Effect of Anandamide on Erythrocyte Survival

Peter J. Bentzen and Florian Lang

Department of Physiology, University of Tübingen

Key Words
Arachidonylethanolamide • Apoptosis • Calcium • Oxidative stress • Cyclooxygenase

Abstract
The endocannabinoid anandamide (Arachidonylethanolamide, AEA) is known to induce apoptosis in a wide variety of nucleated cells. The present study explored whether anandamide induces suicidal death of erythrocytes or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Eryptotic cells are phagocytosed and thus cleared from circulating blood. Triggers of eryptosis include increase of cytosolic Ca²⁺ activity, formation of PGE₂, oxidative stress and excessive cell shrinkage. Erythrocyte Ca²⁺ activity was estimated from Fluo3 fluorescence, phosphatidylserine exposure from annexin V binding, and erythrocyte volume from forward scatter in FACS analysis. Exposure of erythrocytes to anandamide (= 2.5 µM) increased cytosolic Ca²⁺ activity, enhanced the percentage of annexin V binding erythrocytes and decreased erythrocyte forward scatter, effects significantly blunted in the presence of cyclooxygenase inhibitors acetylsalicylic acid (50 µM) or ibuprofen (100 µM) and in the nominal absence of extracellular Ca²⁺. Anandamide further enhanced the stimulating effects of hypertonic (addition of 550 mM sucrose) or isotonic (isosmotic replacement of Cl⁻ with gluconate) cell shrinkage on annexin V binding. The present observations demonstrate that anandamide increases cytosolic Ca²⁺ activity, thus leading to cell shrinkage and cell membrane scrambling of mature erythrocytes.

Introduction
Anandamide, the most important endogenous cannabinoid, has previously been shown to induce apoptosis in a wide variety of cells [1] including liver cells [2-4], colorectal cancer cells [5], transformed glioma cells [6, 7], neurons [8], neuroblastoma [9, 10], neuroglioma [11], PC12 cells [12, 13], blastocysts [14], uterine cervix cancer cells [15], Chinese Hamster Ovary cells [16], prostatic cancer cells [17], endothelial cells [18], dendritic cells [19], lymphocytes [20] and lymphoma cells [10, 21, 22]. On the other hand, cannabinoids have been shown to protect primary neurons, astrocytes and oligodendrocytes from apoptosis [7, 23], to stimulate cell proliferation of cancer cells [24] and to enhance the pro-
liferative effect of erythropoietin [25].

Mechanisms involved in anandamide induced apoptosis of nucleated cells include Ca^{2+} and calpain [8, 10], ceramide [3, 17], lipid rafts [9], G-proteins [26], ROS production [4, 12, 16, 27], p38 kinase pathway [2, 13], Nuclear Factor kappaB [19] and cyclooxygenase 2 [5, 11]. Even though anandamide was able to activate caspases [12, 28], the apoptotic effect of anandamide was not significantly blunted by caspase inhibition [8].

Erythrocytes are similarly able to undergo suicidal cell death or eryptosis [29]. Eryptosis is triggered by activation of Ca^{2+}-permeable erythrocyte cation channels [30-32] and Ca^{2+} entry with subsequent activation of Ca^{2+}-sensitive K^{+} channels, KCl exit, osmotic water loss and cell shrinkage [33, 34]. Ca^{2+} further triggers Ca^{2+}-sensitive scrambling of the cell membrane [35] with phosphatidylserine exposure at the cell surface [30]. Eryptosis is further elicited by ceramide (acylsphingosine) [30-32] and several channel blockers [31, 34].

Phosphatidylserine exposure favors engulfment by macrophages equipped with phosphatidylserine receptors [37] and subsequent elimination from circulating blood [38]. Accordingly, similar to erythrocyte senescence [39-41] and neocytolysis [42], eryptosis leads to disposal of affected erythrocytes [29]. Enhanced eryptosis has been observed following treatment of erythrocytes with PGE_{2} [36], platelet activating factor [44], paclitaxel [45], methylglyoxal [46], chlorpromazine [47], cyclosporine [48], amyloid peptides [49], mercury [50], lead [51] and hemolysin [43]. Moreover, eryptosis is favoured by phosphate depletion [52], iron deficiency [38], Hemolytic Uremic Syndrome [53], sepsis [54], malaria [55], Wilson disease [56], as well as genetic erythrocyte disorders, such as sickle cell anemia, thalassemia and glucose 6 phosphate dehydrogenase deficiency [57]. Eryptosis is inhibited by erythropoietin [58], urea [59], catecholamines [32] and several channel blockers [31, 34].

