Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:347-354

Accepted: June 06, 2011

Stimulation of Suicidal Erythrocyte Death by Benzethonium

Elisabeth Lang[1]*, Kashif Jilani[1]*, Christine Zelenak[1], Venkanna Pasham[1], Diwakar Bobbala[1], Syed M. Qadri[1] and Florian Lang[1]

[1]Department of Physiology, University of Tübingen, Tübingen, *contributed equally and thus share the first authorship

Key Words
Phosphatidylserine • Cell membrane scrambling • Calcium • Cell volume • Eryptosis

Abstract
Benzethonium, an antimicrobial surfactant widely used as preservative of pharmaceuticals, topical wound care product and oral disinfectant, triggers apoptosis of several cell types. The apoptosis is preceded and possibly triggered by mitochondrial depolarization. Even though lacking mitochondria, erythrocytes may similarly undergo suicidal cell death or eryptosis. Hallmarks of eryptosis include cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Eryptosis may be triggered by energy depletion, which leads to increase of cytosolic Ca^{2+}-activity with subsequent Ca^{2+}-sensitive cell shrinkage and cell membrane scrambling. Ca^{2+}-sensitivity is enhanced by ceramide. The present study explored the effect of benzethonium on eryptosis. Cell membrane scrambling was estimated from binding of fluorescent annexin V to phosphatidylserine, cell volume from forward scatter in FACS analysis, cytosolic Ca^{2+}-concentration from Fluo3-fluorescence, hemolysis from hemoglobin release, lactate formation by colorimetry and ceramide utilizing fluorescent antibodies. A 48 hours exposure to benzethonium (=5μM) significantly increased cytosolic Ca^{2+}-concentration, decreased forward scatter and triggered annexin V-binding affecting some 30% of the erythrocytes at 5 μM benzethonium. Only 5% of treated erythrocytes were hemolytic. The effects of benzethonium on annexin V binding were blunted in the nominal absence of Ca^{2+} and in the presence of amiloride (1 mM) but not in the presence of the pancaspase inhibitor zVAD (10 μM). Benzethonium further significantly enhanced the effect of glucose depletion on cytosolic Ca^{2+}-concentration and annexin V-binding, but significantly blunted the effect of glucose depletion on forward scatter. Benzethonium (5 μM) significantly enhanced lactic acid formation but not ceramide abundance. The present observations disclose a novel effect of benzethonium, i.e. triggering of suicidal death of erythrocytes.

Introduction

Benzethonium, a cationic surfactant with bactericidal [1, 2] and fungicidal [3] activity is widely used as preservative of vaccines and drugs [2, 4], for topical

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0282-0347$38.00/0

Accessible online at:
www.karger.com/cpb

Prof. Dr. Florian Lang
Physiologisches Institut der Universität Tübingen
Gmelinstr. 5, 72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618
E-Mail florian.lang@uni-tuebingen.de

wound care [1] as well as for oral disinfection [5, 6]. Recent evidence points to toxicity of benzethonium to human hematopoietic cells, Jurkat cells, neurons, glial cells, conjunctival cells and gingival fibroblasts [2, 4, 5, 7]. Benzethonium triggers cell cycle arrest [5] and suicidal cell death or apoptosis [5, 7, 8], an effect, which may limit its use as antimicrobial agent [2, 4]. On the other hand, in view of its proapoptotic and cytotoxic efficacy in tumor cells, benzethonium has been proposed as broad-spectrum anticancer drug [8]. Benzethonium induced apoptosis is preceded by loss of mitochondrial membrane potential followed by increase of cytosolic Ca^{2+} [8]. Whether mitochondrial depolarization and/or Ca^{2+} entry is required for the toxicity of benzethonium remained elusive.

Erythrocytes may similarly undergo suicidal death or eryptosis, which is, similar to apoptosis of nucleated cells followed by cell membrane scrambling and cell shrinkage [9]. Erythrocytes are, however, devoid of mitochondria and thus, eryptosis in erythrocytes is independent from mitochondrial depolarization. Instead, eryptosis is stimulated by an increase of cytosolic Ca^{2+} concentration, resulting from Ca^{2+} entry through Ca^{2+}-permeable cation channels [10-18]. The channels are activated by several triggers including energy depletion [19]. An increase of cytosolic Ca^{2+} activity is followed by activation of Ca^{2+}-sensitive K^+ channels [20, 21] with subsequent cell shrinkage due to exit of KCl together with osmotically obliged water [22]. In addition, Ca^{2+} triggers cell membrane scrambling with subsequent exposure of phosphatidylserine at the cell surface [18, 23-25]. The Ca^{2+} sensitivity of the machinery accomplishing cell membrane scrambling is increased in the presence of ceramide [26, 27]. Ceramide is generated by a sphingomyelinase, which is in turn stimulated by platelet activating factor [28]. Erythrocyte cell membrane scrambling is further triggered by caspases [25, 29], which can be activated by oxidative stress but are not necessary for the scrambling effect of Ca^{2+} [23, 27, 30].

