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: PK-C, Ca\(^{2+}\)- and phospholipid-dependent protein kinase C; PK-C-1-5, membrane substrates for PK-C; TPA, 12-O-tetradecanoylphorbol 13-acetate; PtdSer, phosphatidylserine; PMSE, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

2 That may also be necessary for PK-C action. Phorbol esters can apparently by-pass this system by activating PK-C directly, probably by binding to the site normally occupied by diacylglycerol (for reviews, see Refs. 1 and 2). Although phorbol esters may not act exclusively through PK-C (e.g. see Refs. 3 and 4), the activation of many cellular processes by these compounds has prompted the speculation that PK-C stimulation is an important intermediary in such systems. Consequently, it is of great interest to identify the targets for this enzyme in various cells. Some substrates have been identified, e.g. both smooth muscle (5) and platelet (6) myosin light chains are phosphorylated by PK-C, and this event may play a role in the platelet release reaction. Other cytoskeletal proteins, e.g. vinculin (7), have also been shown to be targets for PK-C. The enzyme also phosphorylates growth factor receptors, e.g. that for epidermal growth factor (8, 9), and may be responsible for reducing the affinity of those receptors for their ligands (e.g. Ref. 10). In several tissues, including brain, PK-C activity appears to be distributed between cytoplasmic and various membrane compartments (11, 18, 49). In brain, it is clear that both soluble and membrane-bound substrates are available to the enzyme (49). Recently, translocation of PK-C from the cytoplasm to the membrane following phorbol ester or hormone treatment of several cell types has been shown (30-32, 50). However, specific changes in membrane or cytoplasmic protein phosphorylation following such treatments have not been reported.

PK-C has been identified in many mammalian tissues, including blood elements such as platelets, lymphocytes, and neutrophils (1, 11, 18). Human erythrocytes have not been extensively studied, although effects of phorbol esters on protein phosphorylation in both rabbit and avian erythrocytes have been reported (12, 13). Studies on phosphatidylinositol metabolism in erythrocytes have revealed that increased intracellular Ca\(^{2+}\) can stimulate the formation of diacylglycerol (14), an effect mediated by a Ca\(^{2+}\)-sensitive polyphosphoinositide phosphodiesterase (15); thus, the presence of PK-C could have important consequences for red cell responses to Ca\(^{2+}\) elevation. In this report, we demonstrate that human erythrocytes contain PK-C, and the phenomena that result from activation of this enzyme are characterized.

EXPERIMENTAL PROCEDURES

Preparation of Cells, Membrane, and Cytosol Fractions—Human erythrocytes were obtained from freshly donated blood or from outdated blood bank specimens. After extensive washing in HEPES-buffered saline (at least four times with removal of the superficial layer), cells were resuspended at 20% hematocrit in solution A containing 145 mM NaCl, 5 mM KCl, 10 mM Na HEPES (pH 7.4), 0.1 mM Na phosphate, and penicillin/streptomycin (20 units/ml). Cells were stored at 4 °C and used within 2 days. Membranes were prepared by homolysis of packed cells in a 20-fold excess of lysis buffer.
containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.01 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 18,000 rpm for 10 min (Sorvall SS34 rotor), the supernatant was removed and stored on ice, and the ghosts were washed three times further in hemolysis buffer. Ghosts were resuspended to a concentration of 1 mg of protein/ml of lysate buffer and usually used immediately for further manipulation (20).

