Dicoumarol Activates Ca^{2+} -permeable Cation Channels Triggering Erythrocyte Cell Membrane Scrambling

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Key Words

Calcium • Cell volume • Dicoumarol • Eryptosis • Phosphatidylserine • Scrambling

Abstract

Dicoumarol, a widely used anticoagulant, may cause anemia, which may result from enhanced erythrocyte loss due to bleeding or due to accelerated erythrocyte death. Erythrocytes may undergo suicidal death or eryptosis, characterized by cell shrinkage and phospholipid scrambling of the cell membrane. Eryptosis may be triggered by increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$). The present study explored, whether dicoumarol induces eryptosis. $[\text{Ca}^{2+}]_i$ was estimated from Fluo3-fluorescence, cation channel activity utilizing whole cell patch clamp, cell volume from forward scatter, phospholipid scrambling from annexin-V-binding, and hemolysis from haemoglobin release. Exposure of erythrocytes for 48 hours to dicoumarol ($=10 \mu\text{M}$) significantly increased $[\text{Ca}^{2+}]_i$, enhanced cation channel activity, decreased forward scatter, triggered annexin-V-binding and elicited hemolysis. Following exposure to $30 \mu\text{M}$ dicoumarol, annexin-V-binding affected approximately 15%, and hemolysis 2% of treated

erythrocytes. The stimulation of annexin-V-binding by dicoumarol was abrogated in the nominal absence of Ca^{2+} . In conclusion, dicoumarol stimulates suicidal death of erythrocytes by stimulating Ca^{2+} entry and subsequent triggering of Ca^{2+} dependent cell membrane scrambling.

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Introduction

Dicoumarol is a vitamin K antagonist used for anticoagulant therapy [1-4]. The use of dicoumarol is, however, compromised by multiple side effects [5, 6]. Dicoumarol may sensitize cells against the cytotoxic effects of other chemicals [7-9]. On the other hand, dicoumarol may attenuate cytotoxicity [10-13]. Dicoumarol exerts its effects at least in part by interfering with mitochondrial function and stimulation of superoxide release [14-16]. Side effects of dicoumarol include anemia, which is attributed largely to bleeding but may, at least in theory, involve accelerated erythrocyte death [17-19].

Even though lacking mitochondria, erythrocytes may undergo apoptosis-like eryptosis, a suicidal cell

death characterized by cell shrinkage and cell membrane scrambling with exposure of phosphatidylserine at the cell surface [20]. Eryptosis may be triggered by activation of Ca^{2+} -permeable cation channels with subsequent increase of cytosolic Ca^{2+} concentration [21-29]. An increase of cytosolic Ca^{2+} activity is followed by stimulation of Ca^{2+} -sensitive K^+ channels [30, 31] with subsequent exit of KCl along with osmotically obliged water leading to cell shrinkage [32]. Ca^{2+} further triggers phospholipid scrambling of the cell membrane with phosphatidylserine exposure at the cell surface [29, 33-35]. The cell membrane can be sensitized to the scrambling effect of Ca^{2+} by ceramide [36, 37], which is formed by sphingomyelinase [38].

The present study explored, whether dicoumarol triggers eryptosis. To this end, cytosolic Ca^{2+} concentration, cation channel activity, cell volume, and phosphatidylserine exposure have been determined in human erythrocytes in the absence and presence of dicoumarol.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes (aged 1-2 weeks) were kindly provided by the blood bank of the University of Tübingen. Viability of erythrocytes may depend on the donor and the storage time thus causing some interindividual variability. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch. The study was 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's 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, dicoumarol (Sigma, Freiburg, Germany) was added at the indicated concentrations. In Ca^{2+} -free Ringer's solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental conditions, 50 μl cell suspension were washed in Ringer's solution containing 5 mM CaCl_2 and then stained for 20 minutes with Annexin-V-Fluos (1:200 dilution; ImmunoTools, Friesoythe, Germany) under protection from light [39]. 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's solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer's 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's solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer's. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

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.