The present study explored, whether benzethonium stimulates eryptosis and/or modifies the eryptosis following energy depletion.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (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 at 37°C for 48 hours. Where indicated, extracellular glucose was removed or benzethonium (Sigma, Freiburg, Germany) added at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Where indicated, the caspase inhibitor zVAD (Enzo, Lörrach, Germany) was used at a concentration of 10 µM and amiloride (Sigma, Freiburg, Germany) was used at a concentration of 1 mM.

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, 50 µl cell suspension were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-Fluos (1:500 dilution; Roche, Mannheim, Germany) in this solution for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation 50 µl suspension erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Determination of ceramide formation

To determine ceramide abundance at the cell surface, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, 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-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analysed by flow cytometric analysis in FL-1.

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Determination of lactate generation

For the determination of lactate formation, 100 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without 5 µM benzethonium (final hematocrit 10%). After 48 h, the samples were centrifuged (3 min at 400 g, room temperature) and the supernatant was collected. Total lactic acid content in the supernatant was measured by a commercial lactate assay kit (Bioassay systems) according to the manufacturer's instructions.

Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 µl of erythrocytes, incubated in respective experimental solutions, were stained with FITC-conjugated Annexin-V-Fluos (1:250 dilution; Roche, Mannheim, Germany) in 200 µl Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally resuspended in 50 µl of Ringer solution containing 5 mM $CaCl_2$. 20 µl were smeared onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test or unpaired *t* test, as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptotic effects, the same erythrocyte specimens have been used for control and experimental conditions.

Results

Fluo 3 fluorescence was utilized to estimate cytosolic Ca^{2+} concentration. As shown in Fig. 1, the incubation of human erythrocytes in Ringer solution containing benzethonium was followed by an increase of cytosolic Ca^{2+} concentration, an effect reaching statistical significance at 5 µM benzethonium.

Ca^{2+} is known to activate K^+ channels with subsequent exit of KCl and cell shrinkage. Thus, forward scatter was determined to disclose an effect of benzethonium on cell volume. As shown in Fig. 2, exposure of erythrocytes for 48 hours to Ringer solution containing benzethonium was indeed followed by a decrease of forward scatter, an effect reaching statistical significance at 2.5 µM benzethonium.

Ca^{2+} is further known to stimulate cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin V-binding. As illustrated in Fig. 3A and 3B, the percentage of annexin V binding

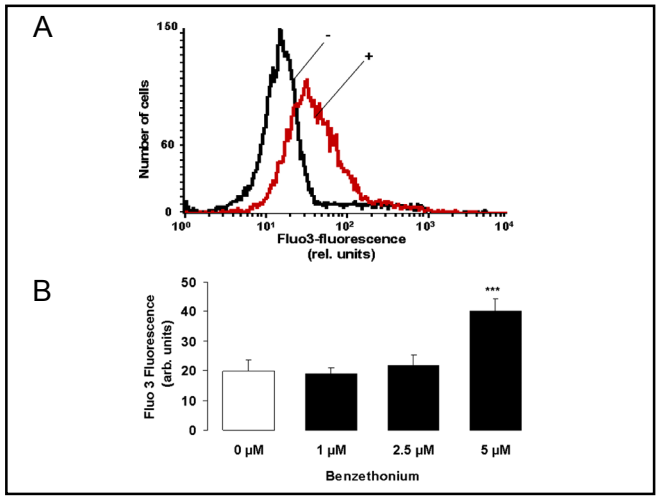

Fig. 1. Effect of benzethonium on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) 1-5 µM benzethonium. *** ($p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA).

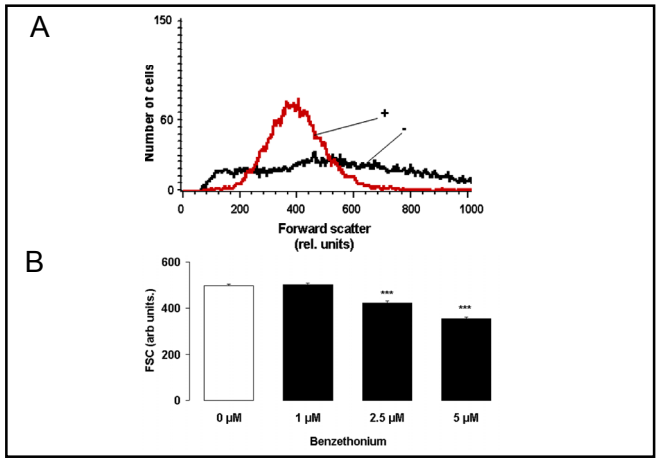

Fig. 2. Effect of benzethonium on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter following incubation for 48 hours in Ringer solution without (white bar) or with (black bars) 1-5 µM benzethonium. *** ($p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA).

erythrocytes was increased following exposure of erythrocytes for 48 hours to Ringer solution containing benzethonium. The effect of benzethonium on annexin V binding reached statistical significance at 2.5 µM benzethonium.