Hitherto nothing is known about effects of anandamide on erythrocyte survival. Thus, the present experiments explored whether anandamide induces suicidal death of erythrocytes and aimed to elucidate underlying mechanisms.

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V).

Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 MgSO_{4}, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_{2}, pH 7.4. Where indicated, chloride was replaced by gluconate or osmolarity increased by addition of 550 mM sucrose.

Anandamide was used at concentrations ranging from 2.5 to 10 µM, ionomycin at a concentration of 1 µM, N-acetylcysteine at 0.1 mM to 1.5 mM, acetylsalicylic acid at 50 µM and ibuprofen at 100 µM. Where applicable, the final concentration of the solvent dimethyl sulfoxide (DMSO) was 0.1%. Ionomycin, N-acetylcysteine, acetylsalicylic acid and ibuprofen were purchased from Sigma (Taufkirchen, Germany), anandamide and the Ca^{2+} dye Fluo-3/AM from Calbiochem (Bad Soden, Germany).

FACS analysis of annexin V binding and forward scatter

FACS analysis was performed as described [60]. After incubation in the presence or absence of anandamide, cells were washed in annexin V binding buffer containing (in mM): 125 NaCl, 10 HEPES/NaOH (pH 7.4), and 5 CaCl_{2}. Erythrocytes were suspended in a solution composed of annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin V buffer (dilution of 1:50). After 10 min. of incubation, samples were finally diluted 1:5 in annexin V binding buffer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analysed by forward scatter and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+} activity

Intracellular Ca^{2+} measurements were performed as described previously [43]. Erythrocytes were loaded with Fluo-3/AM (Calbiochem) by addition of 2 µl of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 1 ml erythrocyte suspension (0.16% hematocrit in Ringer). Cells were incubated at 37°C for 15 min under protection from light. Subsequently, an additional 2 µl aliquot of Fluo-3/AM stock solution was added, and the cells were incubated for 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 g for 5 min at 22°C and washed two times with Ringer solution containing 0.5% bovine serum albumin (Sigma) and one time with Ringer. Fluo-3-AM-loaded erythrocytes were re-suspended in 0.5 ml Ringer solution containing 0.16% hemacrit (Sigma) that contained different concentrations of anandamide, and incubated for different time periods at 37°C. Then, Ca^{2+}-dependent fluorescence intensity was measured by FACS analysis in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a positive control for enhanced Ca^{2+} activity, Fluo-3-labelled erythrocytes were incubated in Ringer containing 1 µM of ionomycin.

Determination of hemolysis

In order to control for hemolysis, cells were suspended (0.3%) and incubated for 24h in Ringer’s solution containing different concentrations of anandamide. After incubation, hemoglobin concentration in the supernatant was determined.
by photometric measurement (Tecan Elisa Reader, at 405 nm). The respective values are expressed in percentage of haemoglobin concentration following complete lysis of the cells by exposure to pure water (set as 100% hemolysis). The absorption of anandamide has been determined concurrently and was subtracted from the absorption of the supernatant.

Statistics

Data are expressed as arithmetic means ± SEM and statistical analysis was made by paired or unpaired t-test or ANOVA, as appropriate.

Results

Phosphatidylserine exposure was determined from binding of FITC-labelled annexin V. Annexin binding in the absence of anandamide was low. Incubation of erythrocytes in Ringer solution for 24 h resulted in an average percentage of annexin V exposing erythrocytes of 1.6 ± 0.1% (n = 4). The addition of anandamide (= 5 µM) increased within 24 h significantly the percentage of annexin V binding cells (Fig. 1A, B).

Anandamide-Induced Erythrocyte Death

Cell Physiol Biochem 2007;20:1033-1042

1035
Cell volume was estimated from forward scatter in FACS analysis after an incubation time of 24 h. The exposure to anandamide (= 2.5 µM) was followed by a dose-dependent significant decrease of the forward scatter pointing to erythrocyte shrinkage (Fig. 2A,B).

