Stimulation of erythrocyte ceramide formation by platelet-activating factor

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Summary

Osmotic erythrocyte shrinkage leads to activation of cation channels with subsequent Ca^{2+} entry and stimulates a sphingomyelinase with subsequent formation of ceramide. Ca^{2+} and ceramide then activate a scramblase leading to breakdown of phosphatidylserine asymmetry of the cell membrane. The mediators accounting for activation of erythrocyte sphingomyelinase and phosphatidylserine exposure remained elusive. The study demonstrates that platelet-activating factor (PAF) is released from erythrocytes upon hyperosmotic cell shrinkage. The experiments further disclose the presence of PAF receptors in erythrocytes and show that PAF stimulates the breakdown of sphingomyelin and the release of ceramide from erythrocytes at isotonic conditions. PAF further triggers cell shrinkage (decrease of forward scatter) and phosphatidylserine exposure (annexin binding) of erythrocytes. The stimulation of annexin-binding is blunted by a genetic knockout of PAF receptors, by the PAF receptor antagonist ABT491 or by inhibition of sphingomyelinase with urea. In conclusion, PAF activates an erythrocyte sphingomyelinase and the then formed ceramide leads to the activation of scramblase with subsequent phosphatidylserine exposure.

Key words: Cell volume, Annexin, Apoptosis, Sphingomyelinase, Phosphatidylserine

Introduction

Osmotic erythrocyte shrinkage activates an amiloride-sensitive, Ca^{2+}-permeable cation channel (Duranton et al., 2002; Lang et al., 2003). The subsequent elevation of intracellular Ca^{2+} activity triggers exposure of phosphatidylserine, membrane blebbing and cell shrinkage (Berg et al., 2001; Bratosin et al., 2001; Lang et al., 2003), events, all typical for apoptosis of nucleated cells (Green et al., 1998; Gulbins et al., 2000). Programmed death of erythrocytes after hyperosmotic shock occurs in the absence of substantial haemolysis and caspase activation (Weil et al., 1998; Berg et al., 2001; Lang et al., 2004a) thereby representing a 'stripped down' cellular system to investigate caspase-independent suicide mechanisms.

Ca^{2+} entry into erythrocytes is regulated at least in part by protein kinase C-dependent pathways (Yang et al., 2000; Andrews et al., 2002) finally leading to breakdown of phosphatidylserine asymmetry by activation of a Ca^{2+} sensitive scramblase (Woon et al., 1999). Because macrophages are equipped with receptors specific for phosphatidylserine (Fadok et al., 2000), erythrocytes exposing phosphatidylserine at their surface are rapidly recognised, engulfed and degraded (Eda et al., 2002). Thus, an increase of cytosolic Ca^{2+} activity could trigger clearance of damaged, 'apoptotic' erythrocytes by phagocytosis.

Phosphatidylserine exposure of osmotically shrunken cells is only partially inhibited by the cation channel inhibitor amiloride or in the nominal absence of Ca^{2+}, pointing to additional mechanisms contributing to scramblase activation of those cells (Lang et al., 2003). In fact, osmotic shrinkage leads to the activation of an erythrocyte sphingomyelinase and the subsequent formation of ceramide (Lang et al., 2004a). Ceramide, a lipid-derived second messenger that is involved in the signalling pathways of apoptosis in several cell types (Goldkorn et al., 1998; Frago et al., 1998; Raisova et al., 2000; Kolesnick et al., 2003; Ogretmen et al., 2004), sensitises the erythrocyte scramblase for cytosolic Ca^{2+} thus triggering phosphatidylserine exposure even without Ca^{2+} entry (Lang et al., 2004a). The mechanisms leading to activation of the sphingomyelinase, however, remain elusive.

Platelet-activating factor (PAF), a phospholipid mediator involved in the regulation of inflammation, thrombosis, atherogenesis and cardiovascular function (Bussolino et al., 1989; Montrucchio et al., 1993; Subbanagounder et al., 1999; Montrucchio et al., 2000; Zimmerman et al., 2002; Haynes, et al., 2002; Goggel et al., 2004), is released from erythrocyte progenitor cells upon increase of cytosolic Ca^{2+} activity (Dupuis et al., 1997). PAF further activates Ca^{2+}-sensitive K^+ channels (Gardos channels) in the erythrocyte cell membrane (Garay et al., 1986) by sensitising them for the stimulating effects of cytosolic Ca^{2+} (Rivera et al., 2002). This study was therefore designed to explore the role of PAF in the

pathways leading to scramblase-mediated breakdown of phosphatidylserine asymmetry in human erythrocytes.

Materials and Methods

Blood cells and purification of erythrocytes

Human whole blood was drawn from healthy volunteers. Informed consent was obtained from all patients 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 using the OptiPure RC quadruple blood pack set equipped with a soft-housing red-cell filter from Baxter (Unterschleissheim, Germany). Briefly, 500 ml of whole blood were automatically mixed with 70 ml CPD buffer containing 3.27 mg/ml citric acid, 26.30 mg/ml sodium citrate, 2.50 mg/ml sodium dihydrogenphosphate dihydrate and 25.50 mg/ml dextrose monohydrate. Blood components were separated by centrifugation at 4795 ***g*** for 10 minutes at 22°C. Plasma, buffy coat and erythrocytes were then pressed into the respective blood bags. During this process SAG-M stabiliser solution containing 8.77 mg/ml NaCl, 9.00 mg/ml dextrose monohydrate, 0.17 mg/ml adenine and 5.25 mg/ml mannitol was added to the erythrocytes and the erythrocytes were passed through the integrated leukocyte depletion filter at room temperature. Aliquots of the erythrocyte concentrates were stored at 4°C until usage.

Animal experiments were made according to German animal protection law and approved by the local authorities. Murine erythrocytes were drawn from PAF-receptor knockout (PAF-$R^{-/-}$) mice and corresponding PAF receptor wild-type (PAF-$R^{+/+}$) littermates by retro-orbital venipuncture and used after purification over Ficoll as described below. PAF-$R^{-/-}$ mice were established using a gene targeting strategy (Ishii et al., 1998). PAF-$R^{-/-}$ mice and the corresponding PAF-$R^{+/+}$ mice have been backcrossed for ten generations onto a C57BL/6N genetic background. Mice were fed with a standard diet and water ad libitum. Female mice at the age of 12 to 13 weeks were used for retro-orbital venipuncture.

Analysis of blood cell numbers in whole blood and in erythrocyte concentrates

Platelet numbers in whole blood, erythrocyte concentrates and also in leukocytes in whole blood were measured in an automated blood cell counter (CellDyn3000; Abbott GmbH, Wiesbaden, Germany). The cells were identified by a combination of their optical and electrical properties in the appropriate counter medium. 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 normalised TrueCount kit from Becton Dickinson (Heidelberg, Germany). The kit is based on the detection of nucleated cells by the fluorescent DNA-intercalator propidium iodide. 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 (WBC).

Furthermore, platelet numbers in whole blood and in erythrocyte concentrates were determined by flow cytometric analysis of thiazole-orange-stained cells (Kienast et al., 1990) using the Retic-Count (Thiazole orange) reagent from Becton Dickinson according to the manufacturer's instructions. Measurements were performed on a FACS-Calibur (Becton Dickinson) and the number of cells in the thrombocyte gate of the respective forward scatter (FSC) versus the intensity of thiazole-orange-fluorescence (FL-1H) dot plots was determined using the CellQuest™ software.

Erythrocytes, platelets and leukocytes of whole blood and erythrocyte concentrates were further quantified in a MDM 905 electronic haematology particle counter (Medical Diagnostics Marx, Butzbach, Germany).

