Protein kinase C mediates erythrocyte “programmed cell death” following glucose depletion

Barbara A. Klarl, Philipp A. Lang, Daniela S. Kempe, Olivier M. Niemoeller, Ahmad Akel, Malgorzata Sobiesiak, Kerstin Eisele, Marlis Podolski, Stephan M. Huber, Thomas Wieder, and Florian Lang

Department of Physiology, University of Tübingen, Tübingen, Germany

Submitted 14 June 2005; accepted in final form 12 August 2005

Glucose depletion of erythrocytes leads to activation of Ca\(^{2+}\)-permeable cation channels, Ca\(^{2+}\) entry, activation of a Ca\(^{2+}\)-sensitive erythrocyte scramblase, and subsequent exposure of phosphatidylserine at the erythrocyte surface. Ca\(^{2+}\) entry into erythrocytes was previously shown to be stimulated by phorbol esters and to be inhibited by staurosporine and chelerythrine and is thus thought to be regulated by protein phosphorylation/dephosphorylation, presumably via protein kinase C (PKC) and the corresponding phosphoserine/threonine phosphatases. The present experiments explored whether PKC could contribute to effects of energy depletion on erythrocyte phosphatidylserine exposure and cell volume. Phosphatidylserine exposure was estimated from annexin binding and cell volume from forward scatter in fluorescence-activated cell sorter analysis. Removal of extracellular glucose led to depletion of cellular ATP, stimulated PKC activity, led to translocation of PKC\(\alpha\), enhanced serine phosphorylation of membrane proteins, decreased cell volume, and increased annexin binding, the latter effect being blunted but not abolished in the presence of 1 \(\mu\)M staurosporine or 50 nM calphostin C. The PKC stimulator phorbol-12-myristate-13-acetate (3 \(\mu\)M staurosporine or 50 nM calphostin C) stimulated Ca\(^{2+}\) entry, increased annexin binding, and increased forward scatter, the latter effects being abrogated by PKC inhibitor staurosporine (1 \(\mu\)M). Fluoro-3 fluorescence measurements revealed that okadaic acid also mimicked the effect of glucose depletion. Recent experiments, however, pointed to the ability of phorbol ester-mediated protein kinase C (PKC) activation to stimulate erythrocyte Ca\(^{2+}\) entry (5) and phosphatidylserine exposure (19). PKC (EC 2.7.1.37) is a family of serine/threonine-specific protein kinases consisting of at least 10 members that require Ca\(^{2+}\), diacylglycerol, or a phospholipid for activation. PKC isoenzymes play an essential role in the regulation of diverse cellular functions including proliferation, differentiation, and apoptosis (45). It is known for a long time that human erythrocytes contain PKC mediating the phosphorylation of cytoskeletal proteins, such as band 4.1, 4.9, and adducin (17), and the human Na\(^{+}/\)H\(^{+}\) antiporter NHE-1 (11). To date, PKC\(\alpha\), PKC\(\xi\), PKC\(\mu\), and PKC\(\zeta\) have been reported to be expressed in erythrocytes (27). Upon activation, they influence cytoskeletal integrity and erythrocyte functions. However, besides the artificial activation of PKC by phorbol esters, no experimental data about the involvement of PKC activation in erythrocyte phosphatidylserine exposure are available.

We hypothesized that PKC may mediate the activation of pathways leading to breakdown of the plasma membrane asymmetry after cellular stress, such as glucose depletion. The present study has been performed to test this hypothesis.

MATERIALS AND METHODS

Cells, solutions, and chemicals. Erythrocytes were drawn from healthy volunteers. Informed consent was obtained from all volunteers and the study has been approved by the Ethical Committee of the Medical Faculty of the University of Tübingen. Erythrocyte concentrates were obtained from whole blood with the use of the OptiPure RC quadruple blood pack set equipped with a soft housing red cell filter from Baxter (Unterschleissheim, Germany). Platelet numbers in erythrocyte concentrates were routinely measured using an automated blood cell counter (CellDyn3000; Abbott, Wiesbaden, Germany). According to these measurements, erythrocyte concentrates contain 2.4 ± 0.2% (n = 22) of the original platelet number of whole blood. Leukocyte numbers in erythrocyte concentrates were quantified by flow cytometric analysis on a Coulter Epics XL (Beckman Coulter, Krefeld, Germany) using the internally normalized TrueCount kit from Becton Dickinson (Heidelberg, Germany). According to these measurements erythrocyte concentrates contain 0.012 ± 0.001% (n = 22) of the original leukocyte number of whole blood and can be

Thus erythrocytes exposing phosphatidylserine at their surface are prone to be eliminated from circulating blood.

