Phorbol 12-myristate 13-acetate-stimulated phosphorylation of erythrocyte membrane skeletal proteins is blocked by calpain inhibitors: possible role of protein kinase M

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Human erythrocytes contain cytosolic protein kinase C (PKC) which, when activated by phorbol 12-myristate 13-acetate (PMA), induces the phosphorylation of the membrane skeletal proteins band 4.1, band 4.9 and adducin. We found that brief treatments of erythrocytes with PMA resulted in a decrease in cytosolic PKC content and in the transient appearance in the cytosol of a Ca^{2+}- and phospholipid-independent 55 kDa fragment of PKC, called PKM. Prolonged treatment with PMA resulted in the complete and irreversible loss of erythrocyte PKC. To investigate the possible role of calpain in this process, the calpain inhibitors leupeptin and E-64 were sealed inside erythrocytes by reversible haemolysis. Both inhibitors prolonged the lifetime of PKC in PMA-treated cells, and leupeptin was shown to block the PMA-stimulated appearance of PKM in the cytosol. Significantly, leupeptin also completely blocked PMA-stimulated phosphorylation of membrane and cytosolic substrates. This effect was mimicked by other calpain inhibitors (MDL-28170 and calpain inhibitor I), but did not occur when other protease inhibitors such as phenylmethanesulphonyl fluoride, pepstatin A or chymostatin were used. In addition, the phosphorylation of exogenous histone sealed inside erythrocytes was also blocked by leupeptin. Immunoblotting showed that leupeptin did not prevent the PMA-induced translocation of PKC to the erythrocyte membrane. Thus inhibition of PKC phosphorylation of membrane skeletal proteins by calpain inhibitors was not due to inhibition of PKC translocation to the membrane. Our results suggest that PMA treatment of erythrocytes results in the translocation of PKC to the plasma membrane, followed by calpain-mediated cleavage of PKC to PKM. This cleavage, or some other leupeptin-inhibitable process, is a necessary step for the phosphorylation of membrane skeletal substrates, suggesting that the short-lived PKM may be responsible for membrane skeletal phosphorylation. Our results suggest a potential mechanism whereby erythrocyte PKC may be subject to continual down-regulation during the lifespan of the erythrocyte due to repeated activation events, possibly related to transient Ca^{2+} influx. Such down-regulation may play an important role in erythrocyte survival or pathophysiology.

INTRODUCTION

The protein kinase C (PKC) enzymes are a family of closely related protein kinases which have slightly different activation requirements and tissue distributions [1]. All of the isoenzymes require phospholipid for activity, and most but not all also require Ca^{2+} [1–4]. Although it is commonly held that the mechanism for PKC activation involves translocation of cytosolic enzyme to the plasma membrane, followed by activation by signal-generated diacylglycerol, or activation of loosely membrane associated PKC [5–7], many aspects of this process remain poorly understood. Two such aspects are: (1) the relationship between PKC membrane association and substrate phosphorylation, and (2) the role or function of intracellular PKC proteolysis. In some instances there is a poor correlation between the redistribution of PKC from the cytosol to the plasma membrane and the phosphorylation of membrane substrates [8,9]. A related issue is the stimulus-induced appearance in many cells of the proteolytically generated fragment of PKC, called PKM. PKM was originally described as a constitutively active fragment of PKC which could be generated *in vitro* [10]. Subsequently, many studies showed that prolonged stimulation of cells, especially with phorbol 12-myristate 13-acetate (PMA), led to the progressive down-regulation of cellular PKC [11–16], and in many cases a proteolytic product identified as PKM has either been shown or inferred (e.g. [11,15–18]. In some instances the prevention of PKM formation by the addition of inhibitors of the protease calpain was shown to block the apparent functions of PKC [19,20]. These studies suggest that in some cell types the generation of PKM may be a key step in the function of PKC.

The human erythrocyte is a useful cell in which to study such phenomena. Erythrocytes contain PKC [21–25] and calpain [26–28], as well as a group of well-characterized membrane-associated substrates for PKC ([21,24]; reviewed in [29]). Addition of PMA to intact erythrocytes results in the phosphorylation of three membrane skeletal proteins, band 4.1, band 4.9 and adducin [21,24,30]. Although it has been assumed that this phosphorylation results from the translocation of PKC to the erythrocyte membrane, this has never been shown directly in the erythrocyte. Studies *in vitro* have shown that PKC phosphorylation of band 4.1 has three effects: (1) it decreases band 4.1 binding to spectrin [31]; (2) it decreases or eliminates the ability of band 4.1 to promote spectrin binding to F-actin [31]; and (3) it prevents band 4.1 binding to one of its membrane attachment sites on the cytoplasmic domain of band 3 [32]. These events are predicted to affect erythrocyte membrane skeletal integrity. PKC

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PKM, protein kinase M fragment of PKC; DMSO, dimethyl sulphoxide; TBS, Tris-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

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phosphorylation has been reported not to affect band 4.9 (dematin)-induced F-actin bundling [33], and it is not known whether it affects the function of adducin.

While studying the mechanism of PKC activation in erythrocytes, we have found that many of the same pathways which are found in nucleated cells also operate in the erythrocyte. Here we have taken advantage of the ease with which erythrocytes can be manipulated to investigate the role of calpain-mediated PKC processing in membrane protein phosphorylation. Our results suggest that a process which is blocked by a variety of calpain inhibitors, possibly proteolysis of PKC, is obligatory for PKC-mediated phosphorylation of erythrocyte membrane proteins. These results provide insights into the mechanism of PKC action in erythrocytes and possibly other cells as well, and have important implications for erythrocyte pathophysiology.

MATERIALS AND METHODS

Materials

Fresh human blood was withdrawn from healthy donors and used within 2 days. [γ-^{32}P]ATP was purchased from DuPont–New England Nuclear. ^{125}I-Protein A and [^{32}P]P_i were purchased from ICN. PMSF (phenylmethanesulphonyl fluoride), leupeptin, pepstatin A, chymostatin, adenosine, PMA, DMSO (dimethyl sulphoxide), phosphatidylserine, diolein, histone (type III), ATP, inosine and E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] were purchased from Sigma. Calpain inhibitor I was purchased from Boehringer Mannheim. MDL-28170 was a gift from Merrell Dow Research Institute, Cincinnati, OH, U.S.A. Phosphocellulose P81 was purchased from Whatman. Nitrocellulose paper was purchased from Bio-Rad. Anti-PKC antiserum (PKC consensus sequence, amino acids 461–472, YRDLKLDNVLLD) was purchased from Research & Diagnostics (Berkeley, CA, U.S.A.).