Solutions

Experiments were performed at 37°C in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)-NaOH, 5 mM glucose, 1 mM $CaCl_2$ pH 7.4. In some experiments, osmolarity was increased to 850 mOsm by adding sucrose. Osmolarity was measured with a VAPRO 5520 vapor pressure osmometer (Wescor, Logan, Utah, USA). 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF16) was from Calbiochem (Schwalbach, Germany) and 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF18) was from Sigma (München, Germany). The purity of PAF16 and PAF18 was >99% and >98%, respectively, as determined by thin-layer chromatography with silica gel G plates and the solvent system chloroform:methanol:water (65:35:6, v/v/v). Stock solutions of PAF16 [1.9 mM in ethanol or 2.0 mM in dimethyl sulfoxide (DMSO)] and PAF18 (1.8 mM in ethanol) were prepared and diluted into Ringer solution to yield the final concentrations. Final concentrations of ethanol and DMSO did not exceed 0.3% and 0.1%, respectively, and appropriate amounts of the solvent were added to controls. Sphingomyelinase (SMase) from *Streptomyces sp.*, D-erythro-*N*-hexanoylsphingosine (C_6-ceramide), the selective PAF antagonist ABT-491 hydrochoride (4-ethynyl-N,N-dimethyl-3-[3-fluoro-4-[(2-methyl-1H-imidazo-[4,5-c]pyridin-1-yl)methyl]benzoyl]-1H-indole-1-carboxamide hydrochloride) and the phospholipase A_2 inhibitor quinacrine were purchased from Sigma. C_6-ceramide was used at concentrations of 20 μM and 50 μM and the final concentration of the solvent DMSO was 0.1%.

ABT-491 hydrochloride (1 μM) and quinacrine (25 μM) were dissolved in Ringer solution with final solvent concentrations of 0.1% aqua dest. and 0.1% DMSO, respectively. As shown previously, sphingomyelinase treatment (0.01 U/ml) of erythrocytes leads to phosphatidylserine exposure and cell shrinkage, both effects being blunted in the absence of extracellular Ca^{2+} (Lang et al., 2004a). The efficacy of quinacrine to block phospholipase A_2 was demonstrated by inhibition of hyperosmotic-shock-induced PGE_2-formation in erythrocytes by 96±9% (n=3). After incubation of up to 6 hours, quinacrine (25 μM) did not induce annexin-binding in erythrocytes [1.9±0.2% (n=6) annexin-positive cells] but significantly reduced annexin-binding after osmotic shock by 41±8% (n=6).

Human red-blood-cell membrane-preparation and western blotting

Expression of the PAF receptor was determined by western blot analysis. For this, erythrocytes were purified [centrifuging whole blood for 25 minutes at 2000 ***g*** over Ficoll (Biochrom KG, Berlin, Germany)], washed three times in PBS and centrifuged at 450 ***g*** for 5 minutes. Erythrocyte preparations were checked for platelet and white blood cell contaminations with an electronic haematology particle counter (MDM 905). Purified erythrocyte preparations contained 2.9±0.2% (n=3) platelets and 4.7±0.3% (n=3) white blood cells of the respective whole blood samples. To remove the haemoglobin, 200 μl of the red-blood-cell (RBC) pellet were haemolysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors [2.5 mM EDTA, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) from Roche Diagnostics (Mannheim, Germany)]. Ghost membranes were pelleted (30,000 ***g*** for 20 minutes at 4°C) and lysed in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholic acid, 1% Triton X-100, 10 μl β-mercaptoethanol. The protein concentration of the samples was determined by using the Bradford

method (Biorad, München, Germany) with bovine serum albumin (BSA; Sigma) as standard. Lysates were separated by 10% SDS-PAGE (50 μg protein per lane), and transferred to Protan BA83 nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany). Protein transfer was controlled by Ponceau Red staining (Fig. 1A). After blocking with 5% nonfat-dried milk at room temperature for 1 hour, the blots were probed overnight at 4°C with a polyclonal goat anti-PAF receptor antibody against the N-terminus of the human PAF receptor (PAF-R (N-17); Santa Cruz Biotechnology, Santa Cruz, California) at 1:200 dilution. The blots were incubated overnight in the presence or absence of 10 μg/ml specific blocking peptide (Santa Cruz Biotechnology) mimicking the N-terminus of the human PAF receptor. After washing, the blots were incubated with a donkey anti-goat antibody (1:2000 dilution) conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature. Antibody-binding was detected with the enhanced chemoluminescence ECL kit (Amersham Biosciences, Freiburg, Germany). To test for contamination of the RBC membrane preparations by platelets, western blot analysis was performed in parallel using a monoclonal mouse anti-human CD61 antibody (Clone PM6/13; dilution 1:200) from Serotec (Oxford, UK) combined with a sheep anti-mouse antibody (1:1000 dilution) conjugated with horseradish peroxidase (Amersham Biosciences).

Determination of PAF release

Erythrocytes (10^8) were treated with 90 μl hypertonic Ringer solution (850 mOsm) or isotonic Ringer solution (300 mOsm) as control. After incubation, lipids were extracted by a modified method of Bligh and Dyer (Lang et al., 2004a). Methanol (150 μl) and chloroform (125 μl) were added to the erythrocyte suspension. Samples were stirred on a vortex mixer and centrifuged at 13,000 ***g*** for 2 minutes. Phase separation was accomplished by the addition of 125 μl chloroform and 125 μl water. The suspension and centrifugation steps were repeated, and 100 μl of the chloroform phases were transferred to scintillation vials and dried under nitrogen. Then, PAF was determined in the PAF [^{3}H] scintillation proximity assay (SPA) system from Amersham Biosciences based on the competition of 1-O-[^{3}H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine ([^{3}H]PAF) with non-radioactive PAF for binding to a specific anti-PAF antibody coupled to fluomicrospheres according to the manufacturer's instructions. [^{3}H]PAF bound to the fluomicrospheres was counted in a β-scintillation counter (Wallac, Freiburg, Germany) and the concentration of PAF in the samples was calculated from a PAF calibration curve (20-1280 pg/tube) which was run in parallel (Fig. 2A).

FACS analysis

FACS analysis was performed as described (Andree et al., 1990). Erythrocyte concentrates (0.3% haematocrit) were treated with different concentrations of PAF16, PAF18 or with other agonists and antagonists as indicated. After incubation, cells were washed in annexin binding buffer containing 125 mM NaCl, 10 mM HEPES pH 7.4 and 5 mM $CaCl_2$. Erythrocytes were stained with Annexin-Fluos (Roche Diagnostics) at a 1:100 dilution. After 15 minutes, samples were diluted 1:5, thoroughly mixed on a vortex mixer to achieve single-cell suspensions, and measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson). Cells were analysed by FSC and intensity of annexin-fluorescence was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

To determine ceramide, a monoclonal antibody-based assay was used that has been validated by other groups (Grassme et al., 2002; Bieberich et al., 2003). After incubation of erythrocyte concentrates (0.3% haematocrit), cells were stained for 1 hour at 4°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) or 1 μg/ml isotype matched pure mouse IgM antibody (Ancell, Bayport, MN, USA) in PBS containing 1% foetal calf serum (FCS) at a dilution of 1:5 as described (Lang et al., 2004a). After three washes with the PBS-FCS, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-FCS. Unbound secondary antibody was removed by repeated washing with PBS-FCS. Samples were then analysed by flow cytometric analysis with a FACS-Calibur. Mean intensity values of FITC-fluorescence in the fluorescence channel FL-1 were determined by using the CellQuest™ software. Isotype-matched pure mouse antibody did not display an increased fluorescence-intensity in FL-1 (data not shown).

Intracellular Ca^{2+} measurements were performed as described (Andrews et al., 2002). Ten millilitres of erythrocyte suspension (0.16% haematocrit) were loaded with the Ca^{2+}-indicator Fluo-3/AM (Calbiochem) by adding 10 μl of a Fluo-3/AM stock solution (2.0 mM in DMSO). After incubation at 37°C for 15 minutes, while shaking vigorously in the dark, an additional 10 μl of Fluo-3/AM was added and incubation continued for a further 25 minutes. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 ***g*** for 3 minutes at 22°C and washed twice with Ringer solution containing 0.5% BSA, and once with plain Ringer solution. Fluo-3/AM-loaded erythrocytes (0.32% haematocrit) were treated with 3.8 μM PAF16, or with 1 μM ionomycin (Sigma) as a positive control to increase intracellular Ca^{2+}. After incubation, cells were analysed by flow cytometry in a FACS-Calibur and intensity of Ca^{2+}-dependent fluorescence was measured in the fluorescence channel FL-1.