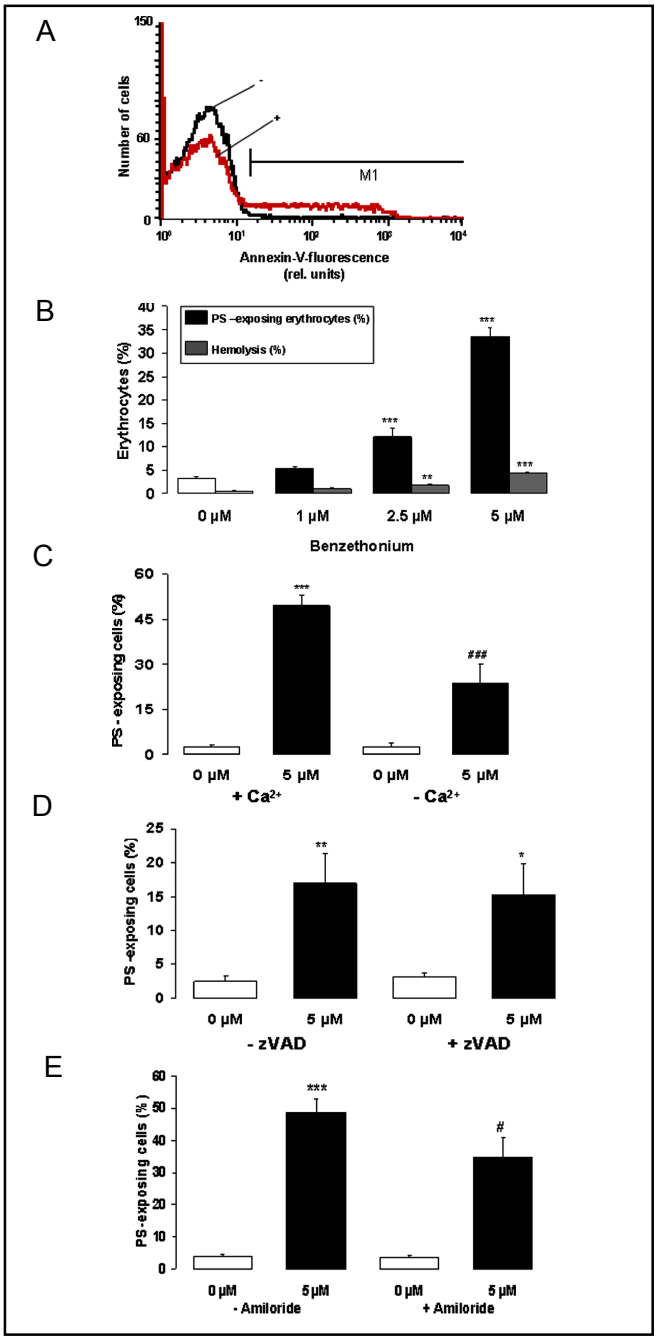

Fig. 3. Effect of benzethonium on phosphatidylserine exposure. A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of erythrocyte annexin V binding following incubation for 48 hours in Ringer solution without (white bar) or with (black bars) presence of 1-5 µM benzethonium. For comparison, arithmetic means ± SEM (n = 4) of the percentage of hemolysis is shown as grey bars. **, *** ($p<0.01$, $p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA). C. Arithmetic means ± SEM (n = 8) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 µM benzethonium in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** ($p<0.001$) indicate significant difference from the absence of benzethonium, ### indicates significant difference ($p<0.001$) from the respective values in the presence of Ca^{2+} (ANOVA). D. Arithmetic means ± SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 µM benzethonium in the absence (left bars, -zVAD) and presence (right bars, +zVAD) of pancaspase inhibitor zVAD (10 µM). *, ** ($p<0.05$, $p<0.01$) indicate significant difference from the absence of benzethonium (ANOVA). E. Arithmetic means ± SEM (n = 5) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 5 µM benzethonium in the absence (left bars, -Amiloride) and presence (right bars, +Amiloride) of amiloride (1 mM). *** ($p<0.001$) indicate significant difference from the absence of benzethonium, # indicates significant difference ($p<0.05$) from the respective values in the absence of amiloride (ANOVA).