Little is known about the signaling linking cell injury to Ca\(^{2+}\) entry and subsequent activation of the scramblase leading to exposure of phosphatidylserine. Recent experiments, however, pointed to the ability of phorbol ester-mediated protein kinase C (PKC) activation to stimulate erythrocyte Ca\(^{2+}\) entry (5) and phosphatidylserine exposure (19). PKC (EC 2.7.1.37) is a family of serine/threonine-specific protein kinases consisting of at least 10 members that require Ca\(^{2+}\), diacylglycerol, or a phospholipid for activation. PKC isoenzymes play an essential role in the regulation of diverse cellular functions including proliferation, differentiation, and apoptosis (45). It is known for a long time that human erythrocytes contain PKC mediating the phosphorylation of cytoskeletal proteins, such as band 4.1, 4.9, and adducin (17), and the human Na\(^{+}/\)H\(^{+}\) antiporter NHE-1 (11). To date, PKC\(\alpha\), PKC\(\xi\), PKC\(\mu\), and PKC\(\zeta\) have been reported to be expressed in erythrocytes (27). Upon activation, they influence cytoskeletal integrity and erythrocyte functions. However, besides the artificial activation of PKC by phorbol esters, no experimental data about the involvement of PKC activation in erythrocyte phosphatidylserine exposure are available.

We hypothesized that PKC may mediate the activation of pathways leading to breakdown of the plasma membrane asymmetry after cellular stress, such as glucose depletion. The present study has been performed to test this hypothesis.

MATERIALS AND METHODS

Cells, solutions, and chemicals. Erythrocytes were drawn from healthy volunteers. Informed consent was obtained from all volunteers and the study has been approved by the Ethical Committee of the Medical Faculty of the University of Tübingen. Erythrocyte concentrates were obtained from whole blood with the use of the OptiPure RC quadruple blood pack set equipped with a soft housing red cell filter from Baxter (Unterschleissheim, Germany). Platelet numbers in erythrocyte concentrates were routinely measured using an automated blood cell counter (CellDyn3000; Abbott, Wiesbaden, Germany). According to these measurements, erythrocyte concentrates contain 2.4 ± 0.2% (n = 22) of the original platelet number of whole blood. Leukocyte numbers in erythrocyte concentrates were quantified by flow cytometric analysis on a Coulter Epics XL (Beckman Coulter, Krefeld, Germany) using the internally normalized TrueCount kit from Becton Dickinson (Heidelberg, Germany). According to these measurements erythrocyte concentrates contain 0.012 ± 0.001% (n = 22) of the original leukocyte number of whole blood and can be
considered virtually free of white blood cells. Aliquots of the erythrocyte concentrates were stored at 4°C until usage. Some variation was encountered between different batches of erythrocytes. Thus all experiments were paralleled by appropriate controls and comparisons have been made to those controls within given batches of erythrocytes. Because no antibiotics were added to avoid possible interactions with the different assay systems, particular care was taken to avoid contamination and all experiments were run with appropriate time controls.

Experiments were performed with erythrocyte concentrates (0.3% hematocrit) at 37°C in a Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 32 HEPES/NaOH, 5 glucose, 1 CaCl2; pH 7.4. For the nominally Ca2+-free solution, CaCl2 was replaced by 1 mM EGTA. For glucose depletion, glucose (5 mM) was omitted from

Fig. 1. Time-dependent energy depletion and phosphatidylserine exposure of erythrocytes after treatment with 2-deoxyglucose (2-DOG), after glucose removal, or after treatment with 2-DOG in combination with glucose removal. A: means ± SE (n = 6) of ATP concentrations of erythrocytes after treatment with Ringer solution either in the absence (control) or presence of 5 mM 2-DOG or glucose-free Ringer either in the absence (0 mM glucose) or presence of 5 mM 2-deoxyglucose (2-DOG + 0 mM glucose). ATP concentration of Ringer-treated erythrocytes was 1.37 ± 0.12 mM (n = 6) and the time-dependent decrease of ATP concentration is given as %control. B: means ± SE (n = 6) of annexin-positive erythrocytes in percentage of the total population after different time points of treatment with Ringer solution either in the absence (control) or presence of 5 mM 2-DOG, or glucose-free Ringer either in the absence (0 mM glucose) or presence of 5 mM 2-DOG (2-DOG + 0 mM glucose).