Determination of cellular ceramide

After stimulation with PAF16, the samples were extracted in chloroform:methanol:HCl (1 M) (100:100:1) and the lower phase was collected and dried. Diacylglycerol was then degraded by alkaline hydrolysis of the samples in 100 μl of 0.1 M methanolic KOH at 37°C for 60 minutes. The samples were extracted again, dried and resuspended in 20 μl of detergent solution [7.5% (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepenta-acetic acid (DETAPAC)]. The samples were then sonicated for 10 minutes in a bath sonicator and the kinase reaction was initiated with 70 μl reaction mix consisting of 10 μl DAG-kinase (Amersham Biosciences) in 5 mM potassium phosphate buffer (pH 7.0) 10% glycerol, 1 mM 2-mercaptoethanol, 0.005 M imidazole/HCl, 0.5 mM DETAPAC (pH 6.6), 50 μl assay buffer [0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM $MgCl_2$ and 2 mM EGTA], 2.8 mM DTT, 5 μM ATP and 10 μCi [^{32}P]gammaATP. The kinase reaction was performed for 30 minutes at room temperature. The reaction was terminated with 1 ml of chloroform:methanol:HCl (1 M) (100:100:1). One hundred and seventy microlitres of a buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES pH 7.2) and 30 μl of a 100 mM EDTA-solution were added. The samples were extracted, the lower phases collected, dried, dissolved in 20 μl of chloroform:methanol (1:1) and separated on a Silica G60 TLC plate with chloroform:methanol:acetic acid (65:15:5). The plate was exposed, ceramide identified by co-migration with an identical standard, scrapped from the plate and quantified by liquid scintillation counting. The amount of ceramide was determined by comparing it with a ceramide standard curve of C_{16}-ceramide (Biozol; Eching, Germany).

Choline-labelling of erythrocytes

Choline-labelling of erythrocytes was performed as described recently (Lang et al., 2004a). Briefly, erythrocyte concentrates (10% haematocrit) were incubated for 72 hours in the presence of 7.4×10^4 Bq/ml [^{3}H]methylcholine chloride (Amersham Biosciences). After labelling, cells were washed twice with Ringer solution, seeded again

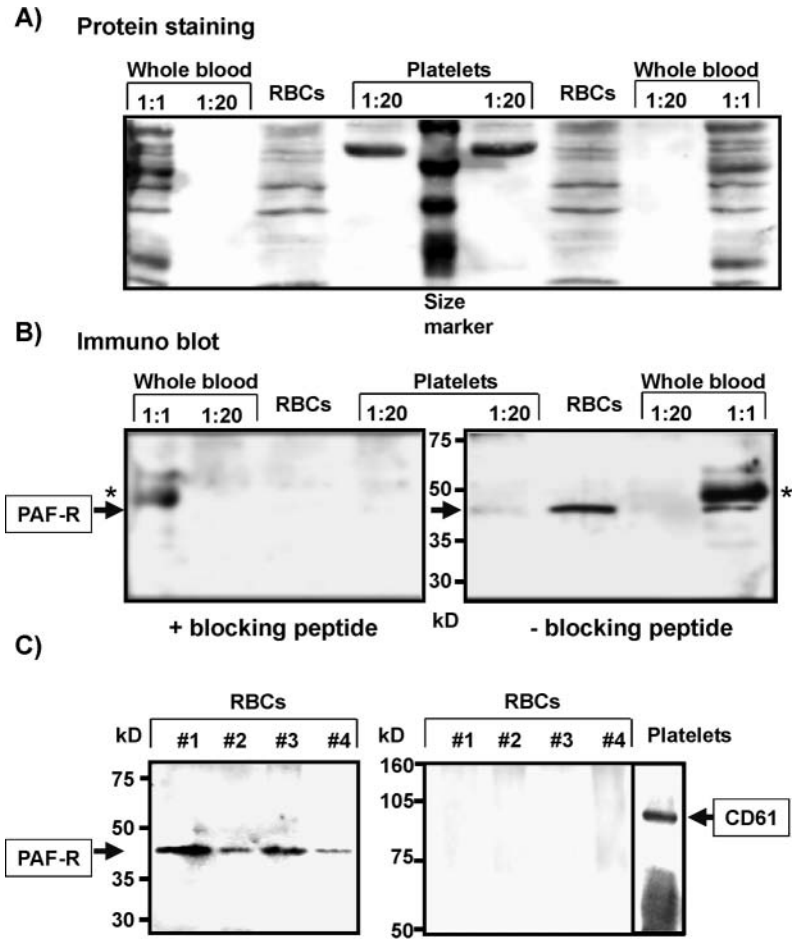

Fig. 1. Expression of the PAF receptor in erythrocytes. (A) Ponceau-Red protein-staining of a western blot of protein extracts from whole blood, diluted whole blood (1:20), purified erythrocytes (RBCs) or diluted platelets (1:20). Fifty microgramms or 2.5 μg (1:20) of protein were loaded per lane. The purified erythrocyte preparation contained 2.9% platelets and 4.7% white blood cells of the original whole blood sample. The molecular mass marker (size marker) is shown in the middle of the blot. (B) Erythrocyte protein extracts were prepared as described in A. Expression of the PAF receptor was then analysed by western blot with a polyclonal goat anti-PAF-receptor antibody in the presence (left) or absence (right) of specific blocking peptide. Positions of molecular mass markers are indicated in the middle. The arrow indicates the position of the PAF receptor. The asterisk indicates a non-specific band in whole blood extracts. (C) Erythrocyte protein extracts from the blood of four different donors (# 1-4) were prepared and 50 μg of protein were loaded per lane. Expression of the PAF receptor was then analysed by western blot with a polyclonal goat anti-PAF-receptor antibody (left blot). Additionally, 50 μg of the same erythrocyte extracts were loaded per lane and a platelet extract was included as positive control (Platelets). Expression of the platelet marker CD61 was analysed by western blot with a monoclonal mouse anti-CD61 antibody (right blot). Positions of molecular mass markers are indicated at the left of each blot. The arrows indicate the positions of PAF receptor (left blot) and CD61 (right blot). Representative immunoblots of three independent experiments are shown.