To explore whether the cell membrane scrambling was paralleled by hemolysis, the hemoglobin release was determined following exposure of the erythrocytes to Ringer solution without or with 1-5 µM benzethonium. As indicated in Fig. 3B, the exposure to benzethonium was indeed followed by hemolysis, an effect reaching statistical significance at 2.5 µM benzethonium. The percentage of hemolytic erythrocytes was, however, one order of magnitude smaller than the percentage of erythrocytes with cell membrane scrambling.

To explore whether benzethonium-induced cell membrane scrambling was dependent on the presence of Ca^{2+}, erythrocytes were exposed to benzethonium in the presence and nominal absence of extracellular Ca^{2+}. As shown in Fig. 3C, the effect of benzethonium on annexin V-binding was indeed blunted in the nominal absence of Ca^{2+}. Furthermore, amiloride (1 mM) also blunted the effect of benzethonium on annexin V-binding (Fig. 3E).

In contrast, benzethonium-induced cell membrane scrambling was not dependent on caspase activation. Accordingly, the annexin V binding was similarly increased by benzethonium in the presence and absence of 10 µM pancaspase inhibitor zVAD (Fig. 3D).

Confocal imaging was performed to visualize eryptosis in the absence and presence of 5 µM benzethonium (Fig. 4). The number of annexin V positive erythrocytes in benzethonium treated cells was indeed higher than in untreated erythrocytes incubated for 48 hours in Ringer solution.

As eryptosis is stimulated by energy depletion paralleled by increase of cytosolic Ca^{2+} activity, decrease of cell volume and increase of cell membrane scrambling,

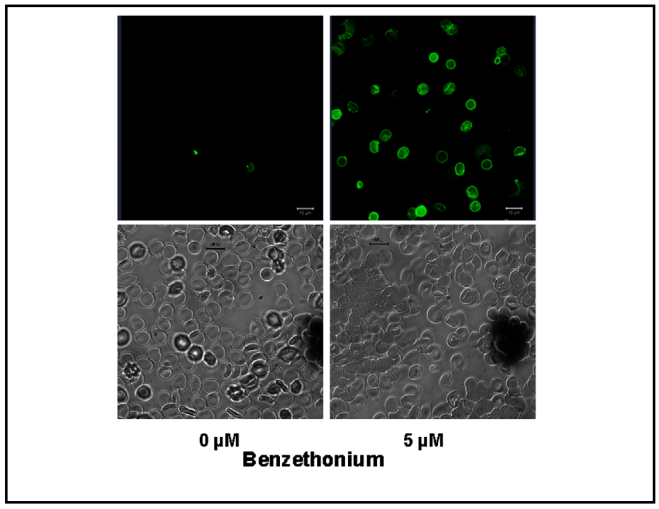

Fig. 4. Confocal images of PS-exposing erythrocytes exposed to benzethonium. FITC-dependent fluorescence (upper panels) and light (lower panels) confocal microscopy of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer solution without (left panels) and with (right panels) 5 µM benzethonium.

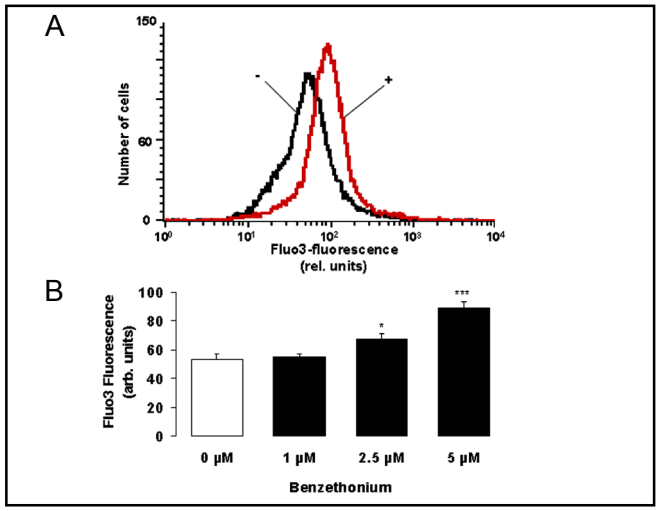

Fig. 5. Effect of benzethonium on erythrocyte cytosolic Ca^{2+} concentration following glucose depletion. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without glucose, without (-, black line) and with the (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without glucose in the absence (white bar) or presence (black bars) of 1-5 µM benzethonium. *, *** ($p<0.05$, $p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA).

an additional series of experiments was performed elucidating the effect of benzethonium on eryptosis following glucose withdrawal.