In Vitro Phosphorylation and Assay of PK-C Activity—Cytosol fractions were incubated in a reaction mixture (final volume 100 μl) containing (final concentrations) cytosol (400-500 μg of protein), 50 mM Tris-HCl (pH 7.4), 5 mM MgSO4, 1 mM Ca EGTA buffer containing 1 mM Ca2+, (1 μM calmodulin) plus PtdSer (background) and Mn free Ca2+ plus PtdSer (optimum previously described methods (18,19). The final preparation consisted of a single major silver-stained protein band (32). Band 4.1 is present at a final concentration of 50 μM, as determined by absorbance at 280 nm, and the synthesized ATP is present at a concentration of 1 μM ATP (specific activity 100-500 mCi/mm,). When added, TPA was present at a final concentration of 50 nM. The reaction was initiated by the addition of ATP and terminated by addition of an EDTA/ATP "stop" solution after 5 min (linear conditions). Samples were spotted onto phosphocellulose paper and processed as described previously (17). In some cases, SDS sample buffer was used to terminate the reaction and histone phosphorylation assessed by counting the appropriate gel bands after electrophoresis. Phosphorylation of membranes (10-20 μg of protein) was carried out under identical reaction conditions.

Intact Cell Phosphorylation—Fresh red cells at 20% hematocrit in solution A were incubated at 37 °C with 0.4 mCi/ml [32P]orthophosphate for 3-8 h to label intracellular ATP pools. Aliquots (250 μl) were then further incubated in microfuge tubes with effectors for various lengths of time, washed once with an iced solution of Tris-buffered saline, pelleted for 20 s in a microfuge, and then immediately hemolyzed with a 20-fold excess of lysing buffer as described above. Cytosol samples were removed, and the membranes were washed twice further; both fractions were then solubilized with SDS sample buffer, and phosphorylated proteins were separated by SDS-7.5% polyacrylamide gel electrophoresis and analyzed by autoradiography and scintillation counting of appropriate bands. Membrane protein concentrations were measured using the Folin reagent and standardized prior to electrophoresis.

Purification of Proteins—PK-C was purified to homogeneity from bovine brain by a new procedure consisting of modifications of previously described methods (18,19). The bulk of the spectrin, actin, and Band 4.1, the band 4.9-rich supernatant, TPA and PtdSer were added at concentrations of 50 nM and 25 μg/ml alone was ineffective, but in combination led to an approximately 3.5-fold stimulation of histone H1 phosphorylation under optimal conditions (i.e. 10-4 M Ca2+). Addition of TPA enhanced enzyme activity at low pCa where PtdSer alone had no effect; this effect was diminishing at higher pCa, although TPA still augmented PtdSer-dependent activity somewhat at optimum pCa. The tumor promoter had only slight effects on histone kinase activity in the absence of PtdSer at all pCa. As expected from previous work in which we demonstrated the absence of calmodulin-dependent protein kinase in human erythrocytes

### RESULTS

**Demonstration of PK-C in Human Erythrocytes—** We used histone H1, which has been found to be the best phosphate acceptor among the histones for brain PK-C (25), to measure PK-C activity in erythrocytes. As shown in Table I, increased phosphorylation of this substrate could be demonstrated on the addition of Ca2+ and PtdSer to cytoplasmic extracts from human erythrocytes in a manner consistent with that previously observed with PK-C in other tissues (11,18,26). Thus, either Ca2+ (10-8-10-4 M) or PtdSer (25 μg/ml) alone was ineffective, but in combination led to an approximately 3.5-fold stimulation of histone H1 phosphorylation under optimal conditions (i.e. 10-4 M Ca2+). Addition of TPA enhanced enzyme activity at low pCa where PtdSer alone had no effect; this effect was diminishing at higher pCa, although TPA still augmented PtdSer-dependent activity somewhat at optimum pCa. The tumor promoter had only slight effects on histone kinase activity in the absence of PtdSer at all pCa. As expected from previous work in which we demonstrated the absence of calmodulin-dependent protein kinase in human erythrocytes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Addition</th>
<th>Histone H1 kinase activity at pCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>None</td>
<td>0.084 0.114 0.102 0.128</td>
</tr>
<tr>
<td>PtdSer</td>
<td>0.094</td>
<td>0.159 0.154 0.578</td>
</tr>
<tr>
<td>TPA</td>
<td>0.283</td>
<td>0.337 0.068 0.063</td>
</tr>
<tr>
<td>Membranes</td>
<td>None</td>
<td>2.91 3.42 3.61 2.65</td>
</tr>
<tr>
<td>(control)</td>
<td>PtdSer</td>
<td>2.79 3.96 3.51 2.72</td>
</tr>
<tr>
<td>Membranes</td>
<td>None</td>
<td>3.27 3.28 3.28 18.9</td>
</tr>
<tr>
<td>(TPA-treated</td>
<td>31.5*</td>
<td>31.7 35.6 16.4</td>
</tr>
<tr>
<td>cells)</td>
<td>PtdSer</td>
<td></td>
</tr>
</tbody>
</table>