Fluo3-dependent fluorescence was employed to determine cytosolic Ca²⁺ activity. As shown in Fig. 3A, anandamide (= 2.5 µM) increased the cytosolic free Ca²⁺ concentration after an incubation time of 6 hours. For comparison, the effect of the Ca²⁺ ionophore ionomycin (1 µM) on the Fluo3-dependent fluorescence is shown. The increase of the cytosolic Ca²⁺ concentration could at least partially account for the stimulation of phosphatidylserine exposure and cell shrinkage following anandamide treatment. In order to further test for a role of Ca²⁺ in the stimulation of phospholipid scrambling, additional experiments were performed comparing the effects of 10 µM anandamide on annexin V binding and forward scatter in the presence and absence of Ca²⁺ in the incubation medium. As shown in Fig 3B, the removal of Ca²⁺ significantly blunted the percentage of annexin V binding erythrocytes after an incubation time of 24 h. Anandamide induced cell shrinkage was likewise significantly reduced by the removal of Ca²⁺ from the medium (Fig 3C).

To examine whether the stimulation of annexin V binding by anandamide was dependent on formation of reactive oxygen species, erythrocytes were preincubated for 60 minutes with several concentrations of the antioxidant N-acetyl cysteine followed by the addition of 10 µM of anandamide with further incubation for 24 hours. The preincubation with N-acetyl cysteine (>0.1 mM) significantly blunted annexin V binding (Fig. 4), but did not significantly blunt the decrease of forward scatter following exposure to anandamide (data not shown).
Fig. 4. Effects of anandamide on erythrocyte phosphatidylserine exposure following preincubation with N-acetylcysteine (NAC). Arithmetic means ± SEM (n = 6) of erythrocyte annexin V binding after preincubation in Ringer solution without (open and closed bar) or with N-acetyl cysteine (grey bars) for 60 minutes at concentrations ranging from 0.1 mM to 1.5 mM followed by the addition of 10 µM of anandamide and continued incubation for 24 hours. * indicates significant difference from Ringer-treated controls (ANOVA; P < 0.05). # indicates significant difference from the absence of preincubation with N-acetyl cysteine (ANOVA; P < 0.05).

Fig. 5. Effects of anandamide on erythrocyte phosphatidylserine exposure and forward scatter in the presence and absence of cyclooxygenase-inhibitors. A. Arithmetic means ± SEM (n = 6) of the annexin V binding of erythrocytes exposed for 24 hours to Ringer solution without (open bars) or with anandamide (closed bars) at a concentration of 10 µM in the absence (Ringer) or presence of cyclooxygenase (COX) antagonists acetylsalicylic acid (ASA, 50 µM) and ibuprofen (Ibu, 100 µM). * indicates significant difference from Ringer-treated controls (ANOVA; P < 0.05). # indicates significant difference from the absence of COX-inhibitors (ANOVA; P < 0.05). B. Arithmetic means ± SEM (n = 6) of the forward scatter of erythrocytes exposed for 24 hours to Ringer solution without (open bars) or with anandamide (closed bars) at concentrations of 10 µM in the absence (Ringer) or presence of COX-antagonists acetylsalicylic acid (50 µM) and ibuprofen (100 µM). * indicates significant difference from Ringer treated controls (ANOVA; P < 0.05). # indicates significant difference from the absence of COX-inhibitors (ANOVA; P < 0.05).

Eryptosis may be triggered by stimulation of PGE₂ formation [43]. Therefore, additional experiments were performed in the presence and absence of the cyclooxygenase inhibitors acetylsalicylic acid (50 µM) and ibuprofen (100 µM). As shown in Fig. 5A/B, both, acetylsalicylic acid (50 µM) and ibuprofen (100 µM) significantly blunted the effect of anandamide on erythrocyte annexin V binding and erythrocyte forward scatter.

Further experiments were performed to elucidate, whether anandamide modified the effect of known triggers of eryptosis, such as hyperosmotic or isotonic cell shrinkage [29]. As shown in Fig. 6, hyperosmotic shock significantly increased the percentage of annexin V binding erythrocytes after an incubation time of 6 h, an effect significantly augmented in the presence of anandamide (= 2.5 µM). The exposure of erythrocytes to hyperosmotic solutions further decreased the forward scatter, an effect reflecting cell shrinkage (Fig. 7). In hypertonic extracellular fluid the forward scatter was similar in the presence and absence of anandamide. Thus, in hypertonic medium anandamide did not further shrink erythrocytes.