Treatment of intact or reversibly haemolysed erythrocytes with PMA

Freshly donated human blood was washed extensively (at least three times with removal of buffy coat) with PBS (5 mM sodium phosphate, pH 8.0, 150 mm NaCl), and resuspended to 20% (v/v) in Buffer A (20 mM $NaHCO_3$, pH 7.5, containing 135 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM adenosine, 5 mM inosine, 12 mM glucose and 200 units/ml penicillin plus 200 μg/ml streptomycin). PMA (from a 1 mM stock in 100% DMSO) at a final concentration of 2 μM, or DMSO (final concn. 0.2%, v/v), was added and the cells were incubated at 37 °C for the times indicated in the Figures. At the indicated times, the erythrocytes were diluted into an excess of ice-cold PBS, washed twice in this same medium, and lysed in 20 vol. of ice cold 5 mM sodium phosphate, pH 8.0, containing 20 μg/ml PMSF and 2 μg/ml leupeptin (lysis buffer). The lysate was centrifuged at 18000 *g* for 15 min, and the supernatant was removed for analysis of PKC activity.

Reversible haemolysis of erythrocytes

This was adapted from the procedure of Ropars et al. [34] with modifications. Freshly prepared erythrocytes were given a final wash in PBS plus 0.5 mM EGTA, followed by pelleting and removal of the supernatant. The pelleted cells were lysed in 2.5 vol. of 10 mM sodium phosphate (pH 7.4)/20 mM $NaHCO_3$/20 mM glucose and in the absence or presence of various protease inhibitors (see the Figure legends). After incubation at 37 °C for 10 min, the lysed cells were resealed by adding 1.5 M KCl/NaCl (8.3:1) to the lysis mixture, to a final concentration of 0.15 M KCl/NaCl (8.3:1). The cells were mixed gently at 37 °C for 30 min, washed three times with ice-cold PBS and resuspended in Buffer A. Control experiments demonstrated that the resealed cells retained 80% of their original packed volume and entrapped 20% of the initial concentration of the inhibitors or other substances added at the time of lysis (which is close to the theoretical prediction, based on dilution during lysis). When examined by phase-contrast microscopy, the resealed cells were seen to have a nearly normal morphology, although 10–20% of the cells were 'cup'-shaped. Ghosts were prepared from these resealed cells by lysis in 20 vol. of ice-cold 5 mM sodium phosphate, pH 8.0, containing 20 μg/ml PMSF and 2 μg/ml leupeptin (lysis buffer), followed by centrifugation at 18000 *g* for 15 min. The supernatant was removed for analysis of PKC activity.

Phosphorylation of reversibly haemolysed erythrocytes

Phosphorylation of membrane proteins

Erythrocytes were reversibly haemolysed as described above, in the presence or absence of leupeptin or other inhibitors (see the Figure legends) added to the lysis medium. These reversibly haemolysed cells were suspended to a final haematocrit of 60% in Buffer A containing 1 mCi of [^{32}P]P_i/ml of packed cells and incubated at 37 °C for 30 min. The cells were treated with PMA or DMSO for 10 min as described above, and then washed twice in ice-cold PBS plus 0.5 mM EGTA. The washed cells were lysed and washed in lysis buffer as described above. The resulting membranes were analysed by autoradiography after gel electrophoresis as described below.

Phosphorylation of histone resealed in erythrocytes

Washed erythrocytes were suspended to a haematocrit of 60% in Buffer A containing 5 mCi of [^{32}P]P_i/ml of packed cells and incubated at 37 °C for 30 min. The cells were then washed with PBS plus 0.5 mM EGTA and reversibly haemolysed as described above in the presence of 5 mg/ml histone (final concn.), with or without 1 mM leupeptin (final concn.) added to the lysis medium. These resealed cells were treated with either 2 μM PMA (in DMSO) or DMSO alone for 10 min at 37 °C, and washed three times in 4 °C PBS plus 0.5 mM EGTA. The packed resealed erythrocytes were lysed as described above. After centrifugation at 18000 *g* for 15 min, portions of the supernatant were subjected to SDS/PAGE followed by autoradiography.

DEAE-cellulose DE-52 chromatography of erythrocyte cytosol

After the various treatments (see the text and Figure legends) erythrocytes were washed with ice-cold PBS, and lysed as described above. After centrifugation, the supernatant was withdrawn and 0.1 volume of 10 × Buffer B (Buffer B: 25 mM Tris/HCl, pH 7.5, containing 5 mM dithiothreitol, 2 mM EGTA, 2 mM EDTA, 40 μM cyclic AMP, 20 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A) was added. The cytosol solution was gently mixed with 15 ml of Whatman DEAE-cellulose DE-52 (pre-balanced with Buffer B) at 4 °C for 90 min. The DE-52 was packed into a 1.5 cm × 20 cm column, washed with Buffer B and eluted with a linear gradient (80 ml) of 0–0.3 M NaCl in Buffer B. Fractions (1.6 ml) were collected and samples were analysed for PKC activity as described below.

PKC assay of erythrocyte cytosol and column fractions

PKC assay on erythrocyte lysates was performed as described by Cohen and Foley [21] with modifications. Intact or reversibly haemolysed erythrocytes treated in various ways (see the Figure legends) were lysed as described above. After centrifugation, the cytosol was diluted to a protein concentration of 2.4 mg/ml, and 0.1 vol. of 10 × Buffer B was added. PKC activity in these lysates and in column fractions was measured as described by Kikkawa et al. [35]. Briefly, 30 μl of cytosol or column fraction was diluted into a final volume of 60 μl containing (final concns.) 20 mM Tris/HCl, pH 7.4, 150 μg/ml histone, 10 mM $MgCl_2$, 40 μM [γ-^{32}P]ATP [(3–5) × 10^6 c.p.m.] and either in 1 mM EGTA (control) or in 1 mM $CaCl_2$, 50 μg/ml phosphatidylserine and 10 μg/ml diolein. The lipids were added after being emulsified by sonication in 20 mM Tris/HCl, pH 7.4. The reaction was allowed to proceed for 10 min at 30 °C, after which the mixture was spotted on to 2 cm × 2 cm strips of phosphocellulose paper [36]. The phosphocellulose papers were then washed in two changes of 4 litres of 75 mM H_3PO_4 and air dried. The ^{32}P content of the dried phosphocellulose papers was determined by liquid-scintillation counting.