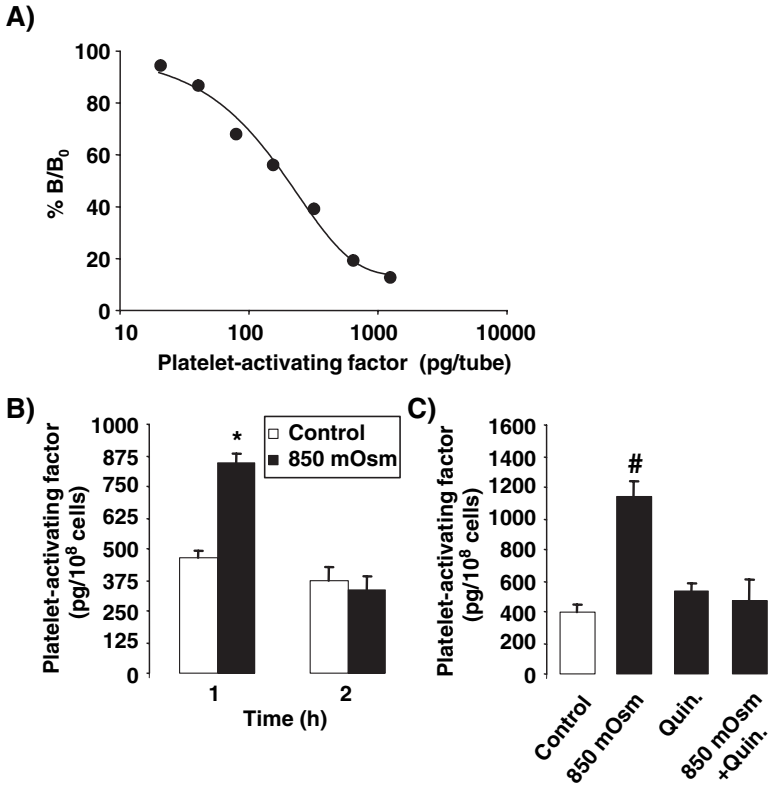

Fig. 2. Increase of PAF by hyperosmotic shock of erythrocytes. (A) Calibration curve of PAF using the [^{3}H] SPA system. The normalised % of bound PAF (% B/B_0) is plotted as a function of the $\log_{10}$ PAF concentration. Data points represent the mean of two determinations with an error less than 5% by using purified PAF as standard (20-1280 pg/tube). (B) Erythrocytes were treated with isotonic Ringer or hypertonic solution (850 mOsm) for 1 or 2 hours. Lipids were then extracted and PAF in the samples was determined with the [^{3}H] SPA system. Arithmetic means ± s.e.m. (n=8-12) of PAF concentrations in erythrocytes exposed to isotonic (control, white bars) or hypertonic (850 mOsm, black bars) extracellular fluid are given in pg/10^8 cells. * indicates significant difference from control (two-tailed t-test. $P \leqslant 0.05$). (C) Erythrocytes were treated with isotonic Ringer or hypertonic solution (850 mOsm) for 1 hour in the presence or absence of 25 μM quinacrine. Arithmetic means ± s.e.m. (n=4) of PAF concentrations in erythrocytes exposed to vehicle-containing isotonic (control) or vehicle-containing hypertonic (850 mOsm) extracellular fluid, to isotonic extracellular fluid in the presence of 25 μM quinacrine (Quin.) or to hypertonic extracellular fluid in the presence of 25 μM quinacrine (850 mOsm + Quin.). Values are given in pg/ 10^8 cells; # indicates significant difference from control (ANOVA, using Dunnett's test as post hoc-test. $P \leqslant 0.05$).

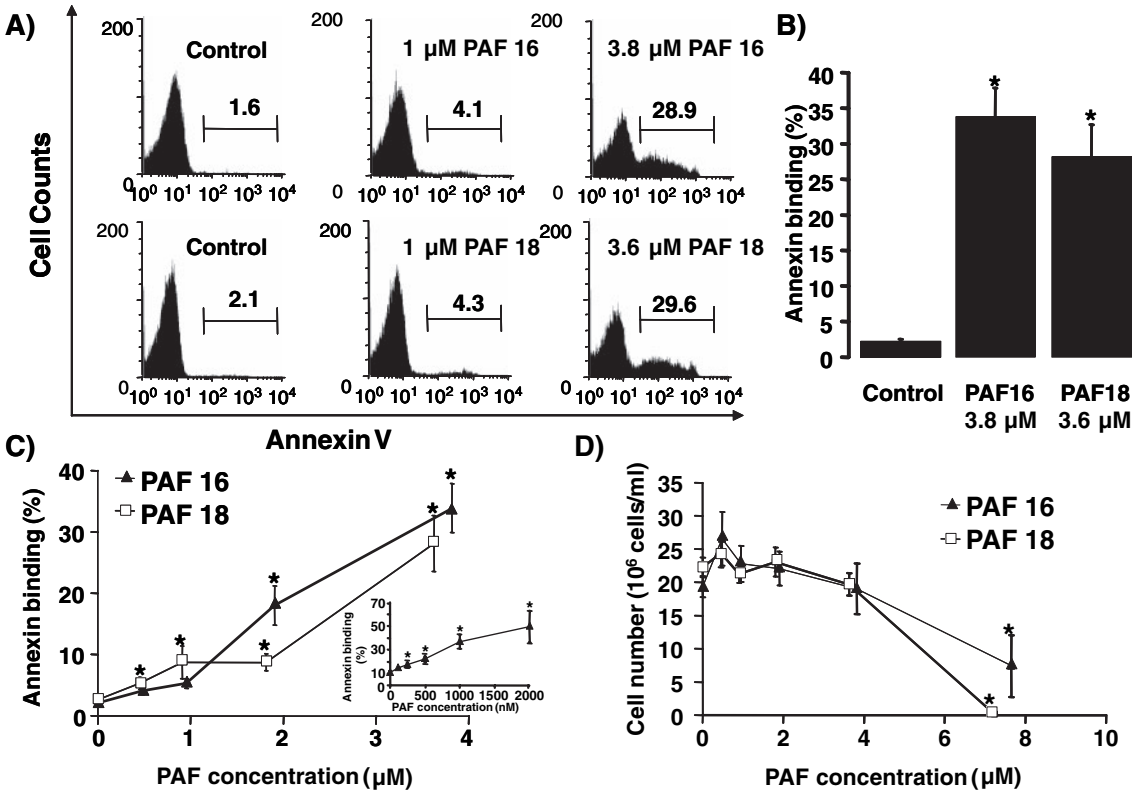

Fig. 3. Stimulation of erythrocyte annexin-binding by PAF and influence of PAF on cell number. (A) Histograms of FITC-annexin-V-binding as obtained by FACS analysis of a representative experiment of erythrocytes incubated for 24 hours in Ringer solution containing 0.3% ethanol as control (control), in Ringer solution + PAF16 (1 µM PAF16; 3.8 µM PAF16) and in Ringer solution + PAF18 (1 µM PAF18; 3.6 µM PAF18). Numbers depict the percentage of annexin-positive cells. (B) FITC-annexin-V-binding in % of the total population as obtained by FACS analysis of erythrocytes after a 24-hour treatment with Ringer solution containing 0.3% ethanol as control (control) or after incubation with 3.8 µM PAF16 or 3.6 µM PAF18 (arithmetic means±s.e.m., n=8). * indicates significant difference from control (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$). (C) FITC-annexin-V-binding of erythrocytes in % of the total population as obtained by FACS analysis (arithmetic means±s.e.m., n=6-8), treated for 24 hours with different concentrations (0.45-3.8 µM) of PAF16 (▲) or PAF18 (□). Controls (no PAF) contained appropriate amounts of ethanol. Additionally, erythrocytes were preincubated with 25 µM quinacrine for 3 hours, and PAF16 (▲) was diluted to the appropriate concentrations (125–2000 nM) from 2 mM DMSO stock solutions. FITC-annexin-V-binding in % of the total population as obtained by FACS analysis (arithmetic means ± s.e.m., n=3-5) is depicted in the insert. * indicates significant difference from controls (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$). (D) Number of erythrocytes seeded at 0.3% haematocrit and treated for 24 hours with different concentrations (0.45-7.6 µM) of PAF16 (▲) or PAF18 (□). The number of erythrocytes (arithmetic means ± s.e.m., n=6-8) after treatment is given in 10^6 cells/ml. Controls (no PAF) contained appropriate amounts of DMSO vehicle. * indicates significant difference from controls (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$).

at 2×10^8 cells/ml in control Ringer solution or in Ringer solution containing 3.8 µM PAF16 or 3.6 µM PAF18. After incubation, cells were washed twice using 1 ml of plain Ringer solution. Lipids were extracted by a modified method of Bligh and Dyer (Lang et al., 2004a) as described above. Of the chloroform phases, 20 µl were taken for scintillation counting, and 50 µl of the chloroform phases were dried under nitrogen and used for sphingomyelin measurements as follows.

Bacterial sphingomyelinase assay

Sphingomyelin was quantified using bacterial sphingomyelinase to release [^{3}H]phosphocholine as described (Jayadev et al., 1994). For this, cellular lipids were resuspended in 100 µl assay buffer (100 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 0.1% Triton X-100). Samples were sonicated and 1 U/ml *Streptomyces sp.* sphingomyelinase (Sigma) was added. Reaction mixtures were incubated for 2 hours at 37°C. Reactions were stopped with 1 ml chloroform/methanol (2:1, v/v). Phase-separation was completed by adding 100 µl water. Sphingomyelin was quantified by counting the upper, aqueous phase, containing the liberated [^{3}H]phosphocholine, and phosphatidylcholine was quantified by drying and counting the lower, organic phase. Where appropriate, sphingomyelin was normalised by using phosphatidylcholine measurements. Blank reactions contained no sphingomyelinase. The radioactivity of control samples reached 1790 dpm/10^9 cells and was set as 100%. Subsequently, sphingomyelin concentration in the samples of PAF-treated cells was calculated as percent (%) of control. Optimization studies illustrated that the above conditions yielded maximal (100%) sphingomyelin hydrolysis (data not shown).