Fig. 2. Stimulation of phosphatidylserine exposure at the erythrocyte surface by glucose depletion in the absence or presence of the kinase inhibitor staurosporine. A: histograms of annexin binding in a representative experiments of erythrocytes incubated for 48 h in Ringer solution (left), or in Ringer solution lacking glucose (right) in the absence (top) or presence (bottom) of 1 μM staurosporine. B: means ± SE (n = 6) of annexin binding of erythrocytes after a 48-h treatment with Ringer solution (left) or glucose-free Ringer (right) either in the absence (solid bars) or presence (open bars) of staurosporine. *P < 0.05, significant difference from Ringer; #P < 0.05, significance to glucose-depleted cells in the absence of staurosporine (ANOVA using Tukey’s test as post hoc test).
the Ringer solution and replaced by NaCl (2.5 mM). Alternatively, glucose-free Ringer solution was supplemented with 5 mM 2-deoxy-glucose from Sigma (Taufkirchen, Germany). Ionomycin was used at a concentration of 1/\mu M, phorbol 12-myristate-13-acetate (PMA) at a concentration of 3/\mu M, okadaic acid at a concentration of 1 and 10/\mu M, staurosporine and K252a at a concentration of 1/\mu M, and calphostin C at concentrations of 2.5–50 nM. The final concentration of the solvent DMSO was 0.1%. Ionomycin, PMA, calphostin C, and staurosporine were purchased from Sigma, and okadaic acid, K252a, and the Ca\textsuperscript{2+} dye Fluo-3 AM were from Calbiochem (Bad Soden, Germany). Staurosporine (1/\mu M) added alone did neither induce significant phosphatidylserine exposure (Fig. 2) nor significant cell shrinkage (Fig. 3).

**Measurement of intracellular Ca\textsuperscript{2+}**. Intracellular Ca\textsuperscript{2+} measurements were performed as described previously (5). Erythrocytes were loaded with Fluo-3 AM (Calbiochem) by addition of 2 \mu l of a Fluo-3 AM stock solution (2.0 mM in DMSO) to 1 ml of erythrocyte suspension (0.16% hematocrit in Ringer). Cells were incubated at 37°C for 15 min under protection from light. Subsequently, an additional 2 \mu l aliquot of Fluo-3 AM was added, and cells incubated for further 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1,000 g for 5 min at 22°C and washed twice with Ringer solution containing 0.5% bovine serum albumin (BSA; Sigma) and once with Ringer. Fluo-3-AM-loaded erythrocytes were resuspended in 1 ml Ringer (0.3% hematocrit) containing okadaic acid (10 \mu M) or ionomycin (1 \mu M) or vehicle alone (0.1% DMSO) and incubated for different time periods at 37°C. Ca\textsuperscript{2+}-dependent fluorescence intensity was then measured by flow cytometric analysis on a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson) in the fluores-

**Fig. 3. Decrease of forward scatter by glucose depletion in the absence or presence of the kinase inhibitor staurosporine.** A: histograms of forward scatter in a representative experiment of erythrocytes incubated for 48 h in Ringer solution (left), or in Ringer solution lacking glucose (right) in the absence (top) or presence (bottom) of 1 \mu M staurosporine. B: means ± SE (n = 10) of forward scatter of erythrocytes after a 48-h treatment with Ringer solution (left) or glucose-free Ringer (right) either in the absence (solid bars) or presence (open bars) of staurosporine. *P ≤ 0.05, significant difference from Ringer; #P ≤ 0.05, significance to glucose-depleted cells in the absence of staurosporine (ANOVA using Tukey’s test as a post hoc test).

the Ringer solution and replaced by NaCl (2.5 mM). Alternatively, glucose-free Ringer solution was supplemented with 5 mM 2-deoxyglucose from Sigma (Taufkirchen, Germany). Ionomycin was used at a concentration of 1 \mu M, phorbol 12-myristate-13-acetate (PMA) at a concentration of 3 \mu M, okadaic acid at a concentration of 1 and 10 \mu M, staurosporine and K252a at a concentration of 1 \mu M, and calphostin C at concentrations of 2.5–50 nM. The final concentration of the solvent DMSO was 0.1%. Ionomycin, PMA, calphostin C, and staurosporine were purchased from Sigma, and okadaic acid, K252a, and the Ca\textsuperscript{2+} dye Fluo-3 AM were from Calbiochem (Bad Soden, Germany). Staurosporine (1 \mu M) added alone did neither induce significant phosphatidylserine exposure (Fig. 2) nor significant cell shrinkage (Fig. 3).