Gel electrophoresis

Samples were electrophoresed in 9% or 9–15%-gradient acrylamide slab gels as described by Hubbard and Lazarides [37]. Gels were dried between two sheets of dialysis membrane and autoradiographed by using Kodak XAR film.

Immunoblotting

Immunoblotting was performed as described by GuptaRoy and Cohen [38] with modifications. Samples from DE-52 column fractions or red-cell ghosts were subjected to gel electrophoresis and transferred on to nitrocellulose membranes [39]. The nitrocellulose membranes were incubated at room temperature for 5–10 min with 5% fat-free dry milk in Tris-buffered saline (TBS; 15 mM Tris/HCl, pH 7.5, 150 mM NaCl; blocking solution). These blots were then incubated overnight at room temperature with anti-PKC-peptide-specific antiserum diluted 1:500 in TBS containing 5% BSA. After four washes with TBS containing 0.1% Tween-20, blots were incubated with blocking solution as above and incubated with ^{125}I-labelled protein A (0.5 μCi/ml) in blocking solution at room temperature for 2 h. The blots were finally washed four times with TBS plus 0.1% Tween-20, air dried and exposed to Kodak XAR film at −80 °C.

Other assays

Protein concentration was determined by the Bradford [40] method with BSA as standard. Lactate dehydrogenase was measured by the method of Beutler [41]. Cytosolic haemoglobin was estimated by measuring the A_{540} of lysates.

RESULTS

PMA-induced down-regulation of erythrocyte PKC by a leupeptin-inhibitable process

While studying the time-dependence of PMA-stimulated phosphorylation of erythrocyte membrane skeletal proteins, we found that prolonged (> 1 h) treatment of erythrocytes with PMA led to nearly complete loss of cytosolic PKC activity. To determine the nature of this PMA-induced PKC loss, erythrocyte cytosols were assayed for PKC activity at various times during PMA treatment. Figure 1(a) shows that during PMA treatment of intact erythrocytes cytosolic PKC activity declined rapidly, was less than 15% of its starting value after 10 min of PMA treatment, and disappeared after about 1 h of exposure to PMA (Figure 1a, continuous line). It was also observed that during PMA treatment there was a transient increase in Ca^{2+}- and phospholipid-independent kinase activity (Figure 1a, broken line).

To confirm that prolonged PMA treatment resulted in the complete loss of erythrocyte cytosolic PKC, cytosols of erythrocytes incubated with either DMSO or PMA at 37 °C for 24 h were subjected to DE-52 chromatography, and fractions were assayed for histone kinase activity in the presence of Ca^{2+}, phosphatidylserine and diolein or in the presence of EGTA. The control cells (DMSO-treated) showed Ca^{2+}- and phospholipid-dependent kinase activity (PKC) which was eluted from the DE-52 column between 0.03 and 0.08 M NaCl (Figure 2a). By contrast, the cytosol from erythrocytes treated for 24 h with PMA did not show any Ca^{2+}- and phospholipid-dependent kinase activity (Figure 2b). This confirms that a 24 h exposure to

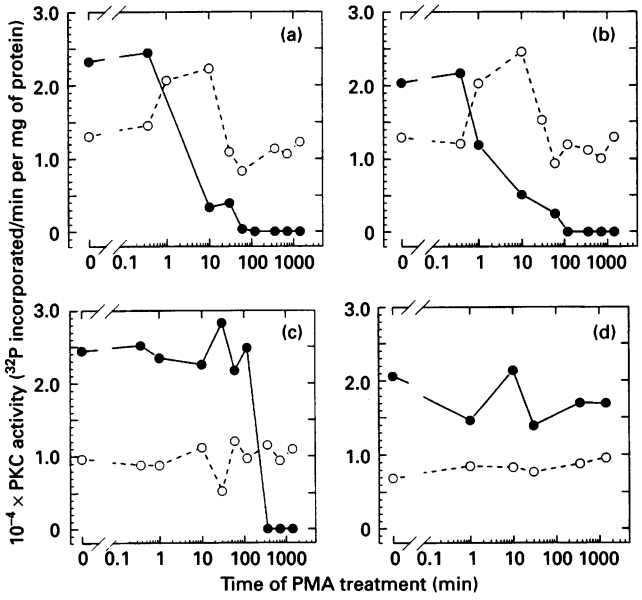

Figure 1 Effect of resealing leupeptin inside erythrocytes on PMA-induced loss of PKC

(**a**) Intact erythrocytes were treated with PMA at 37 °C for 0 s, 22 s, 1 min, 10 min, 30 min, 1 h, 2 h, 6 h, 12 h and 24 h as described in the Materials and methods section. After two washes with ice-cold PBS, cells were lysed and the cytosol was assayed for histone kinase activity (^{32}P incorporation) in the presence of EGTA (○; values shown are corrected for background by subtracting c.p.m. incorporated in the absence of added histone) or Ca^{2+}, phosphatidylserine and diolein (●, which represents the c.p.m. value obtained in the presence of Ca^{2+}, phosphatidylserine and diolein minus the c.p.m. value obtained in the presence of EGTA, i.e. net PKC activity). (**b**) Freshly prepared erythrocytes were reversibly haemolysed as described in the Materials and methods section. The resealed cells were washed with ice-cold PBS, resuspended in Buffer A and incubated with 2 μM PMA at 37 °C for the times indicated as described above. After two washes with PBS, the cells were lysed and the cytosol was assayed for histone kinase activity in the presence of EGTA (○) or Ca^{2+}, phosphatidylserine and diolein (●, which represents the c.p.m. value obtained in the presence of Ca^{2+}, phosphatidylserine and diolein minus the c.p.m. value obtained in the presence of EGTA, i.e. net PKC activity). (**c**) Same as (**b**), except that 1 mM leupeptin was added to the cells during reversible haemolysis, as described in the Materials and methods section. (**d**) Same as (**b**), except 20 μM E-64 was added to the cells during reversible haemolysis, as described in the Materials and methods section. All data points shown are the means of duplicate determinations which agreed to within ±10%.