*To compare the total PK-C activity in the cytosol (i.e. 0.663 - 0.094 = 0.669 pmol/mg of protein/min), with that gained by the membrane after TPA treatment of cells (i.e. 3.15 - 2.79 = 0.36 pmol/mg of protein/min), a conversion factor expressing the packed cell equivalent of equal quantities of protein from cytosol and membrane is needed. This is 53.2 (1 mg of cytosol protein from 3.55 μl of packed cells and 1 mg of membrane protein from 189 μl of packed cells) comparing favorably with the 50.5 ratio obtained from the specific activity values.

**Addition of TPA either alone or in combination with PtdSer to membrane fractions had no effect on histone H1 phosphorylation.**
(17), addition of calmodulin (20 μg/ml) had no effect on histone phosphorylation in this preparation. In view of the very high level of PK-C found in platelets (1), we wished to eliminate the possibility that the low activity measured in red cell extracts represented contamination by small amounts of platelet cytosol. To this end, we passed well-washed erythrocyte preparations over a column of microcrystalline cellulose, a procedure that has been reported to efficiently remove platelets from red cell suspensions (27). Microscopic examinations of the flow-through from this column revealed no detectable platelet contamination, and assay of PK-C activity in extracts from such preparations yielded similar values as those listed in Table I.

Histone H1 was also phosphorylated when added to membrane preparations; however, this reaction was not stimulated by the addition of either PtdSer or TPA at any pCa studied (Table I). Instead, a Ca²⁺-dependent inhibition of histone phosphorylation was observed at pCa 4, the origin of which is not known. Moreover, when the pattern of membrane protein phosphorylation was examined by autoradiography, none of the characteristic substrates for PK-C were observed (see below; Fig. 1) either in the presence of TPA or PtdSer. In fact, histone addition stimulated the phosphorylation of other proteins including β-spectrin by an unknown mechanism (not shown). These results strongly suggest that human erythrocyte membranes normally do not contain a Ca²⁺- or phorbol ester-sensitive PK-C activity.

Translocation of PK-C to the Membrane and Membrane Protein Phosphorylation in Cells Pretreated with TPA—Red cells were incubated with TPA (10–500 nM) for 10 min and then membranes and cytosol were prepared in the usual manner and phosphorylation was assessed in vitro (Figs. 1 and 2). Membranes showed an enhanced phosphorylation of several proteins (Fig. 1), whereas cytosol exhibited a reduced PK-C activity (Fig. 2). The ability of PtdSer (plus Ca²⁺) to stimulate histone H1 phosphorylation in the cytosol fraction decreased in a dose-dependent manner over this range of TPA concentrations (Fig. 2). Endogenous phosphorylation in membranes from cells treated with TPA (500 nM) revealed labeling of a number of protein species that were not labeled in control membranes (Fig. 1, compare lanes 1 and 6). The major new substrates had Mᵣ values of 120,000, 110,000, 80,000, 78,000, and 49,000 (for convenience, we refer to these substrates as PK-C-1–5, respectively) with minor substrates at Mᵣ 70,000 and 53,000 (Fig. 1, lane 6). The Mᵣ 49,000 species (PK-C-5) co-migrated with a protein known as Band 4.9 that was also prominently phosphorylated by the addition of either 8-BrcAMP or the catalytic subunit of cAMP-dependent protein kinase to ghosts from untreated cells (not shown; compare Refs. 28 and 40 where this protein is termed “4.8”). The identity of the two higher Mᵣ proteins (PK-C-1 and -2) is unknown at present (see below). These results suggest that PK-C was translocated from the cytosol to the membrane in TPA-treated cells. Such effects were not seen (a) when cells were treated with an inactive phorbol ester 4a-phorbol dibenzoate, (b) if TPA was added immediately after lysis, or (c) if TPA was added to cells incubated at 4 °C for 10 min. These control experiments show that the translocation event requires the binding of an active phorbol ester to PK-C, that it is not an artifact generated upon lysis of the cells, and that it may require actively metabolizing cells.