**Measurement of intracellular Ca\textsuperscript{2+}**. Intracellular Ca\textsuperscript{2+} measurements were performed as described previously (5). Erythrocytes were loaded with Fluo-3 AM (Calbiochem) by addition of 2 \mu l of a Fluo-3 AM stock solution (2.0 mM in DMSO) to 1 ml of erythrocyte suspension (0.16% hematocrit in Ringer). Cells were incubated at 37°C for 15 min under protection from light. Subsequently, an additional 2 \mu l aliquot of Fluo-3 AM was added, and cells incubated for further 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1,000 g for 5 min at 22°C and washed twice with Ringer solution containing 0.5% bovine serum albumin (BSA; Sigma) and once with Ringer. Fluo-3-AM-loaded erythrocytes were resuspended in 1 ml Ringer (0.3% hematocrit) containing okadaic acid (10 \mu M) or ionomycin (1 \mu M) or vehicle alone (0.1% DMSO) and incubated for different time periods at 37°C. Ca\textsuperscript{2+}-dependent fluorescence intensity was then measured by flow cytometric analysis on a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson) in the fluores-

**Fig. 4. Stimulation of protein kinase C (PKC) activity by glucose depletion and inhibition of PKC activity by staurosporine in vitro.** A: PKC activity of erythrocyte extracts as a function of protein content. Means ± SE (n = 3) of the absorbance at 450 nm (E\textsubscript{450}). B: PKC activity of 20-ng purified PKC as a positive control. The means ± SE (n = 3) of the absorbance at 450 nm (E\textsubscript{450}). C: means ± SE (n = 6–9) of PKC activity of erythrocytes after a 24-h (left) or 48-h (right) treatment with Ringer solution (solid bars) or glucose-free Ringer (open bars). PKC activity is expressed as percentage of control (Ringer-treated erythrocytes). *P ≤ 0.05, significant difference from Ringer-treated erythrocytes (ANOVA using Tukey’s test as post hoc test). D: in vitro PKC activity in 20 \mu g of protein extracted from erythrocytes treated for 48 h with glucose-free Ringer in the presence of different concentrations of staurosporine. Means ± SE (n = 4) of PKC activity are expressed in percentage of control (activity in the presence of vehicle alone).
Annexin binding and forward scatter in FACS analysis. FACS analysis was performed essentially as described (3). After incubation, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES/NaOH, pH 7.4, and 5 CaCl₂. Erythrocytes were stained with Annexin-Fluos (Roche Diagnostics, Mannheim, Germany) at a 1:50 dilution. After 10 min, samples were diluted 1:5 and measured by flow cytometric analysis on a FACS-Calibur (Becton Dickinson). Cells were analyzed by forward scatter as a measure of cell size and annexin-fluorescence intensity was measured in FL-1. As shown previously, decrease of the forward scatter coincides with a reduction of the hematocrit, thereby indicating cell shrinkage (44).

Determination of intracellular ATP. Intracellular ATP concentrations were determined as described (8). After incubation, erythrocytes were washed (3 × 5 min) in phosphate-buffered saline (PBS), centrifuged, and 100 μl of the red blood cell pellet were lysed in distilled