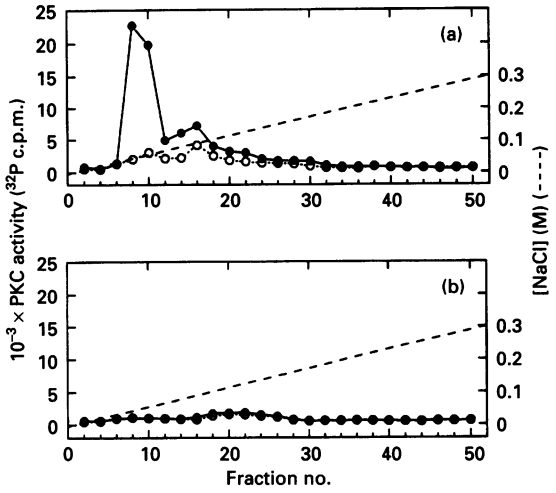

Figure 2 Chromatography of erythrocyte cytosolic PKC after 24 h of PMA treatment

Intact erythrocytes were incubated with DMSO (**a**) or 2 μM PMA (**b**) at 37 °C for 24 h. After two washes with ice-cold PBS, the cells were lysed and the cytosols were subjected to DE-52 ion-exchange chromatography as described in the Materials and methods section. Histone kinase activity of column fractions was assayed in the presence of EGTA (○) or Ca^{2+}, phosphatidylserine and diolein (●). Data points represent individual measurements.

PMA results in a complete loss of PKC from erythrocytes. Since erythrocytes lack protein-synthesis capability, this loss is irreversible.

A possible explanation for the above observations is that PMA caused a proteolytic conversion of PKC into the short-lived PKM, mediated by calpain, as has been reported in human platelets [16] and neutrophils [18]. This explanation would be consistent with the transient appearance of Ca^{2+}- and phospholipid-independent kinase activity seen in Figure 1(a). To test this possibility, we reversibly haemolysed human erythrocytes in the presence of leupeptin, a calpain inhibitor, to allow the leupeptin access to the cell interior. Although the haemolysis resulted in some dilution of cytosolic constituents, measurements showed that the reversibly haemolysed cells retained 32–33 % of cytosolic haemoglobin, lactate dehydrogenase and PKC. These reversibly haemolysed leupeptin-loaded cells were treated with PMA for increasing times, and the cytosols were assayed for kinase activity as described above. By contrast with intact erythrocytes (Figure 1a) and with erythrocytes reversibly haemolysed in the absence of leupeptin (Figure 1b), erythrocytes loaded with leupeptin maintained their PKC content up to about 2 h of PMA treatment, after which time cytosolic PKC disappeared (Figure 1c). This eventual loss of PKC was presumably accompanied by the transient production of PKM, as in Figures 1(a) and 1(b). However, since PKM is detectable in the cytosol for only about 20 min after initiation of PKC down-regulation

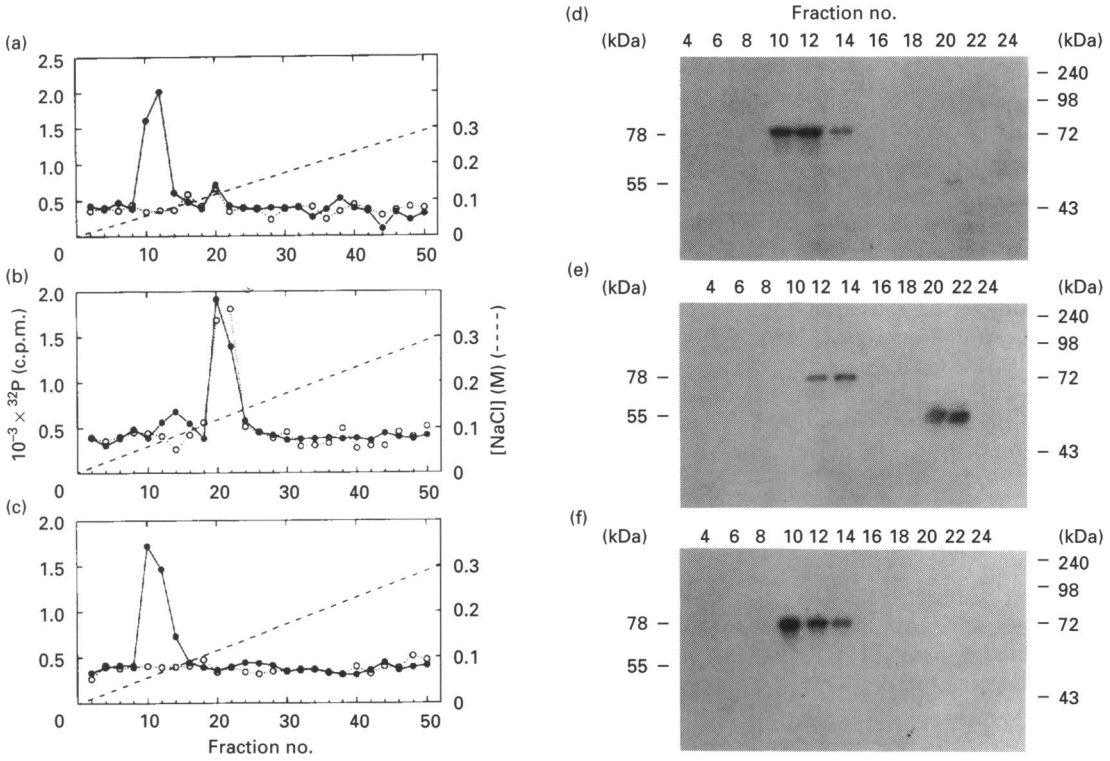

Figure 3 Demonstration of a Ca^{2+}- and phospholipid-independent kinase activity in erythrocyte cytosol after PMA treatment

Erythrocytes were reversibly haemolysed as described in the Materials and methods section in the absence (**a**, **b**, **d**, **e**) or presence (**c**, **f**) of 1 mM leupeptin (final concn.). The resealed cells were washed with ice-cold PBS, suspended in Buffer A and incubated with DMSO (control; **a**, **d**) or 2 μM PMA (**b**, **c**, **e**, **f**) for 10 min. After two washes with PBS, the cells were lysed and the cytosols were subjected to DE-52 ion-exchange chromatography as described in the Materials and methods section. Fractions were assayed for histone kinase activity (**a**, **b**, **c**), or 160 μl of each fraction was subjected to electrophoresis in a 9%-acrylamide gel, followed by immunoblotting with anti-PKC antiserum (**d**, **e**, **f**). Histone kinase activity was assayed in the presence of EGTA (○) or Ca^{2+}, phosphatidylserine and diolein (●). Data points represent individual measurements. Markers for calibration (**d**–**f**) are shown to the right of the gel; kDa values to the left of the gel refer to PKC (78) and PKM (55).

(see Figures 1a and 1b), it was likely to be missed by our 1 h sampling interval.