Determination of cell numbers

Erythrocyte concentrates (0.3% haematocrit) were incubated with increasing concentrations of PAF16, PAF18 or Triton X-100. After incubation, the cell number was determined using a haemocytometer.

Statistics

Data are expressed as arithmetic means ± s.e.m. and statistical

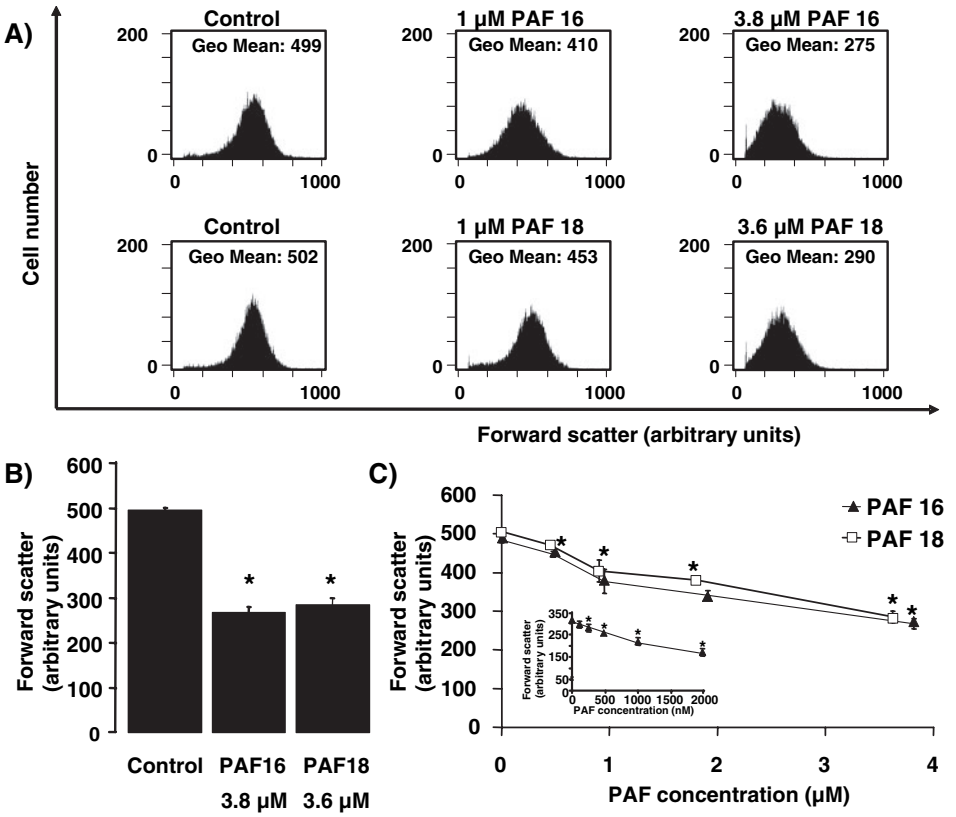

Fig. 4. Effect of PAF on erythrocyte cell volume. (A) Histograms of forward-scatter in FACS analysis of a representative experiment of erythrocytes incubated for 24 hours in Ringer solution containing 0.3% ethanol as a control (control), in Ringer solution + PAF16 (1 µM PAF16; 3.8 µM PAF16) and in Ringer solution + PAF18 (1 µM PAF18; 3.6 µM PAF18). Numbers depict the geometric mean of the forward scatter of the cell population. (B) Erythrocyte forward scatter in FACS analysis after a 24-hour treatment with Ringer solution containing 0.3% ethanol (control) and after incubation with 3.8 µM PAF16 or 3.6 µM of PAF18 (means ± s.e.m., n=8). * indicates significant difference from control (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$). (C) Forward scatter in FACS analysis of erythrocytes (means ± s.e.m., n=6-8), treated for 24 hours with different concentrations (0.45-3.8 µM) of PAF16 (▲) or PAF18 (□). Controls (0 µM PAF) contained appropriate amounts of ethanol. Additionally, erythrocytes were preincubated with 25 µM quinacrine for 3 hours and PAF16 (▲) was diluted to the appropriate concentrations (125-2000 nM) from 2 mM DMSO stock solutions. Forward scatter in FACS analysis (means ± s.e.m., n=3-5) is depicted in the insert and is given in arbitrary units. * indicates significant difference from controls (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$).

analyses were made by using paired or unpaired t-tests, or by using ANOVA with Dunnett's or Tukey's tests as the post-hoc test, where appropriate.

Results

Recently, evidence was provided that erythrocytes undergo a specialised form of programmed cell death but the physiological regulators remained largely unknown (Berg et al., 2001; Bratosin et al., 2001). Because PAF is one of the main mediators of thrombosis and atherogenesis, we hypothesised that PAF and its receptor have a functional role in erythrocytes. To test for PAF-receptor expression in erythrocytes, western blot analysis of membrane extracts from Ficoll-separated erythrocytes, platelets and whole blood was performed by using an anti-human PAF-receptor antibody. Indeed, an immunoreactive band migrating at the expected size of 40 kDa was detected in platelets, purified erythrocytes (RBCs) as well as in whole blood (Fig. 1B, right blot). The antibody-derived signal at 40 kDa vanished after the addition of a tenfold excess of specific blocking-peptide (Fig. 1B, left blot). The band also disappeared when whole blood was diluted by a factor of 20. Thus, when the RBC preparations were depleted of platelets by a factor of 34 and of leukocytes by a factor of 21, the immunoreactive band in the RBC preparation could not originate from platelet or leukocyte contaminations. In a second set of experiments, western blot analysis of RBC extracts from four healthy donors showed again PAF-receptor expression (Fig. 1C, left blot) whereas RBC extracts were free of the immunoreactive platelet marker CD61 (Fig. 1C, right blot).

To investigate the physiological role of PAF in a well defined cellular environment with negligible amounts of other cell types, erythrocyte concentrates were prepared with OptiPure RC quadruple blood pack sets. Erythrocyte concentrates used in this study contained 2.4±0.2% (n=22) of the original platelet number and 0.012±0.001% (n=22) of the original leukocyte number of the respective whole blood. Unless otherwise stated, all experiments described below were performed with highly purified erythrocyte concentrates.

The results from a scintillation proximity assay show that, erythrocyte concentrates contain significant amounts of PAF, reaching 460±29 pg/10^8 cells (n=12; Fig. 2B). The amount of PAF was significantly increased to 840±75 pg/10^8 cells (n=12) by an 1-hour hyperosmotic shock. The elevated PAF concentration returned to baseline levels after 2 hours of hyperosmotic shock (Fig. 2B). As shown in Fig. 2C, the release of PAF was abrogated in the presence of phospholipase A_2 inhibitor quinacrine. Taken together, the data on PAF-receptor expression and PAF formation suggest that PAF signalling plays a role in RBC (patho-)physiology, at least after osmotic stress.