Fig. 6. Forward scatter after exposure of erythrocytes to PMA and okadaic acid. A: histograms of forward scatter in a representative experiment of erythrocytes incubated for 24 h in Ringer solution (top), or in Ringer solution containing 1 μM okadaic acid (bottom) either in the absence (left) or presence of 3 μM PMA (right). B: means ± SE (n = 10) of forward scatter of erythrocytes after a 24-h treatment with Ringer solution (left) or with Ringer solution containing PMA (right) either in the presence of okadaic acid (solid bars) or absence (open bars) of okadaic acid. *P ≤ 0.05, significant difference from Ringer. #P ≤ 0.05, significance to the presence of okadaic acid alone (ANOVA using Tukey’s test as post hoc test).
PKC activity assay. Erythrocyte concentrates (5% hematocrit) were incubated for 24 or 48 h in the presence or absence of 5 mM glucose. After incubation, cells were collected by centrifugation at 1,100 g for 5 min, and washed once with 1 ml PBS. Then, 150-μl lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM EGTA, 1% Triton X-100 and a cocktail of protease inhibitors (Roche Diagnostics) was added. The samples were incubated for 30 min on ice and cell debris was pelleted at 22,000 g, 4°C for 15 min. Protein concentration of the clear supernatant was determined by the Bradford method (Bio-Rad, Munich, Germany) with BSA (Sigma) as a standard. PKC activity in erythrocyte extracts was measured using the StressXpress PKC Kinase Activity Assay Kit from Stressgen, which was purchased from Biomol (Hamburg, Germany). To this end, 5–20 μg cellular protein in a kinase assay dilution buffer were added to PKC substrate microtiter plates and the reaction was initiated by the addition of 2 mM ATP. After incubation for 60 min at 30°C, the reaction was stopped by emptying the contents of each well. Forty microliters of phosphospecific substrate antibody were then added, samples were incubated for 45 min at room temperature, and washed four times with wash buffer. The samples were incubated for another 30 min on ice and centrifuged at 1,100 g for 5 min. Protein concentration of the supernatant was determined with the Bradford method. After being blocked with 5% nonfat dried milk at room temperature for 1 h, the blots were probed overnight at 4°C with a monoclonal anti-PKCα antibody. Positions of molecular weight markers are indicated at the left of each blot. The arrows indicate the position of PKCα. A: cytosolic (left blot) or membrane (right blot) erythrocyte protein extracts were obtained as described in (A) and probed with the use of a monoclonal anti-phosphoserine antibody. Representative immunoblots from 3 independent experiments are shown in A and B.

Fig. 7. PMA and okadaic acid induce PKCα translocation and enhance the level of serine phosphorylation of erythrocyte membrane proteins. A: after treatment for 24 h with Ringer solution (Co), or Ringer solution containing 3 μM PMA, 1 μM okadaic acid (Oka), or 3 μM PMA + 1 μM okadaic acid (PMA+Oka), cytosolic (left blot) or membrane (right blot) erythrocyte protein extracts were prepared and 1 μg (cytosol) or 50 μg (membrane) of protein were loaded per lane. Translocation of PKCα was then analyzed by Western blot using a monoclonal anti-PKCα antibody. Positions of molecular weight markers are indicated at the left of each blot. The arrows indicate the position of PKCα. B: cytosolic (left blot) or membrane (right blot) erythrocyte protein extracts were obtained as described in (A) and probed with the use of a monoclonal anti-phosphoserine antibody. Representative immunoblots from 3 independent experiments are shown in A and B.

water and proteins were precipitated by HClO4 (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated KHCO3 solution. All manipulations were performed at 4°C to avoid ATP degradation. After dilution of the supernatant, the ATP concentration of the aliquots was determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics) and a luminometer (Biolumat LB9500, Berthold, Bad Wildbad, Germany). After incubation, cells were collected by centrifugation at 1,100 g, 4°C for 5 min, and washed once with 1 ml PBS. Then, 150-μl lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM EGTA, 1% Triton X-100 and a cocktail of protease inhibitors (Roche Diagnostics) composed of 10 μg/ml peptatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.1 mM PMFS were added. The samples were incubated for 30 min on ice and cell debris was pelleted at 22,000 g, 4°C for 15 min. Protein concentration of the clear supernatant was determined by the Bradford method (Bio-Rad, Munich, Germany) with BSA (Sigma) as a standard. PKC activity in erythrocyte extracts was measured using the StressXpress PKC Kinase Activity Assay Kit from Stressgen, which was purchased from Biomol at 1:200 or at 1:1,000 dilution, respectively. After being blocked with 5% nonfat dried milk at room temperature for 1 h, the blots were probed overnight at 4°C with a monoclonal anti-PKCα antibody (clone 3) from BD Biosciences Pharmingen (Heidelberg, Germany) or a monoclonal anti-phosphoserine antibody from Biomol at 1:200 or at 1:1,000 dilution, respectively. After being washed, the blots were incubated for 1 h at room temperature with a sheep anti-mouse IgG antibody (1:1,000 dilution) conjugated with horseradish peroxidase from Amersham Biosciences (Freiburg, Germany). After being washed, antibody binding was detected with the enhanced chemoluminescence ECL kit from Amersham Biosciences.
RESULTS