The loss of kinase activity from leupeptin-loaded cells after the 2 h time point is likely to be due to either a degradation or a loss of leupeptin from the resealed cells. The delay in PKC loss in the leupeptin-loaded reversibly haemolysed cells was not due to reversible haemolysis itself, since Figure 1(b) shows that cells reversibly haemolysed in the absence of leupeptin manifest nearly the same kinetics of PKC loss and PKM appearance as intact cells which were not reversibly haemolysed (Figure 1a). In an additional experiment (Figure 1d) the compound E-64, which is an irreversible inhibitor of calpain [42], was used instead of leupeptin. In this case, cytosolic PKC activity of PMA-treated cells remained constant for the entire time of the experiment (24 h). These results suggest that a leupeptin (or E-64)-inhibitable process was involved in PMA-induced loss of PKC.

In order to explore further the nature of this leupeptin-inhibitable process, cytosols of reversibly haemolysed erythrocytes were subjected to DE-52 chromatography. Fractions were assayed for kinase activity and for PKC antigen by immunoblotting with an anti-peptide antiserum against a consensus sequence within the C4 region (catalytic domain) of PKC [1]. Figure 3(a) shows that the cytosol from reversibly haemolysed control cells (without leupeptin) contained a major peak of Ca^{2+}- and phospholipid-dependent kinase activity (Figure 3a, fraction 10), which was eluted from the DE-52 column between 0.04 and 0.1 M NaCl, and correspond to a 78 kDa protein band on a Western blot (Figure 3d, fractions 10–14). After a 10 min PMA treatment of reversibly haemolysed erythrocytes (without leupeptin), the peak of cytosolic Ca^{2+}- and phospholipid-dependent kinase activity was dramatically decreased (Figure 3b). This decrease was accompanied by an increase in a peak of Ca^{2+}- and phospholipid-independent kinase activity eluted at fraction 20. Western blotting of these fractions (Figure 3e) confirmed the loss of the 78 kDa band from fractions 10–14 and showed an increase in a 55 kDa band in fractions 20–22 following PMA treatment (Figure 3e, fractions 20–22). Figure 3(c) and 3(f) show that the PMA-induced decrease in Ca^{2+}- and phospholipid-dependent kinase activity and the concomitant increase in Ca^{2+}- and phospholipid-independent kinase activity and the 55 kDa PKC immunoreactive band were blocked by preloading the erythrocytes with leupeptin. Cytosol from these cells had a distribution of kinase activity on the DE-52 column similar to that from reversibly haemolysed cells which had not been treated with PMA. These results suggest that PMA-induced loss of PKC in human erythrocytes is associated with a proteolytic conversion of PKC into a Ca^{2+}- phospholipid-independent kinase by a leupeptin-inhibitable process.

PMA-stimulated erythrocyte skeletal protein phosphorylation is blocked by inhibitors of calpain

To determine whether leupeptin has any effect on the PMA-stimulated phosphorylation of erythrocyte cytoskeletal proteins, erythrocytes were reversibly haemolysed and resealed in the presence or absence (control) of leupeptin. These cells were briefly incubated in $[^{32}P]P_i$ to generate intracellular [^{32}P]ATP and then treated with PMA. After 10 min, membranes were prepared and analysed for ^{32}P incorporation by gel electrophoresis and autoradiography. Figure 4, lane 1, shows membranes from cells reversibly haemolysed in the absence of leupeptin followed by metabolic labelling. Lane 2 shows membranes from cells reversibly haemolysed in the absence of leupeptin followed by metabolic labelling and treatment with PMA. Membranes in lane 2 show enhanced phosphorylation of band 4.1, band 4.9 and adducin,

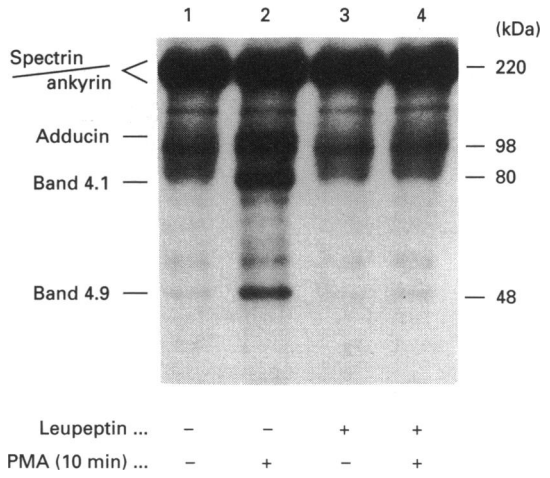

Figure 4 Inhibition of PMA-induced membrane skeletal phosphorylation by leupeptin in metabolically labelled erythrocytes

Erythrocytes were reversibly haemolysed as described in the Materials and methods section in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 mM leupeptin. After resealing, the cells were metabolically labelled in the presence of $[^{32}P]P_i$ for 30 min, followed by an additional 10 min incubation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of PMA (see the Materials and methods section for details). The cells were washed in ice-cold saline and membranes were prepared and electrophoresed in a 9%-acrylamide gel. The gel was dried and exposed to Kodak XAR film. Each lane contains 20 μg of membrane protein. Markers for calibration are shown to the right of the gel.

just as do cells which have never been reversibly haemolysed [21,22,24]. Thus, reversibly haemolysed cells still manifest PMA-stimulated membrane skeletal phosphorylation. Figure 4, lanes 3 and 4, show membranes from cells reversibly haemolysed in the presence of leupeptin followed by metabolic labelling and no PMA treatment (lane 3) or PMA treatment (lane 4). Lane 4 shows that inclusion of leupeptin in the reversibly haemolysed cells completely blocks PMA-stimulated phosphorylation of the membrane skeletal proteins. Lane 3 shows that inclusion of leupeptin in the reversibly haemolysed cells has no effect on the basal non-PMA-stimulated phosphorylation of other membrane proteins. This shows that leupeptin is not a non-specific inhibitor of phosphorylation. Moreover, we tested the effects of leupeptin on PKC activity in cytosol (lacking membranes) from normal erythrocytes, using histone as a substrate. Leupeptin, up to a concentration of 2 mM, had no effect on PKC activity under these conditions (results not shown; see the Discussion section).

A dose–response curve (not shown) indicated that the concentration of added leupeptin required for half-maximal inhibition of band 4.1 phosphorylation was 320 μM. Since control experiments showed that the concentration of leupeptin in the resealed cells was only 20% of that added, this translates into a half-maximal inhibitory concentration of 64 μM in the resealed cells.