Electrophysiology

Patch clamp recordings were performed at room temperature. The patch electrodes were made of borosilicate glass capillaries (150 TF-10, Clark Medical Instruments) using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 17 to 20 M Ω were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA). For current measurements, erythrocytes were held at a holding potential (V_h) of -10 mV, 200 ms pulses from -100 to +100 mV were applied in increments of +20 mV. The original current traces are depicted without filtering (acquisition frequency of 3 kHz). The currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current-voltage relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solutions, and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch [40]. Data were corrected for liquid junction potentials. For whole cell recording, the pipette solutions consisted of (in mM): 125 Na-gluconate, 10 NaCl, 1 MgCl_2 , 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4). The NaCl Ringer's bath solution contained (in mM): 145 NaCl, 5 KCl, 2 MgCl_2 , 1 CaCl_2 , 5 glucose, 10 HEPES/NaOH (pH 7.4). Cl^- free bath solution contained (in mM): 150 Na-gluconate, 10 HEPES/NaOH (pH 7.4). The NMDG-Cl and CaCl_2 bath solutions contained (in mM): 180 NMDG or 100 CaCl_2 , 10 HEPES (titrated with NMDG or CaOH_2 to pH 7.4). Chemicals were obtained from Sigma (Taufkirchen, Germany) and were of the highest grade available. Experiments were performed with or without a 3-6 hours exposure to dicoumarol. Sustained exposure resulted in eryptosis, which rendered the performance of patch clamp recordings difficult.

Fig. 1. Effect of dicoumarol on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) the presence of 30 μM dicoumarol. B. Arithmetic means $\pm$ SEM ($n = 16$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer's solution without (white bar) or with (black bars) 1-30 μM dicoumarol. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of dicoumarol (ANOVA).

Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 μl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated Annexin-V-Fluos (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μl Ringer's solution containing 5 mM CaCl_2 . Then the erythrocytes were washed twice and finally resuspended in 50 μl of Ringer's 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 $\pm$ SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test or t-test, as indicated in the figure legends. n denotes the number of different erythrocyte specimens studied. The batches of erythrocytes differed moderately in their susceptibility to eryptosis. Thus, the control values were not identical in all series of experiments. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch.

Fig. 2. Effect of dicoumarol on cation channels in human erythrocytes. A. Mean ($\pm$ SEM) of I/V relationships in erythrocytes incubated for 3-6 hours at 37 $^{\circ}\text{C}$ in Ringer bath solution in the absence (open squares, $n = 18$) or presence (closed triangles, $n = 11$) of 30 μM dicoumarol. Currents were recorded with the Na-gluconate pipette and Ringer ($\pm$ 30 μM dicoumarol) bath solutions. B. Mean ($\pm$ SEM) of I/V relationships (as in A) for the currents recorded in 180 mM NMDG-Cl bath solution ($n = 18$ for control and $n = 11$ for 30 μM dicoumarol treated cells). C. Mean ($\pm$ SEM) of I/V relationships (as in A) for the currents recorded in 100 mM CaCl_2 bath solution ($n = 16$ for control and $n = 10$ for 30 μM dicoumarol treated cells). D. Mean ($\pm$ SEM) of I/V relationships (as in A) for the currents recorded in 150 mM Na-gluconate bath solution ($n = 16$ for control and $n = 11$ for 30 μM dicoumarol treated cells). E. Mean ($\pm$ SEM, $n = 11-18$) conductance (as calculated from the data in (A, B) for the inward currents by linear regression) in the absence (open bars) and presence (closed bar) of 30 μM dicoumarol. *, ** indicate significant difference ($p < 0.05$, $p < 0.001$) from the respective value in the absence of dicoumarol (t-test).