To deplete erythrocytes from ATP they were treated for different time periods with the glycolysis inhibitor 2-deoxyglucose (2-DOG) either in the presence or absence of 5 mM glucose. As shown in Fig. 1A, treatment of erythrocytes with 2-DOG alone led to a steady decline of intracellular ATP levels reaching ~50% of control after 48 h. Energy depletion was faster when the cells were starved by removal of glucose from the medium. The most rapid decline of ATP was observed reaching 2-DOG alone led to a steady decline of intracellular ATP levels after 6 h of incubation (Fig. 1A). Parallel measurements of annexin binding revealed that induction of “programmed erythrocyte cell death” clearly lagged behind energy depletion (Fig. 1B). According to these measurements, an increase of phosphatidylserine-exposing cells was first observed after 24 h and was best seen after 48 h, irrespective of the accelerated rate of ATP depletion after addition of 2-DOG. Removal of glucose (for 24 or 48 h) was used in the following experiments as the standard procedure to study the effects of energy depletion in more detail.

Energy depletion by removal of extracellular glucose for 48 h significantly increased the number of annexin binding cells (Fig. 2, A and B). Interestingly, the elevated number of phosphatidylserine exposing cells was blunted albeit not fully abolished by 1 μM staurosporine (Fig. 2, A and B), pointing to the involvement of PKC in the triggering of phosphatidylserine exposure following glucose depletion. Removal of glucose also reduced the forward scatter in FACS analysis reflecting cell shrinkage (Fig. 3A; top right histogram). Similar to its effect on phosphatidylserine exposure, 1 μM staurosporine significantly inhibited energy depletion-induced shrinkage by 24% (Fig. 3B).

To further investigate the role of PKC in erythrocyte “programmed cell death,” kinase activity assays were performed. As shown in Fig. 4A, erythrocyte extracts indeed contained significant amounts of PKC activity. More importantly, incubation of erythrocytes in glucose-free Ringer for 24 and 48 h significantly increased PKC activity by 31 and 65%, respectively (Fig. 4C). The enhanced kinase activity in cell extracts from glucose-depleted erythrocytes was staurosporine-sensitive, i.e., inhibited by staurosporine in vitro (Fig. 4D). These data were confirmed using the specific PKC inhibitor calphostin C (15). As illustrated in Fig. 5A, calphostin C inhibited glucose depletion-induced phosphatidylserine exposure with an IC50 of 12 nM. Similarly, enhanced PKC activity was concentration-dependently blunted by calphostin C (Fig. 5B).

Additional experiments were performed to explore whether enhanced PKC-dependent protein phosphorylation and/or reduced protein dephosphorylation triggers phosphatidylserine exposure of erythrocytes. As shown in Fig. 5, C and D, activation of PKC by the phorbol ester PMA (3 μM) and inhibition of phosphoserine/threonine phosphatases by okadaic acid (1 μM) both increased the number of annexin-binding proteins, pointing to a role of PKC in the regulation of phosphatidylserine exposure.

Statistics. Data are expressed as arithmetic means ± SE and statistical analysis was made by paired or unpaired two-tailed t-test or ANOVA, as appropriate. *P ≤ 0.05 was considered statistically significant.
erythrocytes. The combined application of PMA and okadaic acid led to an increase of annexin binding, which was significantly larger than the effect of either PMA or okadaic acid alone (Fig. 5D). The effect of PMA and okadaic acid on annexin binding was paralleled by a significant decrease of forward scatter (Fig. 6, A and B). The effect of okadaic acid on the forward scatter was significantly enhanced (although to a lesser extent compared with annexin binding) by PMA, and maximal cell shrinkage was observed after combined application of the phosphatase inhibitor and the kinase activator (Fig. 6B; right bar). Thus stimulation of protein phosphorylation by PKC and inhibition of phosphoserine/threonine phosphatases favor erythrocyte “programmed cell death.”