To determine whether the inhibition of PMA-stimulated phosphorylation was specific to inhibitors of calpain, several other protease inhibitors were loaded into erythrocytes by reversible haemolysis. As in Figure 4, these cells were briefly incubated in $[^{32}P]P_i$ to generate intracellular [^{32}P]ATP and then treated with PMA. After 10 min, membranes were prepared and analysed for ^{32}P incorporation by gel electrophoresis and autoradiography. Figure 5 shows that, of the other inhibitors tested, only those inhibitors which affect calpain {leupeptin, MDL-28170 (Cbz-Val-Phe; [43]) and calpain inhibitor I}, but not those

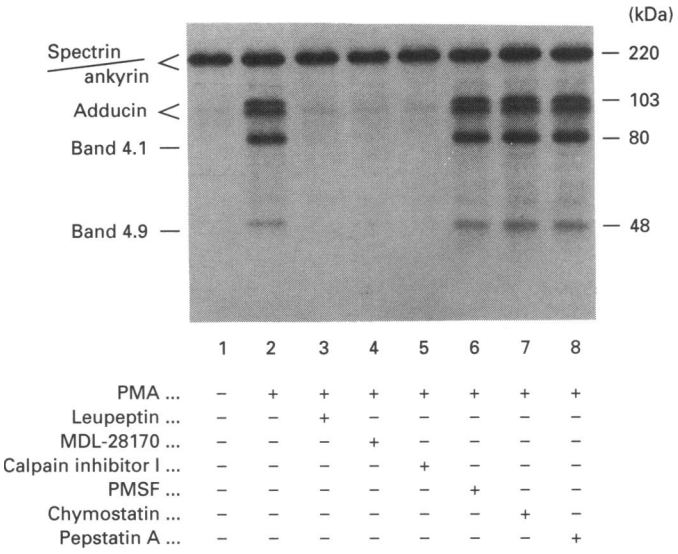

Figure 5 Inhibition of membrane skeletal phosphorylation by calpain inhibitors in metabolically labelled erythrocytes

Erythrocytes were reversibly haemolysed as described in the Materials and methods section in the absence (lane 1) or presence of (final conc. given) 1 mM leupeptin (lane 3), 100 μM MDL-28170 (lane 4), 34 μg/ml calpain inhibitor I (lane 5), 0.5 mg/ml PMSF (lane 6), 0.5 mg/ml chymostatin (lane 7), or 0.5 mg/ml pepstatin A (lane 8). After resealing, the cells were metabolically labelled in the presence of [^{32}P]P_i for 30 min, followed by an additional 10 min incubation in the absence (lane 1) or presence (lanes 2–8) of PMA (see the Materials and methods section for details). The cells were washed in ice-cold saline and membranes were prepared and electrophoresed in a 9%-acrylamide gel. The gel was dried and exposed to Kodak XAR film. Each lane contains 20 μg of membrane protein. Markers for calibration are shown to the right of the gel.

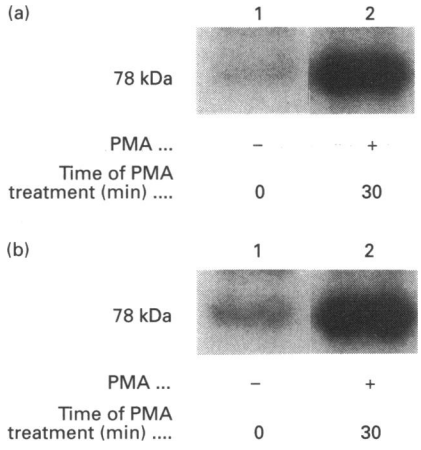

Figure 6 PKC immunoblot of erythrocyte ghosts after PMA treatment

Erythrocytes were reversibly haemolysed in the absence (**a**) or presence (**b**) of 1 mM leupeptin (final concn.) as described in the Materials and methods section. The cells were then suspended in Buffer A and incubated with 2 μM PMA for 30 min. After two washes with PBS, the cells were lysed and the ghosts were subjected to electrophoresis in a 9%-acrylamide gel and transferred on to nitrocellulose paper as described in the Materials and methods section. The blots were probed with anti-PKC antiserum as described in the Materials and methods section. Each lane contains 120 μg of membrane protein.

which affect serine (PMSF, chymostatin) or aspartic (pepstatin A) proteases, inhibited PMA-stimulated phosphorylation of proteins 4.1 and 4.9.

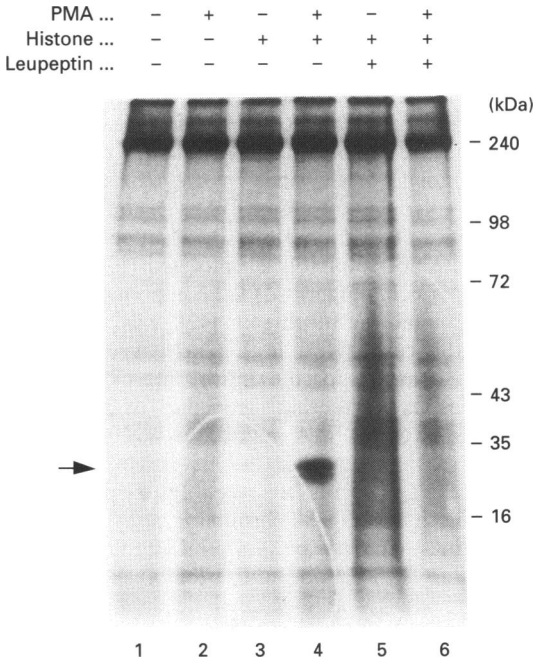

Figure 7 Inhibition of PMA-stimulated phosphorylation of cytosolic histone by leupeptin in metabolically labelled erythrocytes

Erythrocytes were metabolically labelled with [^{32}P]P_i and reversibly haemolysed as described in the Materials and methods section in the presence (lanes 3–6) or absence (lanes 1 and 2) of 5 mg/ml histone, with (lanes 5 and 6) or without (lanes 3 and 4) 1 mM leupeptin. The resealed cells were treated with either DMSO (lanes 1, 3, 5) or 2 μM PMA (lanes 2, 4, 6) for 10 min at 37 °C. Erythrocytes were lysed, membranes were removed by centrifugation and cytosolic proteins were subjected to electrophoresis in a 9–15%-acrylamide gradient gel and autoradiography as described in the Materials and methods section. The arrow shows the position of histone as determined by Coomassie Blue staining. Each lane contains 200 μg of cytosolic protein. Markers for calibration are shown to the right of the gel.