Next, we tested whether direct application of PAF to the culture medium mimics the effect of hyperosmotic shock on phosphatidylserine exposure. Treatment of erythrocyte concentrates with 3.8 µM PAF16 led to a steep increase of annexin-binding cells, which reflects exposure of phosphatidylserine (Fig. 3A,B). In a separate set of control

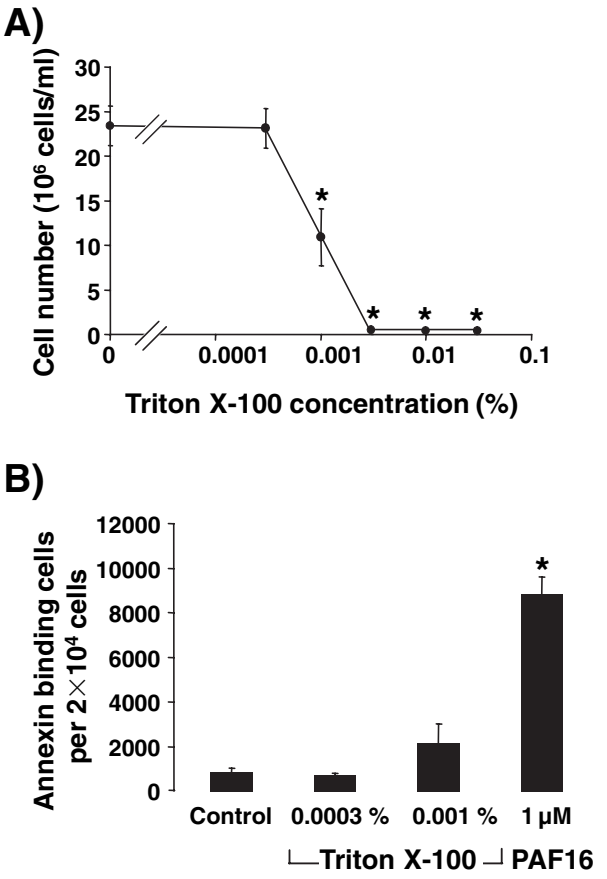

Fig. 5. Influence of Triton X-100 on cell number and erythrocyte-annexin-binding. (A) Number of erythrocytes seeded at 0.2% haematocrit and treated for 24 hours with different concentrations (0.0003%-0.03%) of Triton X-100 (●). The number of erythrocytes after treatment is given in 10^6 cells/ml (arithmetic means ± s.e.m., n=6). Controls (0% Triton X-100) were treated with Ringer solution. * indicates significant difference from controls (ANOVA, using Dunnett's test as post-hoc test; $P\leqslant0.05$). (B) Number of FITC-Annexin-V-binding cells per 20,000 cells as obtained by FACS analysis of erythrocytes after a 24-hour treatment with Ringer solution or after incubation with 0.0003% or 0.001% Triton X-100 (arithmetic means ± s.e.m., n=6). As a positive control, erythrocytes were treated with 1 µM PAF16 diluted from a 2 mM DMSO stock solution. * indicates significant difference from vehicle-treated control (ANOVA using Dunnett's test as post hoc test; $P\leqslant0.05$).

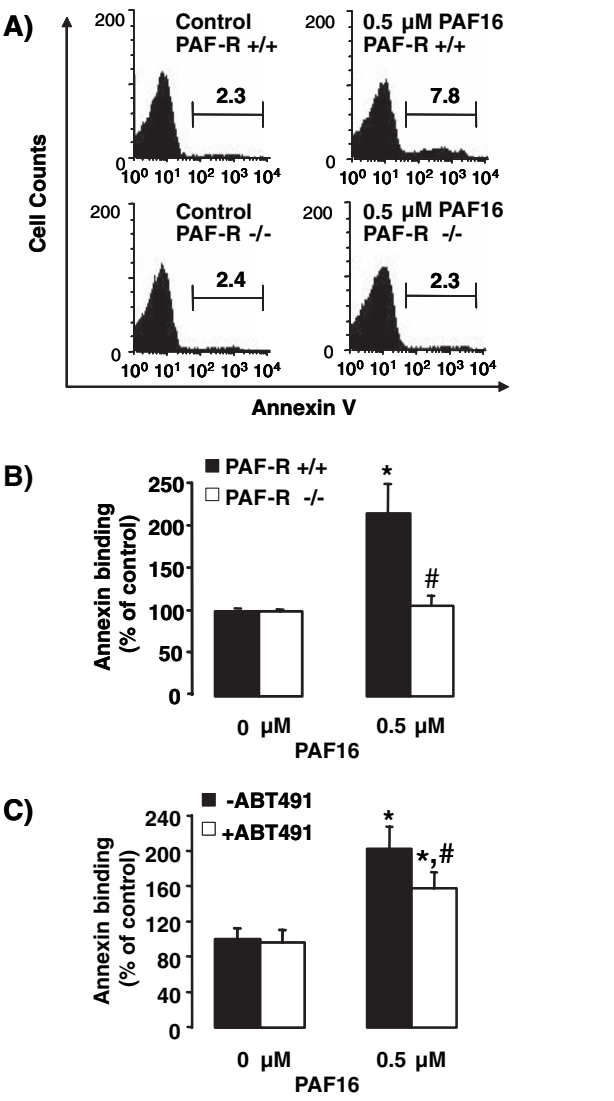

Fig. 6. Knockout of PAF-receptor abrogates and treatment with the PAF-receptor antagonist ABT491 blunts PAF-induced stimulation of erythrocyte-annexin-binding. (A) Histograms of FITC-Annexin-V-binding as obtained by FACS analysis in a representative experiment of murine erythrocytes from a wild-type mouse (PAF-$R^{+/+}$; upper histograms) or a PAF receptor knockout mouse (PAF-$R^{-/-}$; lower histograms) incubated for 3 hours in Ringer solution containing DMSO vehicle (left histograms, control) or in Ringer solution containing 0.5 µM PAF16 (right histograms). Numbers give the percentage of annexin-positive cells. (B) FITC-Annexin-V-binding in % of control as obtained by FACS analysis of murine erythrocytes from PAF-$R^{+/+}$ (black bars) or PAF-$R^{-/-}$ mice (white bars) after a 3-hour treatment with Ringer solution containing DMSO vehicle (0 µM), or after incubation with 0.5 µM PAF16 (arithmetic means ± s.e.m., n=10). * indicates significant difference from the respective control erythrocytes; # indicates significant difference from PAF-treated erythrocytes of PAF-$R^{+/+}$ mice (ANOVA, using Tukey's test as post-hoc test; $P\leqslant0.05$). (C) FITC-Annexin-V-binding in % of control as obtained by FACS analysis of human erythrocytes (arithmetic means ± s.e.m., n=8), treated for 24 hours with Ringer solution containing DMSO vehicle (0 µM) or 0.5 µM PAF16 in the absence (black bars) or presence (white bars) of 1 µM ABT491. * indicates significant difference from the respective control erythrocytes; # indicates significant difference from PAF-treated erythrocytes in the absence of ABT491 (ANOVA, using Tukey's test as post-hoc test; $P\leqslant0.05$).

experiments, the increase of annexin-binding cells following a 24-hour treatment with 2 µM PAF16 (compared with 0.1% DMSO as control) in erythrocyte concentrates and in the corresponding whole-blood suspensions was similar [fourfold (n=8) and fourfold (n=8), respectively]. Therefore, PAF is an inducer of erythrocyte scramblase and the presence of platelets or white blood cells does not significantly modify the PAF-induced effect.

The PAF analogues PAF16 and PAF18 were similarly effective (Fig. 3B) showing activity in the range of 500 nM to 4 µM (Fig. 3C). However, erythrocyte numbers remained constant up to these concentrations (Fig. 3D), indicating that phosphatidylserine exposure occurred in the absence of haemolysis. However, exposure of the cells to higher concentrations of PAF16 or PAF18 (7.6 or 7.2 µM,

respectively) led to substantial haemolysis (Fig. 3D). Pretreatment of erythrocyte concentrates with 25 μM quinacrine – to reduce endogenous PAF formation and the use of DMSO to dissolve PAF16 further – sensitised the cells for PAF16-induced phosphatidylserine exposure. In those experiments, a significant effect of PAF16 was observed at 250 nM and treatment with 2 μM PAF16 induced phosphatidylserine exposure in 50±13% (n=4) of the cells (Fig. 3C, insert).