Isotonic cell shrinkage could be accomplished by Cl⁻ removal. Thus, additional experiments have been performed on the effects of anandamide following Cl⁻ removal. As illustrated in Fig. 8, Cl⁻ removal significantly

Anandamide-Induced Erythrocyte Death
increased the percentage of annexin V binding erythrocytes after an incubation time of 24h, an effect significantly augmented in the presence of anandamide (= 2.5 µM). Cl⁻ removal further decreased the forward scatter, again pointing to erythrocyte shrinkage (Fig. 9). Similar to the observations during hyperosmotic shock, the decrease of forward scatter following Cl⁻ removal was not augmented in the presence of anandamide. Accordingly, anandamide did not further shrink erythrocytes in the absence of Cl⁻.
Discussion

The present study discloses a novel effect of the endocannabinoid anandamide, i.e. the triggering of eryptosis. The concentrations required for this effect are almost three orders of magnitude higher than those in circulating plasma of healthy individuals but are well in the range of local concentrations. The plasma anandamide concentrations approach in healthy individuals 4 nM but may be substantially higher in disease states such as acute hepatitis [2]. It has been estimated that local concentrations may approach values as high as 60 µM in disease [2].

The present experiments further allow some insight into the mechanisms mediating the anandamide effect. As evidenced from Fluo3 fluorescence, anandamide...
stimulates Ca\(^{2+}\) entry after 6 h of incubation. The increase of cytosolic Ca\(^{2+}\) is expected to activate Ca\(^{2+}\)-sensitive K\(^{+}\) channels with subsequent K\(^{+}\) exit, hyperpolarization, and exit of Cl\(^{-}\) and osmotically obliged water, eventually leading to cell shrinkage [33, 34]. Moreover, Ca\(^{2+}\) may modify the cytoskeleton [61, 62] and activate a wide variety of Ca\(^{2+}\)-sensitive enzymes such as transglutaminase [63], phospholipases [64], calpain [63], protein kinases as well as phosphatases [65, 66]. The degradation of membrane proteins by calpain leads to cell membrane blebbing, another hallmark of eryptosis [67, 68]. Anandamide induced apoptosis of nucleated cells similarly involves Ca\(^{2+}\) and calpain [8, 10].

The effect of anandamide is blunted in the nominal absence of extracellular Ca\(^{2+}\), a finding further highlighting the role of Ca\(^{2+}\) in anandamide induced phospholipid scrambling of erythrocyte cell membranes.

The Ca\(^{2+}\) permeable erythrocyte cation channels are activated by the cycloxygenase product PGE\(_2\) [43]. Accordingly, the formation of PGE\(_2\) largely accounts for the stimulation of the cation channels and subsequent eryptosis following osmotic shock [43]. Thus, we have tested for effects of anandamide in the presence of cyclooxygenase inhibitors acetylsalicylic acid and ibuprofen. As a matter of fact, both drugs significantly blunted the effect of anandamide on phospholipid scrambling and forward scatter. The anandamide induced apoptosis of nucleated cells has previously been shown to depend on cyclooxygenase 2 [5, 11]. Moreover, in nucleated cells anandamide treatment has been shown to trigger the release the PGE\(_2\) precursor arachidonic acid [69].

Triggers of eryptosis further include oxidative stress [57]. Preincubation of erythrocytes with the anti-oxidant N-acetyl cysteine indeed significantly blunted the anandamide induced phosphatidylserine exposure, suggesting a role of reactive oxygen species in the anandamide induced phosphatidylserine exposure of erythrocytes. Previous studies [4, 12, 16, 27] suggested that the pro-apoptotic effect of anandamide in nucleated cells is in part due to the generation of reactive oxygen species.

Taken together, the present observation suggest that anandamide stimulates the formation of PGE\(_2\), which in turn activates the Ca\(^{2+}\) permeable cation channels leading to entry of Ca\(^{2+}\) with subsequent phospholipid scrambling and activation of Ca\(^{2+}\)-sensitive K\(^{+}\) channels, KCl exit, water loss and cell shrinkage [33, 34].

The phosphatidylserine exposure at the cell surface favours phagocytotic removal of apoptotic or eryptotic cells by phagocytes equipped with phosphatidylserine receptors [70], which allow engulfment of defective cells [37]. Thus, phosphatidylserine exposing erythrocytes are rapidly sequestered from circulating blood [38].

In conclusion, anandamide does not only trigger apoptosis of nucleated cells but similarly induces eryptosis, the suicidal death of erythrocytes. Anandamide is effective at least in part through cyclooxygenase dependent stimulation of Ca\(^{2+}\) entry. The effects observed in erythrocytes may be similarly operative in nucleated cells.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic and Jasmin Bühringer. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/13-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research), the Else-Übelmesser-Stiftung and the Biomed program of the EU (BMH4-CT96-0602).

References

Anandamide-Induced Erythrocyte Death

Cell Physiol Biochem 2007;20:1033-1042