We next addressed whether the inhibition of phosphorylation by the calpain inhibitors could be due to an inhibition of PMA-induced translocation of PKC to the erythrocyte membrane. Reversibly haemolysed erythrocytes were treated with PMA for various times and membranes from these cells were analysed for PKC content by immunoblotting. Figure 6 shows that at 30 min ghosts from reversibly haemolysed PMA-treated erythrocytes prepared in the absence (Figure 6a) or presence (Figure 6b) of leupeptin had an increased amount of a 78 kDa PKC immunoreactive band as compared with non-PMA-treated controls. The amount of membrane-associated PKC antigen appeared similar in both cases. In both cases, the amount of the 78 kDa PKC immunoreactive band on the membrane was maximal and identical at the earliest time point tested (22 s; results not shown). Thus, even though PMA caused the translocation of PKC to the membranes of leupeptin-loaded cells to the same extent as in control cells, the leupeptin-loaded cells nevertheless exhibited no enhancement in the phosphorylation of bands 4.1 or 4.9 (Figures 4 and 5). This result shows that the leupeptin inhibition of phosphorylation was not due to an inhibition of PMA-induced translocation of PKC to the erythrocyte membrane, and that membrane association of PKC itself is not sufficient for phosphorylation of membrane substrates.

Finally, we addressed the question of whether the inhibition of PMA-stimulated phosphorylation by leupeptin was specific to proteins of the erythrocyte membrane skeleton, or whether phosphorylation of other substrates, such as cytosolic proteins,

would also be affected. Preliminary results showed that erythrocytes contained no readily detectable cytosolic substrates for PKC (compare Figure 7, lanes 1 and 2). Consequently we prepared reversibly haemolysed erythrocytes loaded with an exogenous substrate, histone. Figure 7, lane 4, shows that PMA treatment of these cells led to the phosphorylation of cytosolic histone (designated by the arrow to the left of lane 1). Lane 6 shows that, if the cells were resealed in the presence of leupeptin, histone phosphorylation was blocked. Control experiments (results not shown) showed that equivalent amounts of histone were trapped in the cells regardless of whether leupeptin was included. We conclude that leupeptin blocks PMA-stimulated phosphorylation of both membrane-associated and cytosolic substrates by PKC.

DISCUSSION

Our results show that activation of erythrocyte PKC by PMA results in a sequence of events which is common to many cell types. As has been shown previously in the erythrocyte, PMA induces translocation of the PKC to the plasma membrane and phosphorylation of specific membrane protein substrates. However, our results also show that brief PMA treatment results in the appearance in the cytosol of an approx. 55 kDa fragment of PKC. On the basis of its size, elution from a DE-52 column, and Ca^{2+}- and phospholipid-independent kinase activity, this is probably the catalytic domain of PKC, commonly referred to as PKM. Moreover, we found that prolonged treatment with PMA results in the complete disappearance of both PKC and PKM from erythrocytes. We found that this disappearance could be delayed or prevented by the inclusion of the calpain inhibitor leupeptin, or other calpain inhibitors, inside erythrocytes by reversible haemolysis. Surprisingly, we also found that sealing leupeptin inside erythrocytes not only inhibited PMA-induced PKC cleavage, but also completely inhibited the phosphorylation of membrane and cytosolic PKC substrates.

Our data suggest that a process blocked by calpain inhibitors is necessary for PMA-stimulated phosphorylation to occur. One process which is affected by such inhibitors is calpain cleavage of PKC. Inhibition of PKC cleavage might block phosphorylation if the active form of the PKC enzyme in the erythrocyte is the catalytically active PKM fragment, which has only a finite lifetime in the cell before being degraded by an as-yet uncharacterized pathway. Further work will be needed to define precisely the role of PKM in the phosphorylation of erythrocyte membrane skeletal proteins.

A possible alternative explanation is that leupeptin, or the other calpain inhibitors used, are inhibiting PKC by some mechanism unrelated to calpain. However, addition of leupeptin to erythrocyte cytosol (lacking membranes), or addition of leupeptin to ghosts prepared from PMA-treated erythrocytes, has no inhibitory effect on PKC mediated phosphorylation (see the Results section and [21]). Thus leupeptin is effective only when it is present inside erythrocytes at the time of PMA stimulation. Moreover, inclusion of leupeptin inside erythrocytes followed by metabolic labelling with $[^{32}P]P_i$ results in no inhibition of the non-PKC-related phosphorylation of membrane proteins (Figure 4), i.e. leupeptin is not a non-specific inhibitor of phosphorylation. Another possibility is that the calpain inhibitors were blocking the calpain-induced cleavage of substrate proteins, and that for some reason such cleavage is necessary for phosphorylation to occur. This explanation is unlikely, since: (1) we were unable to detect proteolysis of bands 4.1 or 4.9 in PMA-treated erythrocytes by Western blotting (results not shown); (2) our data show that the phosphorylated bands 4.1 and 4.9 co-migrate with the native uncleaved proteins in SDS gels; and (3) leupeptin also inhibited PMA-stimulated phosphorylation of cytosolic histone, and there was no evidence of any proteolysis of histone. Finally, our results in Figure 6 show that leupeptin has no inhibitory effect on the PMA-induced translocation of PKC to the plasma membrane.

Our results suggest that membrane association of PKC itself is not sufficient for phosphorylation of substrate proteins, since in the presence of leupeptin no phosphorylation results, even though PMA induced PKC translocation to the plasma membrane (compare Figures 4 and 6). What could account for the apparent inability of PKC to phosphorylate membrane skeletal proteins, even though it has translocated to the membrane? One possibility is that membrane-associated PKC cannot gain access to the appropriate substrates because it is bound to a membrane domain which is somehow separate from the membrane skeletal substrates. According to this hypothesis, calpain-induced cleavage of membrane-associated PKC could result in the release of soluble PKM, which could gain access to substrate proteins (e.g. [44]) or possibly re-bind to the membrane at another site. Another possibility is that the erythrocyte PKC isoenzyme(s) responsible for phosphorylation must be proteolysed before they can recognize their substrate proteins. Precedent for such an effect is found in the case of PKC-ϵ, which phosphorylates histone IIIs effectively only after its regulatory domain has been cleaved [45,46]. The specific PKC isoenzymes responsible for the phosphorylation of erythrocyte membrane proteins remain to be determined.

The involvement of leupeptin-inhibitable processes or calpain in PKC-mediated signalling has been demonstrated in several cell types. Leupeptin has been shown to inhibit hexamethylene-bisacetamide-induced differentiation of murine erythroleukaemia cells, presumably by blocking the production of PKM [20]. Similarly, leupeptin blocks PMA-induced phosphorylation of 48 kDa and 20 kDa proteins in neutrophils [19,47], although in this case both PKC and the 20 kDa substrate protein are susceptible to leupeptin-inhibitable proteolysis. Monoclonal antibodies to calpain have also been shown to result in decreased exocytosis by neutrophils, possibly related to the inhibition of PKM-induced phosphorylation of cytoskeletal proteins [48].