It was also observed that the phosphorylation of the exogenous substrate histone H1 was increased in membranes derived from cells exposed to TPA (Table I). Simultaneously, a diminution of the phosphorylation of the five major membrane PK-C substrates was observed (not shown), suggesting that exogenous histone competed successfully with endogenous membrane substrates for PK-C. A quantitative comparison between the loss of PK-C from the cytosol seen upon maximal TPA treatment of intact cells with the additional amount of histone H1 kinase activity appearing in the membrane fraction under identical conditions yielded closely corresponding values (Table I, legend).

The PK-C-like activity in membranes from cells treated with the tumor promoter was expressed fully in the absence of exogenous Ca²⁺ and PtdSer, and the addition of these effectors failed to further stimulate the phosphorylation of either endogenous substrates (Fig. 1, lanes 6 and 7) or exogenous histone H1 (Table I). Raising EGTA in the reaction mixture to 5 mM decreased activity by only 50% (reversible on readdition of Ca²⁺); thus, it appears that membrane-bound PK-C is relatively independent of added Ca²⁺ or PtdSer. Attempts were made to elute PK-C from its membrane binding site(s). Neither high (1 M NaCl) nor low (<1 mM total salt) ionic strength treatments diminished membrane-associated enzyme activity; on the other hand, we were able to release a histone H1 kinase activity from the membrane of TPA-treated cells with 0.1% Nonidet P-40 (not shown).

These results strongly suggest that TPA treatment leads to the migration of PK-C activity from the cytosol to the plasma membrane and that this activity is responsible for the subsequent increase in membrane phosphorylation.

Phosphorylation of Membrane Proteins by Exogenous PK-C—In order to confirm that the enhanced membrane phosphorylation seen in the experiments described above was...
indeed due to a PK-C-like enzyme in the erythrocyte, attempts were made to duplicate the phosphorylation pattern seen in membranes from TPA-treated cells by the addition of exogenous purified bovine brain PK-C to membranes from untreated cells. Indeed, the pattern produced under these conditions was virtually identical to that seen in membranes from phorbol ester-treated cells: the same five major substrates became phosphorylated (Fig. 1, compare lanes 5 and 6). (It should be noted that we observed a substantial difference in the ability of exogenous PK-C to phosphorylate Band 4.1 in membranes from fresh red cells versus those obtained from outdated blood bank specimens, phosphorylation being superior in the stored cells. The other PK-C substrates were not affected, and the origin of the effect is unknown.) These results imply a close relationship between red cell PK-C and the enzyme from brain.

**TPA-stimulated Phosphorylation in Intact Erythrocytes**—Erythrocytes were incubated with $^{32}$P and then exposed to TPA at various concentrations. After incubation with the effector, cells were hemolyzed, and membranes and cytosol were analyzed independently for phorbol ester-mediated changes in protein phosphorylation. As shown in Fig. 3A, the pattern of membrane phosphorylation after TPA treatment closely resembled that seen after addition of PK-C to isolated membranes in vitro or that found on phosphorylation of isolated membranes from TPA-treated cells (Fig. 1). Thus, the phosphorylation of PK-C-1-5 was markedly stimulated in a dose-dependent manner by TPA. A number of less prominent substrates were also evident in these experiments, particularly those of Mr 70,000, 63,000, 60,000, and 53,000 (unlabeled arrowheads, Fig. 3A). However, analysis of spectrin, ankyrin, and Band 3 revealed no significant change in labeling following TPA incubation.