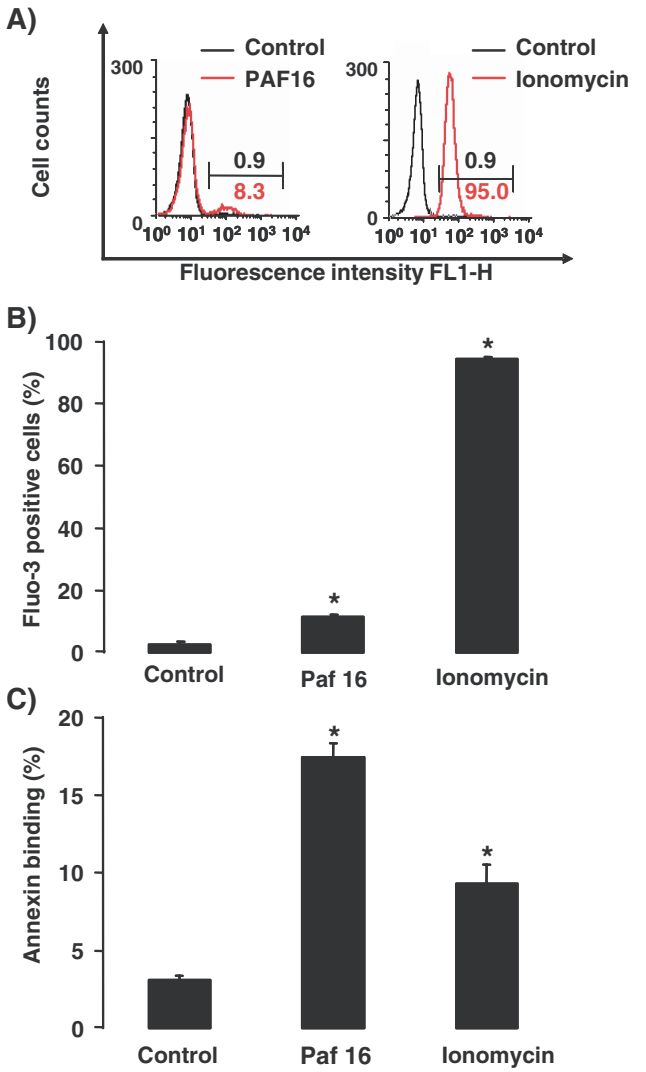

Fig. 7. Intracellular Ca^{2+}-activity and annexin-binding of erythrocytes following treatment with PAF and ionomycin. Erythrocytes were loaded with Fluo-3/AM and intracellular Ca^{2+} was determined by FACS analysis, as described in Materials and Methods, after a 30-minute treatment with 3.8 μM PAF16 or 1 μM ionomycin. Additionally, annexin-binding was determined in parallel by FACS analysis. (A) Representative histograms of Ca^{2+}-dependent fluorescence in FL1 of vehicle-treated erythrocytes (black line), of PAF16-treated erythrocytes (red line) or of ionomycin-treated erythrocytes (red line). Numbers give the percentage of Fluo-3 positive cells. (B) Arithmetic means ± s.e.m. (n=4) of Fluo-3-positive cells in % of the total population of vehicle-treated erythrocytes (control), PAF-treated erythrocytes (PAF16) or ionomycin-treated erythrocytes (ionomycin). * indicates significant difference from control erythrocytes (ANOVA, using Dunnett's test as post-hoc test; $P \leq 0.05$). (C) Arithmetic means ± s.e.m. (n=4) of FITC-Annexin-V-binding in % of the total population of vehicle-treated erythrocytes (control), PAF-treated erythrocytes (PAF16) or ionomycin-treated erythrocytes (ionomycin). * indicates significant difference from control erythrocytes (ANOVA, using Dunnett's test as post-hoc test; $P \leq 0.05$).

Treatment with PAF also led to a decrease of the forward-scatter (Fig. 4A) reflecting cell shrinkage. Again, PAF16 and PAF18 were similarly effective (Fig. 4B,C) and the susceptibility of erythrocytes towards PAF16-induced cell shrinkage was increased by pretreatment with 25 μM quinacrine (Fig. 4C, insert).

Using Triton X-100, we excluded that annexin-binding in response to PAF treatment is simply because of detergent-like disturbance of the erythrocyte membrane. Whereas Triton X-100 clearly led to haemolysis (Fig. 5A), neither non-lytic concentrations (0.0003%) nor lytic concentrations (0.001%) of Triton X-100 significantly increased the number of annexin-binding erythrocytes (Fig. 5B).

To test whether the observed effects of PAF are because of PAF-receptor-dependent signalling, experiments were performed with erythrocytes from wild-type (PAF-$R^{+/+}$) and PAF-receptor knockout (PAF-$R^{-/-}$) mice. Similar to human erythrocytes, treatment of erythrocytes from PAF-$R^{+/+}$ mice with 0.5 μM PAF16 led to significant exposure of phosphatidylserine (Fig. 6A, upper right). Compared to vehicle-treated controls, Annexin-binding of PAF-$R^{+/+}$ erythrocytes was enhanced two-fold (n=10) after treatment with PAF16 (Fig. 6B). By contrast, PAF-$R^{-/-}$ erythrocytes were insensitive towards treatment with PAF16 (Fig. 6A, lower right and Fig. 6B). To further explore PAF-induced annexin binding, the PAF receptor antagonist ABT491 was used in human erythrocyte concentrates. As shown in Fig. 6C, 1 μM ABT491 significantly blunted PAF16-induced annexin-binding by approximately 44%.

Exposure of phosphatidylserine in erythrocytes is triggered by two mechanisms: (1) by activation of a Ca^{2+}-permeable cation channel, and (2), by elevation of ceramide levels following sphingomyelinase activation. We first investigated the role of the cation channel. Treatment of erythrocyte concentrates with PAF16 led to an increase of intracellular Ca^{2+} activity in a small but significant population of the cells (Fig. 7A,B). This was accompanied by enhanced annexin-binding (Fig. 7C). However, the effect of PAF16 on intracellular Ca^{2+} was small compared with the effect of the Ca^{2+}-ionophore ionomycin. However, PAF16 induced stronger binding of annexin than ionomycin (Fig. 7C). Thus, the elevation of intracellular Ca^{2+} could not fully account for the PAF-induced effects. Next, we tested whether PAF influences the formation of ceramide. Exposure of erythrocytes to PAF16 (3.8 μM) or PAF18 (3.6 μM) increased their binding to an anti-ceramide antibody (Fig. 8A) and the mean fluorescence of anti-ceramide FITC-staining was significantly increased compared with control erythrocytes [two-fold and two-fold, respectively (Fig. 8B). As a positive control, erythrocytes were incubated for 5 minutes in the presence of 1 U/ml sphingomyelinase leading to a similar fluorescence shift (Fig. 8A,B; SMase). To ascertain ceramide formation after treatment with PAF, ceramide levels were also measured biochemically, by using DAG kinase. According to these measurements, the ceramide levels increased from 200 ± 15 pmol/10^6 erythrocytes (n=3) in control cells to 350 ± 25 pmol/10^6 erythrocytes (n=3) after 18 hours, and to 390 ± 28 pmol/10^6 erythrocytes (n=3) after 24 hours of PAF treatment.