The inhibition of PKC cleavage by leupeptin has been studied in some detail [49]. Evidence suggests that the active, membrane-associated, form of PKC is susceptible to calpain cleavage, whereas the inactive form is not [10]. Thus we may hypothesize that the complete loss of erythrocyte cytosolic PKC resulting from prolonged PMA treatment is due to a process of continual recruitment of cytosolic PKC to the plasma membrane, where it is eventually cleaved by calpain. Our data (Z. Ai and C. M. Cohen, unpublished work) suggest, however, that after PMA treatment of erythrocytes there is only a small fraction of total cellular PKC ($\sim 5\%$) which is associated with the membrane at any time. This can also be seen from Figure 1, which shows that cytosolic PKC activity manifests no detectable decrease at the earliest sampling after PMA treatment of intact or reversibly haemolysed cells (Figure 1), even though membrane-associated PKC antigen was found to be elevated to near-maximal levels at that time (results not shown). Thus degradation of cytosolic PKC may result from the PMA-induced recruitment of PKC to a relatively small number of membrane binding sites, where it is cleaved and eventually lost from the membrane. By this mechanism the continued presence of PMA would ultimately lead to the loss of all cytosolic PKC after its transient appearance on the membrane.

A similar effect has been observed in neutrophils, in which it has been shown that Ca^{2+}-activation induces association of both

PKC and calpain with the plasma membrane, where both are activated [50,51]. The membrane-associated PKC is apparently cleaved by membrane-associated calpain, releasing active PKM [50,51]. As in the erythrocyte, stimulation of neutrophils by PMA results in the transient appearance of PKM, followed by its complete degradation and, ultimately, in complete disappearance of both PKC and PKM [18,52]. In neutrophils, inhibition of calpain enhances the appearance of membrane PKC, and decreases or blocks generation of PKM [48,52]. Neutral serine proteases have been implicated in the further degradation of neutrophil PKM [52].

Erythrocytes contain cytosolic calpain [26–28], which, in the presence of micromolar Ca^{2+}, binds to the plasma membrane, where it is active [53]. Cytosolic calpain activity is thought to be blocked by the endogenous calpain inhibitor calpastatin [54]. The process of calpain activation is still poorly understood (for reviews, see [28,29]), and it is unclear whether addition of PMA to erythrocytes induces PKM formation due to a change in susceptibility of PKC to calpain attack, or whether calpain itself is somehow activated by PMA. Further work will be needed to explain the apparently selective cleavage of PKC by calpain. Erythrocyte membrane skeletal proteins are excellent substrates for calpain (reviewed in [29]), yet are unaffected by the same process which results in PKC cleavage.

An issue which remains to be resolved is the relationship of our results in intact or resealed erythrocytes with results obtained with PKC and isolated erythrocyte membranes and proteins. Wolf et al. [55] have shown that purified rat brain PKC will bind to erythrocyte inside-out vesicles and will phosphorylate band 4.1. There was no suggestion that calpain-mediated proteolysis was required for that phosphorylation to take place. Moreover, we and others have shown previously that purified bands 4.1 and 4.9 can be phosphorylated in solution by purified rat brain or erythrocyte PKC [22,31–33], again with no evidence of calpain involvement. Finally, we have shown here that PKC in erythrocyte cytosol (lacking membranes) is not inhibited by up to 2 mM calpain added in solution. Several possible explanations for these apparent discrepancies need to be investigated. For example, it may be that erythrocyte membranes contain factors which prevent intact membrane-associated PKC, but not PKM, from phosphorylating membrane or cytosolic substrates. If such factors were lost upon preparation of inside-out vesicles, then added PKC would be able to phosphorylate membrane substrates, as shown by Wolf et al. [55]. It is also possible in the studies cited above that some PKM was generated during the activation of PKC, and that this PKM was responsible for the phosphorylation. Further studies will be needed to resolve these issues.

PMA-induced down-regulation of PKC has been observed in a variety of cells (see the Introduction). The unique feature of this process in erythrocytes is that the down-regulation must be irreversible, since erythrocytes lack the capacity to synthesize proteins. Although it is unknown whether other modes of activation of erythrocyte PKC would also result in such down-regulation, the possibility that this is the case would be of some importance to erythrocyte physiology. Thus, any event which results in the activation of erythrocyte PKC may at the same time result in the partial depletion of the intracellular pool of PKC. Since human erythrocytes survive for approx. 120 days in the circulation, it is possible that they could experience multiple PKC activation events during their lifespan. Thus the progressive down-regulation of PKC may be involved with erythrocyte senescence. Indeed, there is some evidence that both senescent and abnormal erythrocytes have decreased cytosolic PKC activity (see below).

Although there are no known physiological agonists which induce PKC activation in erythrocytes, it has been shown that elevation of intracellular Ca^{2+}, by use of a Ca^{2+} ionophore, will result in activation of erythrocyte PKC [25]. Since erythrocytes have been shown to experience transient elevations in intracellular Ca^{2+} induced by shear stress [56,57], it is possible that PKC may repeatedly be activated, and therefore progressively depleted, during the lifespan of the erythrocyte. The presence of small amounts of PKC on the membranes of non-PMA-treated erythrocytes seen in Figure 6 may be due to such effects. Consistent with this idea, fractionation of erythrocytes by density has shown that dense fractions, which contain the most senescent cells, have lower cytosolic PKC activity than the light fractions [58]. Similarly, sickle erythrocytes, which may experience greater than normal Ca^{2+} leakage, have also been shown to have decreased cytosolic PKC activity [59]. Because PKC affects the association of erythrocyte cytoskeletal proteins *in vitro* [23,32], it is possible that a progressive deficit of cytosolic PKC could contribute to the pathophysiology of erythrocyte senescence or sickle cell disease. Additional studies are needed to test these ideas directly.

In summary, our results suggest that a leupeptin-inhibitable process, possibly calpain-mediated proteolysis of PKC, is a necessary step in the phosphorylation of substrates by erythrocyte PKC. A similar phenomenon may occur in other cells types as well. Because of its simplicity, the erythrocyte is an ideal system for further exploration of this and other molecular aspects of PKC activation. Moreover, PKC activation and possible down-regulation may play key roles in the function and pathophysiology of the erythrocyte.

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