The increased labeling of all proteins was half-maximal at 40 nM TPA (e.g. Fig. 2) and took about 45 min to reach a maximum at saturating levels (1 $\mu$M) of the phorbol ester. Once again, 4a-phorbol didecanoate, a compound that is inactive as a tumor promoter or as an activator of PK-C in vitro, did not stimulate phosphorylation in the intact cell. TPA had no effect on protein phosphorylation when exposed to prelabeled cells at 4 °C.

In contrast to events at the membrane, only a single major TPA-stimulated phosphoprotein of Mr 140,000 was apparent when cytosol fractions were examined in these intact cell experiments (Fig. 3B).
PROTEIN KINASE C IN ERYTHROCYTES

FIG. 3. Stimulation of phosphorylation of membrane and cytosolic proteins in intact prelabeled erythrocytes by TPA. Erythrocytes were labeled with 32P as described under "Experimental Procedures" and then incubated with various concentrations of TPA for 10 min. Membranes (A) and cytosol (B) were then prepared and analyzed separately by SDS-7.5% polyacrylamide gel electrophoresis and autoradiography. The positions of the major TPA-stimulated substrates in the membrane fraction (at left: PK-C-1-5) are shown as well as four minor substrates of M, 70,000, 63,000, 61,000, and 53,000 indicated by unlabeled arrowheads. Note that PK-C-1, -2, and -5 are also phosphorylated in membranes from untreated cells (A, left lane). A single major substrate was detected in the cytosol fraction (B, arrowhead).

DISCUSSION

The results presented in this report demonstrate the existence in the human erythrocyte of an enzymatic activity strongly resembling PK-C. Our criteria for this conclusion are the dependence of histone H1 kinase activity on the presence of both Ca2+ and PtdSer and the ability of TPA to stimulate activity at very low pCa, properties that have previously been observed with the purified bovine brain enzyme (26, 29). The physical resemblance between the enzyme from the erythrocyte and that found in other tissues must await purification of the enzyme from red cells; preliminary experiments in this laboratory show that the erythrocyte enzyme has a similar elution profile to the brain enzyme on various chromatographic matrices. PK-C was observed in the cytosol fraction of control cells, but we could detect no Ca2+- or PtdSer- or TPA-stimulated histone H1 kinase activity in well-washed ghost preparations. Thus, membranes from normal red cells probably contain little, if any, PK-C activity. This conclusion is supported by the lack of phosphorylation of the five characteristic PK-C substrates in such membrane preparations. It is possible, however, that the Ca2+- and phorbol ester-unresponsive histone H1 kinase activity seen in


these membranes (Table I) represents a PK-C-like enzyme that has lost its ability to be regulated by these agents. A calculation of the approximate number of PK-C molecules/red cell can be made using the following assumptions: taking the specific activity of pure PK-C to be 1 nmol/mg of protein/min (measured with histone H1 (18)) and the specific activity of PK-C in erythrocyte cytosol to be 0.535 pmol/mg of protein/min under optimal conditions (Table I; this figure was obtained with 30 µl of a 20-fold dilute cytosol preparation calculated to represent 1.5 µl or 1.2 x 10^7 of original packed cells), a number of approximately 300/cell can be obtained. This number could be smaller if the specific activity of pure PK-C were an underestimate or larger if the specific activity in the cytosol were an underestimate.