According to Fluo 3 fluorescence, removal of glucose for 48 hours was followed by a marked increase of cytosolic Ca^{2+} concentration, an effect significantly augmented in the presence of benzethonium (Fig. 5). The effect of benzethonium reached statistical significance at ≥ 2.5 µM drug concentration.

According to forward scatter, glucose withdrawal was followed by a decrease of cell volume. As shown in Fig. 6, exposure of erythrocytes for 48 hours to glucose depletion was followed by a decrease of forward scatter. Somewhat surprisingly, benzethonium reversed the decrease of forward scatter, an effect reaching statistical significance at ≥ 1 µM drug concentration.

According to annexin V binding, glucose withdrawal triggered cell membrane scrambling with phosphatidylserine exposure at the cell surface. As shown in Fig. 7, the percentage of annexin V binding erythrocytes was increased following exposure of erythrocytes for 48 hours to glucose-free Ringer solution, an effect further augmented in the presence of benzethonium. The effect reached statistical significance at ≥1 µM benzethonium.

A further series of experiments revealed that benzethonium (5 µM) significantly enhanced lactic acid formation in erythrocytes following a 48 hours exposure pointing to enhanced flux through the glycolytic pathway (Fig. 8).

Additional experiments were performed to investigate the effect of benzethonium on ceramide abundance in erythrocytes. As a result, the ceramide-dependent fluorescence intensities were similar in the absence (18.1 ± 1.3 a.u., n = 4) and in the presence (20.2 ± 1.9 a.u., n = 4) of 5 µM benzethonium.

Discussion

The present observations reveal that benzethonium triggers cell membrane scrambling in human erythrocytes. Moreover, benzethonium augments the erythrocyte cell membrane scrambling following energy depletion. The concentration required to trigger eryptosis is similar to that inducing apoptosis of nucleated cells [5]. The concentrations of benzethonium required for inducing suicidal erythrocyte death are only slightly higher than those achieved *in vivo*, which have been reported to approach approximately 2 µM [31].

The effect of benzethonium on cell membrane scrambling results at least in part from an increase of cytosolic Ca^{2+} concentration, which is well known to trigger erythrocyte membrane scrambling [18, 23, 25].

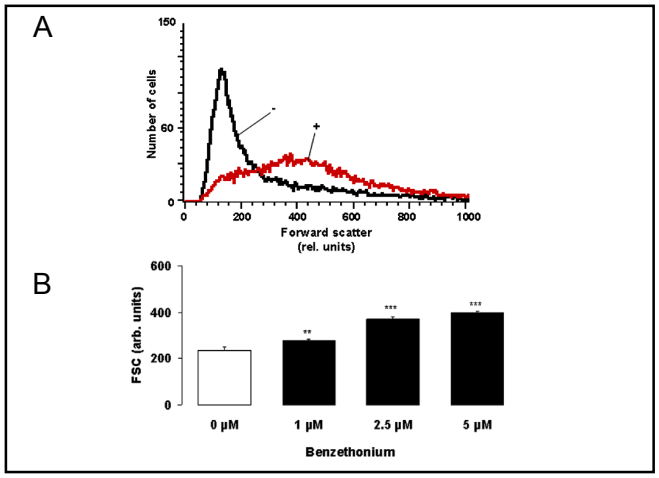

Fig. 6. Effect of benzethonium on erythrocyte forward scatter following glucose depletion. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without glucose, without (-, black line) and with the (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter following incubation for 48 hours in the absence of glucose in the absence (white bar) or presence (black bars) of 1-5 µM benzethonium. **, *** ($p<0.01$, $p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA).

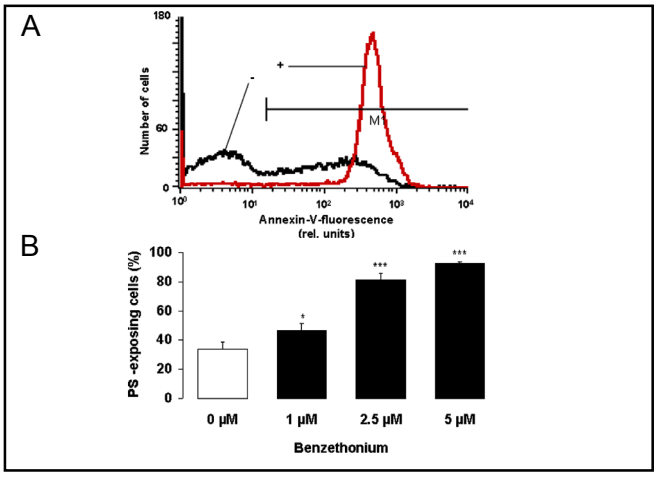

Fig. 7. Effect of benzethonium on phosphatidylserine exposure following glucose depletion. A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without glucose, without (-, black line) and with (+, red line) presence of 5 µM benzethonium. B. Arithmetic means ± SEM (n = 12) of erythrocyte annexin V binding following incubation for 48 hours in the absence of glucose in the absence (white bar) or presence (black bars) of 1-5 µM benzethonium. *, *** ($p<0.05$, $p<0.001$) indicate significant difference from the absence of benzethonium (ANOVA).