Next, Western blot analyses were carried out to elucidate the PKC isoforms involved in the erythrocyte death pathway. Figure 7A depicts a complete translocation of PKCα from the cytosol to the membrane after stimulation with PMA. Partial translocation of PKCα was also observed after okadaic acid treatment, whereas the combination of PMA and okadaic acid again led to complete translocation of the kinase. In accordance with an enhanced kinase activity, translocation of PKCα coincided with hyperphosphorylation of distinct membrane protein bands as detected by the use of an anti-phosphoserine antibody. As expected, the highest level of hyperphosphorylation was observed after treatment with the kinase activator PMA in
combination with the phosphatase inhibitor okadaic acid (Fig. 7B).

We now explored whether the same events were triggered after glucose depletion. Indeed, removal of glucose from the medium led to partial translocation of PKCζ from the cytosol to the membrane, an effect which was enhanced by PMA (Fig. 8A). More importantly and despite reduced ATP levels (see Fig. 1A), incubation of erythrocytes in glucose-free medium led to enhanced serine phosphorylation of membrane proteins (Fig. 8B; right plot, lanes 0Glc and PMA+0Glc). As further shown in Fig. 8C, hyperphosphorylation of membrane proteins coincided with an increase of annexin binding. Thus it appears safe to conclude that activation of PKC by translocation plays a role in erythrocyte death signaling after glucose depletion.

The following experiments aimed to clarify the effects of the phosphatase inhibitor okadaic acid on erythrocyte programmed cell death. Similar to the broad-spectrum kinase inhibitor K252a (not shown), the nonselective PKC inhibitor staurosporine at a concentration of 1 µM fully abolished the effect of okadaic acid on annexin binding (Fig. 9, A and B). As shown in Fig. 9C, calphostin C (15 nM) significantly blunted okadaic acid-induced annexin binding by 72%, thereby confirming the inhibitory effect of staurosporine. Furthermore, staurosporine also abrogated the effect of okadaic acid on forward scatter (Fig. 10, A and B) and calphostin C significantly inhibited okadaic acid-induced erythrocyte shrinkage (Fig. 10C). Taken together, these data indeed point to the stimulation of erythrocyte programmed cell death by protein phosphatase inhibition, which is abrogated by suppression of PKC activity.

Finally, the mechanism of okadaic acid-induced erythrocyte death was further investigated by measuring intracellular Ca²⁺ after challenge with the phosphatase inhibitor. According to Fluo-3 fluorescence, exposure of erythrocytes to okadaic acid increased the percentage of cells with enhanced cellular Ca²⁺ activity by a factor of 4.4 (Fig. 11, A and B). As a positive control, the Ca²⁺ ionophore ionomycin similarly increased cytosolic Ca²⁺ activity (Fig. 11, A and B). Thus, inhibition of protein phosphatases mimics the effect of protein kinase activators, e.g., phorbol esters, on intracellular Ca²⁺.

DISCUSSION

The present experiments provide evidence for a role of PKC in the regulation of programmed death of erythrocytes, a cellular mechanism that is characterized by breakdown of the plasma membrane asymmetry, by shrinkage, and by activation of proteolytic enzymes, e.g., calpains (6, 37). These changes of cellular physiology occur in the absence of substantial hemolysis and a simple lytic mechanism of phosphatidylserine exposure has been ruled out (38). Furthermore, previous studies (36, 44) showed that cellular loss of monovalent ions (K⁺ and Cl⁻) are a prerequisite for erythrocyte shrinkage and phosphatidylserine exposure. Programmed death of erythrocytes has recently been named eryptosis to distinguish this form of cell death from apoptosis of nucleated cells (31). Our observations confirm the previous studies demonstrating the effect of phorbol esters on Ca²⁺ entry (4) and phosphatidylserine exposure (19). In addition, we show here that the effects of phorbol esters are mimicked by inhibition of phosphatases with okadaic acid (see Figs. 5, 6, and 11). The effects of okadaic acid are fully abrogated by the PKC inhibitor staurosporine (see Figs. 9 and 10). Thus Ca²⁺ entry and erythrocyte phosphatidylserine exposure are regulated by a delicate balance of protein phosphorylation and dephosphorylation.