Treatment of erythrocytes with TPA resulted in the translocation of PK-C from the cytoplasm to the membrane. This conclusion is based on the observations that histone H1 kinase activity decreased in the cytosol from cells treated with increasing TPA concentrations and that phosphorylation in isolated ghosts from the same cells increased simultaneously. The phosphorylation of both a characteristic subset of five endogenous membrane proteins and of added histone H1 was stimulated substantially in membranes from TPA-treated cells (Fig. 1 and Table I). The elevated histone kinase activity found in the membrane accounted quantitatively for that lost from the cytosol (Table I legend). Membrane-bound PK-C did not require added Ca^{2+} or PtdSer for full activity, but activity was reduced by 50% when the EGTA concentration in the assay medium was increased to 5 mM. These results indicate that PK-C translocates to the membrane after TPA treatment of whole cells and is capable of phosphorylating a characteristic subset of membrane proteins in a "Ca^{2+}-insensitive" manner. A possible explanation for the independence of activity on added cofactors is that the membrane-associated enzyme retains bound phorbol ester and Ca^{2+} and that membrane components fulfill the role of the lipid cofactor; Ca^{2+} must be in a form that is relatively inaccessible to added chelator. It is also conceivable that during the translocation process the enzyme becomes less Ca^{2+}-sensitive, e.g. some may be proteolytically cleaved to a Ca^{2+}-insensitive form (cf. Ref. 48). The enzyme must be tightly bound, as it could not be released with chelators or with either high or low ionic strength solutions, but was only liberated on treatment of the membrane with nonionic detergent. This observation suggests that the enzyme may bind hydrophobically to the inner leaflet of the phospholipid bilayer, and it is interesting to note that PtdSer is restricted to this face of the membrane in erythrocytes (for review, see Ref. 42).

Translocation of PK-C-like activity has previously been seen in cultured cells (30) and pancreatic acini (31) in response to TPA and more recently in pituitary cells exposed to gonadotropin-releasing hormone (32) and T-lymphocyte clones treated with interleukin-2 (50). Apart from the lymphocyte, the effects in these cell types were not as complete as found here in the erythrocyte. Moreover, this is the first report of specific changes in membrane protein phosphorylation after PK-C translocation. The rapidity of the effect raises questions as to the mechanism of the translocation event: evidently TPA induces a change in PK-C that allows it to bind to the membrane, but whether a specific transport process is involved is not understood. At present, we can only say that the process is considerably slowed at 4 °C, perhaps implying a metabolic component (i.e. the temperature coefficient of a purely diffusional process would be insufficient to explain the magnitude of the translocation delay seen in cells at 4 °C).

Confirmation that the phosphorylation events seen in isolated membranes from phorbol ester-treated cells were indeed mediated by an endogenous PK-C activity came from experiments in which exogenous highly purified PK-C from bovine
Protein Kinase C in Erythrocytes

FIG. 5. Phosphorylation of purified erythrocyte membrane proteins by PK-C. Ankyrin (ANK) (10 μg) and proteins 4.1 and 4.9 (5 μg) were purified from membranes as described under "Experimental Procedures" and either incubated with purified brain PK-C (0.2 μg) in the presence (+) or absence (−) of 10−4 M Ca2+ (PtdSer (25 μg/ml) was present in these tubes) or with the catalytic subunit of CAMP-dependent protein kinase (C at 0.3 μg, 1 mM EGTA also added). At left, the Coomassie Blue staining profile of the purified proteins is shown (PS); at right, the autoradiogram (AR).

brain was added to control membranes. The pattern of phosphorylation seen here, i.e. increased phosphorylation of the five characteristic proteins, PK-C-1-5, was identical to that seen in membranes from TPA-treated cells (Fig. 1). That these membrane phosphorylation events could take place in the intact cell was verified by prelabeling cells with 32P i and then exposing them to TPA. Subsequent isolation of membranes from phorbol ester-treated cells again showed phosphorylation of proteins PK-C-1-5, as well as a number of minor bands. Only one prominent cytosolic substrate was found in these intact cell experiments. It is well-established that PK-C is a major cellular target for phorbol esters (1, 26, 33) although it may not be the sole site of action of these compounds (3, 4). In the erythrocyte, it seems likely that the effects of TPA are entirely due to PK-C activation, given the similarity in protein phosphorylation patterns that emerged from the three different experimental manipulations described here.