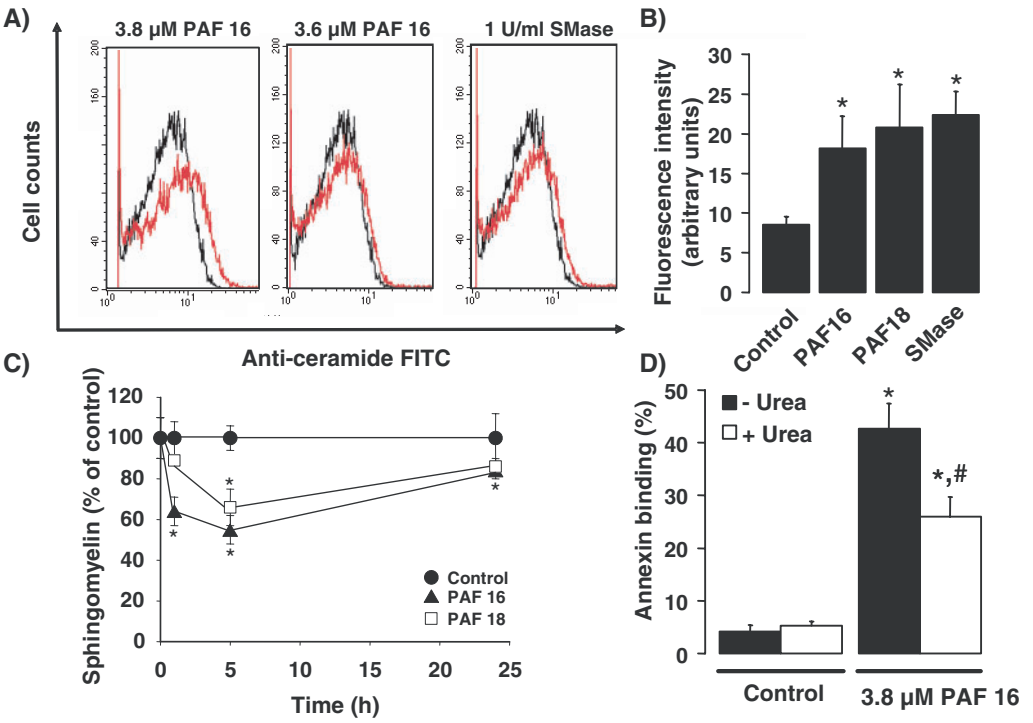

Fig. 8. Induction of ceramide formation and sphingomyelin breakdown in erythrocytes following PAF treatment and inhibition of PAF-induced annexin-binding of erythrocytes by urea. (A) Histograms of anti-ceramide FITC-coupled-antibody-binding as obtained by FACS analysis in a representative experiment of erythrocytes incubated for 24 hours in Ringer solution containing 0.3% ethanol (all histograms; black lines), in Ringer solution + 3.8 µM PAF16 (left histogram; red line), in Ringer solution + 3.6 µM PAF18 (middle histogram; red line) or in Ringer solution + 1 U/ml SMase (right histogram; red line). (B) Fluorescence intensity of anti-ceramide FITC-coupled antibody (in arbitrary units) as obtained by FACS analysis of erythrocytes after a 24-hour treatment with Ringer solution containing appropriate amounts of ethanol (control) or after incubation with 3.8 µM PAF 16, 3.6 µM PAF18 or after 1 U/ml SMase (means ± s.e.m., n=8). * indicates significant difference from control (ANOVA, using Dunnett's test as post-hoc test. $P \leqslant 0.05$). (C) Erythrocytes were labelled with [^{3}H]methylcholine chloride for 72 hours. Then, erythrocytes were exposed to 3.8 µM PAF16 (▲) or 3.6 µM PAF18 (□) for different periods of time. Control erythrocytes (●) were treated in parallel with Ringer solution containing 0.3% ethanol. After incubation, sphingomyelin was determined with bacterial sphingomyelinase. Sphingomyelin levels are given in % of control (means ± s.e.m., n=3). * indicates significant difference from control (ANOVA, using Dunnett's test as post-hoc test; $P \leqslant 0.05$). (D) FITC-Annexin-V-binding in % of the total population as obtained by FACS analysis of erythrocytes after a 24-hour treatment with Ringer solution containing 0.3% ethanol vehicle (control) or after incubation with 3.8 µM PAF16 in the absence (black bars) or presence (white bars) of 600 mM urea (arithmetic means ± s.e.m., n=8). * indicates significant difference from the respective control; # indicates significant difference from PAF16-treated erythrocytes in the absence of urea (ANOVA, using Tukey's test as post-hoc test; $P \leqslant 0.05$).

Furthermore, determination of the cellular sphingomyelin contents revealed that PAF-induced ceramide formation was paralleled by sphingomyelin breakdown. A 5-hour incubation with PAF16 or PAF18 led to a decrease of sphingomyelin levels, approaching 55±7% (n=3) and 66±9% (n=3), respectively, of the sphingomyelin content in control erythrocytes (Fig. 8C). To further verify the role of ceramide in scramblase activation, we treated erythrocyte concentrates with increasing amounts of a cell-permeable ceramide analogue C_6-ceramide. In accordance with a previous report (Lang et al., 2004a), 20 µM and 50 µM of C_6-ceramide significantly increased annexin-positive cells from 1.0 ± 0.5% (n=9) in control cells to 24.5 ± 4.2% (n=9) and 64.3 ± 5.4% (n=9), respectively.

We have recently shown that addition of 600 mM urea inhibits hyperosmotic-shock-induced ceramide formation by interfering with sphingomyelinase activity (Lang et al., 2004b). We thus used urea to demonstrate the link between PAF-induced ceramide formation and PAF-induced phosphatidylserine exposure. Urea did not influence annexin-binding of control cells, whereas the addition of urea to PAF-treated erythrocytes significantly inhibited PAF-induced annexin-binding by 40% (Fig. 8D).

Discussion

This study demonstrates that osmotic shock triggers the transient release of PAF from erythrocytes, an effect abrogated by the phospholipase A_2 inhibitor quinacrine. PAF returns to baseline levels after 2 hours, indicating that excessive PAF concentrations are detoxified by the cells. Along those lines, it has been shown that erythrocytes express phospholipase A_2 (Shin et al., 2002) as well as PAF acetylhydrolase (Stafforini et al., 1993).

Independently from the source of the mediator, extracellular PAF triggers the breakdown of phosphatidylserine asymmetry in erythrocytes. We show here, that purified erythrocytes contain significant levels of the PAF receptor, a finding that is in contrast to a previous report failing to demonstrate specific binding of [^{3}H]PAF to erythrocyte plasma membranes (Hwang et al., 1983). In this study, the presence and functional significance of the PAF receptor in erythrocytes is shown in western blots and FACS analysis, respectively. Most importantly, we show the inefficacy of PAF in erythrocytes that lack the PAF receptor and the blunting of the PAF effect in the presence of the PAF receptor antagonist ABT491, which highlights the significance of the PAF receptor.

The PAF-induced effect on cellular phosphatidylserine exposure in highly-purified erythrocyte concentrates is at least partially because ceramide formation is activated, presumably catalysed by an intracellular sphingomyelinase. Nevertheless, it is possible that some residual white blood cells contribute to ceramide formation, e.g. by secretion of a Zn^{2+}-

dependent sphingomyelinase as described by Schissel et al. (Schissel et al., 1996). Because erythrocytes that expose phosphatidylserine may adhere to other cells (Eda et al., 2002), triggering of phosphatidylserine exposure might facilitate cell-cell interactions and thus formation of cell aggregates. Indeed, PAF has been shown to foster adherence of erythrocytes to endothelial cells (Sultana et al., 1999), to be involved in the formation of platelet-erythrocyte aggregates (Sirolli et al., 2001) and to mediate the systemic cardiovascular shock following antibody infusion (Tanaka et al., 2002). Moreover, it has been shown to participate in the vascular obstruction by dehydrated sickle cells (Kaul et al., 2000; Kaul et al., 2001).

However, exposure of phosphatidylserine at the cell surface is thought to stimulate the uptake of erythrocytes by macrophages (Romero et al., 1999). Accordingly, breakdown of phosphatidylserine asymmetry might be important for erythrocyte ageing, which is paralleled by an increase of cytosolic Ca^{2+} activity (Romero et al., 1999; Kiefer et al., 2000). Beyond that, any erythrocyte disorder that facilitates cell shrinkage, such as sickle cell anaemia (Joiner, 1993; Wu et al., 2003), thalassaemia (Mach-Pascual et al., 1996) or iron deficiency (Jolobe, 2000), would be expected to favour phosphatidylserine exposure at the erythrocyte surface, an effect which has indeed been observed in a previous study (Lang et al., 2002). It is noteworthy that the plasma levels of several pro-inflammatory cytokines, including PAF, are enhanced in sickle cell anemia (Rivera et al., 2002).

High concentrations of urea as they prevail in kidney medulla, do not induce phosphatidylserine exposure or interfere with the integrity of the erythrocytes. On the contrary, urea protects erythrocytes against hyperosmotic shock, an effect proven to be due to direct inhibition of erythrocyte sphingomyelinase (Lang et al., 2004b). Accordingly, blocking the sphingomyelinase with 600 mM of urea blunted the effect of PAF on phosphatidylserine exposure.

In conclusion, osmotic shrinkage of erythrocytes leads to the release of PAF, which in turn activates an erythrocyte sphingomyelinase leading to ceramide formation. Ceramide then stimulates erythrocyte scramblase leading to phosphatidylserine exposure of the shrunken erythrocytes. This mechanism might be important for the clearance of defective erythrocytes and for the adherence of affected erythrocytes with subsequent impairment of microcirculation.

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