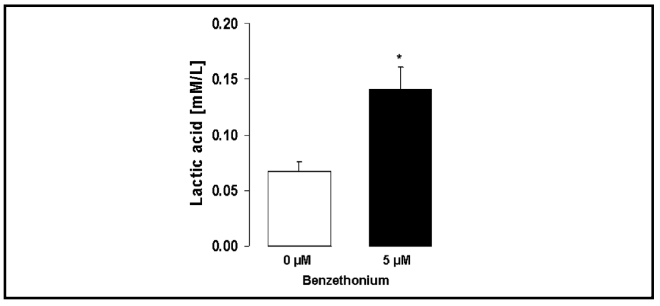

Fig. 8. Benzethonium-sensitive lactic acid formation in erythrocytes. Arithmetic means ± SEM (n = 4) of lactic acid formation in erythrocytes following incubation for 48 hours in the absence (white bar) or presence (black bar) of 5 µM benzethonium. * ($p<0.05$) indicates significant difference from the absence of benzethonium (t- test).

Benzethonium further augments the increase of cytosolic Ca^{2+} concentration following energy depletion. Most likely, benzethonium stimulates Ca^{2+} entry. The erythrocyte Ca^{2+} channels have previously been shown to involve TRPC6 [13].

As erythrocytes are devoid of mitochondria, the stimulation of Ca^{2+} entry does not require prior depolarization of the mitochondrial membrane. Whether or not Ca^{2+} entry and subsequent apoptosis in nucleated cells are critically dependent on mitochondrial depolarization remains to be tested.

Increased erythrocyte Ca^{2+} concentrations are further expected to activate Ca^{2+} sensitive K^+ channels [20, 21] with subsequent exit of K^+, hyperpolarisation of the cell membrane, exit of Cl^- and osmotically obliged water [22]. As a matter of fact, benzethonium decreases the forward scatter, a finding reflecting cell shrinkage. On the other hand, somewhat surprisingly benzethonium significantly blunts the shrinking effect of energy depletion. Moreover, benzethonium stimulates hemolysis, an observation similarly pointing to a swelling effect of the drug, even though the percentage of hemolysis is only a small fraction of the percentage of cells undergoing eryptosis following benzethonium treatment. The present observations do not allow safe conclusions regarding the swelling effect of benzethonium. Possibly, the substance stimulates entry of Na^+, which is extruded by the Na^+/K^+ ATPase in the presence of glucose but not during energy depletion. Enhanced Na^+ entry would be expected to stimulate the Na^+/K^+ ATPase thus enhancing ATP consumption followed by enhanced ATP generation from glycolysis.

Higher benzethonium concentrations were required for a statistically significant effect on Fluo3 fluorescence than on annexin V binding and forward scatter. This seeming discrepancy may reflect the relatively low sensitivity of Fluo3 fluorescence, which may preclude the identification of minor changes of cytosolic Ca^{2+} activity large enough to stimulate cell membrane scrambling and

activate the Ca^{2+} sensitive K^+ channels. Both, cell membrane scrambling (Annexin V binding) and cell volume (forward scatter) can be determined with relatively high precision.

The effect of benzethonium on eryptosis may compound eryptosis resulting from other causes. Enhanced eryptosis is observed in several clinical disorders [9], such as iron deficiency [32], phosphate depletion [33], Hemolytic Uremic Syndrome [34], sepsis [35], sickle cell disease [36], malaria [37-41], Wilson's disease [41] and possibly metabolic syndrome [42]. Moreover, benzethonium may potentiate the eryptotic effect of other xenobiotics and endogeneous substances [43-49].

Consequences of accelerated eryptosis include development of anemia [9] and adherence of phosphatidylserine-exposing erythrocytes to the vascular wall with subsequent derangement of microcirculation [50-54]. Eryptotic erythrocytes may further stimulate blood clotting [50, 55, 56].

In conclusion, the present study reveals a novel effect of benzethonium, i.e. the stimulation of eryptosis and the augmentation of eryptosis during energy depletion. The effect may contribute to the toxic effect of the substance.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Sari Rübe. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/6-1 and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research). The authors state that they have no conflict of interest to disclose.