More importantly, the present observations expand the previous knowledge by the demonstration that glucose depletion of erythrocytes leads to stimulation of staurosporine- and calphostin C-sensitive PKC activity (see Fig. 4 and Fig. 5, respectively), an effect that has also been observed in coronary endothelial monolayers (43). Interestingly, enhanced phosphorylation of proteins occurred at decreased ATP levels. This is in accordance with an earlier study (55) demonstrating that cellular ATP depletion leads to activation of PKC in a human biliary cell line. Activation of PKC was confirmed by Western blot analysis demonstrating glucose depletion-induced translocation of the kinase to erythrocyte cell membranes (see Fig. 8). Staurosporine and calphostin C further blunt the effects of energy depletion or okadaic acid treatment on erythrocyte phosphatidylserine exposure and cell shrinkage (see Figs. 2, 3, 5, 9, and 10), thereby functionally linking these events to PKC activation. Our observations therefore suggest that PKC participates in the regulation of eryptosis after energy depletion or phosphatase inhibition. However, staurosporine only partially blunts the stimulating effect of glucose deprivation on annexin binding, contrasting its full effect on the annexin binding after treatment with okadaic acid. Thus energy depletion apparently stimulates annexin binding not exclusively by activation of PKC. Along those lines, previous studies have established that hyperosmotic shock triggers two independent cellular mecha-
nisms fostering activation of erythrocyte scramblase, the activation of a Ca\(^{2+}\)-permeable channel leading to entry of Ca\(^{2+}\) (21, 30, 32) and the activation of a sphingomyelinase with subsequent formation of ceramide, which sensitizes the erythrocyte scramblase for Ca\(^{2+}\) (33).

The exposure of phosphatidylserine at the cell surface favors the binding to respective phosphatidylserine receptors expressed by macrophages (26, 28, 41). Binding to these receptors triggers engulfment and subsequent degradation of the affected erythrocytes (9, 23, 48). Thus erythrocytes exposing phosphatidylserine at their surface will be cleared from circulating blood. Moreover, those erythrocytes may bind to receptors in the vascular wall and thus impede microcirculation (4, 5, 24, 47, 50, 51). Along those lines, we observed enhanced trapping of erythrocytes in renal medulla after ischemia of the mouse kidney (34). Phosphatidylserine-exposing cells may further participate in hemostasis (4, 7, 39, 47, 54).

As has been established earlier, red cells are to some extent always permissible to Ca\(^{2+}\), a phenomenon referred to as the “calcium pump leak” (25). Besides its effect on the scramblase, increase of cytosolic Ca\(^{2+}\) could thereby modify the cytoskeleton (46, 52, 53), activate a transglutaminase leading to cross-linking of proteins (2, 13), and stimulate phospholipases (1, 49), protein kinases, and phosphatases (16, 42), as well as proteases such as calpain (2). The degradation of membrane proteins by calpain may participate in the machinery leading to erythrocyte death (6, 12, 18, 32, 35, 36, 40, 57) or may be involved in erythrocyte senescence.

In conclusion, the present observations provide several lines of evidence, i.e., functional experiments using different PKC inhibitors, PKC translocation, and activity assays, and serine phosphorylation studies, for the involvement of PKC in the regulation of erythrocyte programmed cell death. Stressors like glucose depletion lead to activation of PKC, which in turn activates the cation channels presumably by direct phosphorylation of the channel proteins. The subsequent entry of Ca\(^{2+}\) leads to activation of the Ca\(^{2+}\)-sensitive scramblase, phosphatidylserine exposure, and thus eryptosis. However, the incomplete inhibitory effect of staurosporine on annexin binding of glucose-depleted cells points to further mechanisms involved in the triggering of erythrocyte scramblase.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of E. Faber and the meticulous preparation of the manuscript by Tanja Loch and Lejla Subasic. The authors thank Dr. Marc Waidmann (University of Tübingen, Germany) for kindly providing highly purified erythrocyte concentrates.

GRANTS

This study was supported by Deutsche Forschungsgemeinschaft Grant La 315/4–3, La 315/6–1, and La315/13–1, the Center for Interdisciplinary Clinical Research Grant 01 KS 9602, and the Biomed program of the European Union Grant BMH4–CT96–0602.

REFERENCES

25. Etzion Z, Tiffert T, Bookchin RM, and Lew VL. Effects of deoxygenation on active and passive Ca\textsuperscript{2+} transport and on the cytoplasmic Ca\textsuperscript{2+} levels of sickle cell anemia red cells. *J Clin Invest* 92: 2489–2498, 1993.