Three of the five major membrane substrates seen in the present study are relatively well-characterized species: these are the doublet comprising protein 4.1 (PK-C-3 and -4) and protein 4.9 (PK-C-5). We confirmed that these proteins in an isolated form were substrates for purified PK-C. In addition, both proteins were shown for the first time using purified components to be substrates for the catalytic subunit of cAMP-dependent protein kinase (Fig. 5) as has been demonstrated previously by cAMP addition to ghosts or whole cells (28, 40). We are currently evaluating the site specificity of the phosphorylation of these two proteins to determine if different sites are being phosphorylated by the two enzymes.3 Proteolytic digestion experiments are consistent with the hypothesis that the sites phosphorylated by addition of TPA to intact cells are similar to those phosphorylated upon addition of exogenous PK-C to isolated membranes (Fig. 4). Band 4.1 is known to be an important component of the erythrocyte skeleton (for review, see Refs. 34–36); it augments the interaction between spectrin and actin (36–38) and contributes to the attachment of the spectrin-actin network to the inner face of the plasma membrane (38, 39) via its interaction with glycophorin and Band 3 (39). In a recent study, Ling and Sapirstein (13) noted the phosphorylation of Band 4.1 and a protein of M, 100,000 when rabbit erythrocytes were exposed to TPA. It is not known whether the higher M, species corresponds to either PK-C-1 or -2 described here, but our results with protein 4.1 are in agreement with their observations. Protein 4.1 is present in cells other than erythrocytes (e.g. Ref. 43 and 44) and has been shown to be a phosphoprotein in other tissues such as lens (45). Thus far, evidence for PK-C involvement in protein 4.1 phosphorylation in other tissues has not been presented. Protein 4.9 has recently been claimed to be an actin-bundling protein (22). To date, no effects of phosphorylation on any of the functional properties of either of these proteins have been documented; thus, it will be of great interest to determine whether phosphorylation by PK-C alters the function of either protein.

The other major substrates for PK-C in the erythrocyte
Protein Kinase C in Erythrocytes

membrane are two minor proteins of $M_r$, 120,000 an 110,000 (PK-C-1 and -2). These proteins are barely visible by Coomassie Blue staining of SDS-polyacrylamide gels and to our knowledge have been little previously studied (although it is possible that the protein termed "3.1" by Luna et al. (41) is equivalent to PK-C-2 and it is likely that the phosphorylated peripheral proteins termed "2.8" and "2.9" by Johnson et al. (51) are equivalent to PK-C-1 and -2. Moreover, the latter authors showed marked phosphorylation of these proteins in control erythrocytes as we have confirmed here (Fig. 3), suggesting that they may be substrates for other red cell protein kinases.). These two proteins behave in a similar manner to proteins 4.1 and 4.9 when the membrane is extracted by either alkali or nonionic detergent, suggesting that they may be substrates for other red cell protein kinases.

Addendum—After submission of this paper, Witters et al. (52) reported on the phosphorylation of the purified human erythrocyte glucose transporter ($M_r$, 50,000–60,000) by bovine brain PK-C. They also found phosphorylation of the transporter and proteins of $M_r$, 80,000 (Band 4.1) and $M_r$, 125,000 (possibly PK-C-1 and/or or -2 of this paper) on addition of TPA to prelabeled intact erythrocytes.

Acknowledgments—We would like to thank Dr. Ted Steck for his support and comments on the manuscript. We also thank Melanie Tyler, Leslie Whitted, and Kenneth Zuckerman for technical assistance.

REFERENCES

29. Yamamishii, J., Takai, Y., Kikkawa, K., Sano, K., Castagna, M.,
Protein Kinase C in Erythrocytes

16029