References

1 Bearden DT, Allen GP, Christensen JM: Comparative *in vitro* activities of topical wound care products against community-associated methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother 2008;62:769-772.

2 Geier DA, Jordan SK, Geier MR: The relative toxicity of compounds used as preservatives in vaccines and biologics. Med Sci Monit 2010;16:SR21-SR27.

3 Ichikawa T, Yano Y, Fujita Y, Kashiwabara T, Nagao K: The enhancement effect of three sugar alcohols on the fungicidal effect of benzethonium chloride toward Candida albicans. J Dent 2008;36:965-968.

4 Braun S, Werdehausen R, Gaza N, Hermanns H, Kremer D, Kury P, Hollmann MW, Stevens MF: Benzethonium increases the cytotoxicity of S(+)-ketamine in lymphoma, neuronal, and glial cells. Anesth Analg 2010;111:1389-1393.

5 Nomura Y, Bhawal UK, Nishikiori R, Sawajiri M, Maeda T, Okazaki M: Effects of high-dose major components in oral disinfectants on the cell cycle and apoptosis in primary human gingival fibroblasts *in vitro*. Dent Mater J 2010;29:75-83.

6 Tsubura S, Mizunuma H, Ishikawa S, Oyake I, Okabayashi M, Katoh K, Shibata M, Iizuka T, Toda T, Iizuka T: The effect of Bacillus subtilis mouth rinsing in patients with periodontitis. Eur J Clin Microbiol Infect Dis 2009;28:1353-1356.

7 Enomoto R, Suzuki C, Ohno M, Ohasi T, Futagami R, Ishikawa K, Komae M, Nishino T, Konishi Y, Lee E: Cationic surfactants induce apoptosis in normal and cancer cells. Ann NY Acad Sci 2007;1095:1-6.

8 Yip KW, Mao X, Au PY, Hedley DW, Chow S, Dalili S, Mocanu JD, Bastianutto C, Schimmer A, Liu FF: Benzethonium chloride: a novel anticancer agent identified by using a cell-based small-molecule screen. Clin Cancer Res 2006;12:5557-5569.

9 Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M: Eryptosis, a window to systemic disease. Cell Physiol Biochem 2008;22:373-380.

10 Bernhardt I, Weiss E, Robinson HC, Wilkins R, Bennekou P: Differential effect of HOE642 on two separate monovalent cation transporters in the human red cell membrane. Cell Physiol Biochem 2007;20:601-606.

11 Duranton C, Huber SM, Lang F: Oxidation induces a Cl^- dependent cation conductance in human red blood cells. J Physiol 2002;539:847-855.

12 Duranton C, Huber S, Tanneur V, Lang K, Brand V, Sandu C, Lang F: Electrophysiological properties of the Plasmodium Falciparum-induced cation conductance of human erythrocytes. Cell Physiol Biochem 2003;13:189-198.

13 Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM: TRPC6 contributes to the Ca^{2+} leak of human erythrocytes. Cell Physiol Biochem 2008;21:183-192.

14 Foller M, Sopjani M, Koka S, Gu S, Mahmud H, Wang K, Floride E, Schleicher E, Schulz E, Munzel T, Lang F: Regulation of erythrocyte survival by AMP-activated protein kinase. FASEB J 2009;23:1072-1080.

15 Huber SM, Gamper N, Lang F: Chloride conductance and volume-regulatory nonselective cation conductance in human red blood cell ghosts. Pflugers Arch 2001;441:551-558.

16 Kaestner L, Christophersen P, Bernhardt I, Bennekou P: The non-selective voltage-activated cation channel in the human red blood cell membrane: reconciliation between two conflicting reports and further characterisation. Bioelectrochemistry 2000;52:117-125.

17 Kaestner L, Bernhardt I: Ion channels in the human red blood cell membrane: their further investigation and physiological relevance. Bioelectrochemistry 2002;55:71-74.

18 Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. Cell Death Differ 2003;10:249-256.

19 Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M, Eisele K, Podolski M, Huber SM, Wieder T, Lang F: Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. Am J Physiol Cell Physiol 2006;290:C244-C253.

20 Bookchin RM, Ortiz OE, Lew VL: Activation of calcium-dependent potassium channels in deoxygenated sickled red cells. Prog Clin Biol Res 1987;240:193-200.

21 Brugnara C, de Franceschi L, Alper SL: Inhibition of Ca^{2+}-dependent K^+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. J Clin Invest 1993;92:520-526.

22 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca^{2+}-activated K^+ channels in human erythrocyte apoptosis. Am J Physiol Cell Physiol 2003;285:C1553-C1560.

23 Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. Cell Death Differ 2001;8:1197-1206.

24 Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F: Dependence of Plasmodium falciparum *in vitro* growth on the cation permeability of the human host erythrocyte. Cell Physiol Biochem 2003;13:347-356.

25 Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. Cell Death Differ 2001;8:1143-1156.

26 Lang F, Gulbins E, Lang PA, Zappulla D, Foller M: Ceramide in suicidal death of erythrocytes. Cell Physiol Biochem 2010;26:21-28.

27 Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T: Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. Cell Death Differ 2004;11:231-243.

28 Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrossek V, Ishii S, Shimizu T, Waidmann M, Hessler G, Huber SM, Lang F, Wieder T: Stimulation of erythrocyte ceramide formation by platelet-activating factor. J Cell Sci 2005;118:1233-1243.

29 Mandal D, Moitra PK, Saha S, Basu J: Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. FEBS Lett 2002;513:184-188.

30 Weil M, Jacobson MD, Raff MC: Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. J Cell Sci 1998;111:2707-2715.

31 Xue Y, Zhang S, Tang M, Zhang T, Wang Y, Hieda Y, Takeshita H: Comparative study on toxic effects induced by oral or intravascular administration of commonly used disinfectants and surfactants in rats. J Appl Toxicol 2011;in press.

32 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. FASEB J 2006;20:368-370.

33 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. Pflugers Arch 2004;448:471-477.

34 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. J Mol Med 2006;84:378-388.

35 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. J Mol Med 2007;85:273-281.

36 Lang PA, Kasinathan RS, Brand VB, Duranton C, Lang C, Koka S, Shumilina E, Kempe DS, Tanneur V, Akel A, Lang KS, Foller M, Kun JF, Kremsner PG, Wesselborg S, Laufer S, Clemen CS, Herr C, Noegel AA, Wieder T, Gulbins E, Lang F, Huber SM: Accelerated clearance of Plasmodium-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. Cell Physiol Biochem 2009;24:415-428.

37 Siraskar B, Ballal A, Bobbala D, Foller M, Lang F: Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice. Cell Physiol Biochem 2010;26:347-354.

38 Bobbala D, Alesutan I, Foller M, Huber SM, Lang F: Effect of anandamide in Plasmodium Berghei-infected mice. Cell Physiol Biochem 2010;26:355-362.

39 Foller M, Bobbala D, Koka S, Huber SM, Gulbins E, Lang F: Suicide for survival-death of infected erythrocytes as a host mechanism to survive malaria. Cell Physiol Biochem 2009;24:133-140.

40 Koka S, Bobbala D, Lang C, Boini KM, Huber SM, Lang F: Influence of paclitaxel on parasitemia and survival of Plasmodium berghei infected mice. Cell Physiol Biochem 2009;23:191-198.

41 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Hefter H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. Nat Med 2007;13:164-170.

42 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO_2 increases? J Cardiometab Syndr 2008;3:30-34.

43 Bhavsar SK, Bobbala D, Xuan NT, Foller M, Lang F: Stimulation of suicidal erythrocyte death by alpha-lipoic acid. Cell Physiol Biochem 2010;26:859-868.

44 Bhavsar SK, Eberhard M, Bobbala D, Lang F: Monensin induced suicidal erythrocyte death. Cell Physiol Biochem 2010;25:745-752.

45 Braun M, Foller M, Gulbins E, Lang F: Eryptosis triggered by bismuth. Biometals 2009;22:453-460.

46 Eberhard M, Ferlinz K, Alizzi K, Cacciato PM, Faggio C, Foller M, Lang F: FTY720-induced suicidal erythrocyte death. Cell Physiol Biochem 2010;26:761-766.

47 Mahmud H, Foller M, Lang F: Arsenic-induced suicidal erythrocyte death. Arch Toxicol 2009;83:107-113.

48 Mahmud H, Mauro D, Foller M, Lang F: Inhibitory effect of thymol on suicidal erythrocyte death. Cell Physiol Biochem 2009;24:407-414.

49 Mahmud H, Mauro D, Qadri SM, Foller M, Lang F: Triggering of suicidal erythrocyte death by amphotericin B. Cell Physiol Biochem 2009;24:263-270.

50 Andrews DA, Low PS: Role of red blood cells in thrombosis. Curr Opin Hematol 1999;6:76-82.

51 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. Br J Haematol 1999;107:300-302.

52 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. Blood 2003;101:4625-4627.

53 Pandolfi A, Di Pietro N, Sirolli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M: Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. J Cell Physiol 2007;213:699-709.

54 Wood BL, Gibson DF, Tait JF: Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. Blood 1996;88:1873-1880.

55 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arterioscler Thromb Vasc Biol 2007;27:414-421.

56 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci 2005;62:971-988.