Results

Cytosolic Ca^{2+} concentration in human erythrocytes was determined utilizing Fluo 3 fluorescence in FACS analysis. As illustrated in Fig. 1A and 1B, a 48 hours exposure to dicoumarol increased cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), an effect reaching statistical significance at 20 μM dicoumarol concentration. At higher concentration (30 μM) dicoumarol increased $[\text{Ca}^{2+}]_i$ (by $17.0 \pm 3.3\%$, $n = 4$) within 1 hour.

Whole cell patch-clamp experiments were performed to elucidate whether dicoumarol influences cation ion channel activity. Replacing Ringer bath

Fig. 3. Effect of dicoumarol on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) the presence of 30 μ M dicoumarol. B. Arithmetic means $\pm$ SEM (n = 16) of the erythrocyte forward scatter following incubation for 48 hours in Ringer's solution without (white bar) or with (black bars) 1-30 μ M dicoumarol. *** (p<0.001) indicate significant difference from the absence of dicoumarol (ANOVA).

Fig. 4. Confocal images of PS-exposing erythrocytes exposed to dicoumarol. Confocal microscopy of FITC-dependent fluorescence (upper panels) and light microscopy (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 hours incubation in Ringer's solution without (left panels) and with (right panels) 30 μ M dicoumarol.

solution with Na-gluconate markedly increased, whereas replacing Na^+ with NMDG or Ca^{2+} decreased currents in untreated control cells when recorded with Na-gluconate in the pipette solution. In addition, substitution of Na^+ by the larger and less permeable cation NMDG $^+$ shifted the reversal potentials of the current-voltage-relationships (I/V) from ~ 0 mV in Ringer bath (Fig. 2 A) to about -60 mV (Fig. 2 B). The data suggest the following rank of cation permselectivity of the channels in control cells: $\text{Na}^+ > \text{Ca}^{2+} \gg \text{NMDG}^+$. As illustrated in Fig. 2, dicoumarol (30 μ M) treatment indeed significantly enhanced the cation conductance irrespective to the cation used in the bath solutions. The cation conductance following Cl-removal was also found to be more than 2 times higher in cells treated with dicoumarol than in control cells (0.482 nS for dicoumarol-treated and 0.213 nS for control cells, respectively).

An increase of cytosolic Ca^{2+} concentration were expected to stimulate Ca^{2+} sensitive K^+ channels leading to K^+ exit, hyperpolarisation, Cl^- exit and thus to cell shrinkage by cellular loss of KCl and osmotically obliged water. Cell volume was thus determined

utilizing forward scatter in FACS analysis. As shown in Fig. 3A and 3B, a 48 hours exposure of erythrocytes to dicoumarol decreased the forward scatter, an effect reaching statistical significance at 10 μ M dicoumarol. Thus, dicoumarol exposure was followed by cell shrinkage.

An increase of cytosolic Ca^{2+} concentration was further expected to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes were thus identified by annexin V binding. As apparent from Fig. 4, dicoumarol exposure increased the percentage of annexin V binding erythrocytes. The percentage of phosphatidylserine exposing erythrocytes was quantified in FACS analysis. As illustrated in Fig. 5A and 5B, the percentage of annexin V binding erythrocytes was higher following a 48 hours exposure of erythrocytes to Ringer's solution containing dicoumarol than following a 48 hours exposure of erythrocytes to Ringer's solution without dicoumarol. The difference of the percentage annexin V binding erythrocytes between the presence and absence of dicoumarol reached statistical significance at ~ 10 μ M dicoumarol.

Fig. 5. Effect of dicoumarol on phosphatidylserine exposure and erythrocyte membrane integrity. A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer's solution without (-, black line) and with (+, red line) presence of 30 μM dicoumarol. B. Arithmetic means $\pm$ SEM ($n = 16$) of erythrocyte annexin V binding following incubation for 48 hours in Ringer's solution without (white bar) or with (black bars) the presence of 1–30 μM dicoumarol. For comparison, arithmetic means $\pm$ SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. *, **, *** ($p < 0.05$, $p < 0.001$, $p < 0.001$) indicate significant difference from the absence of dicoumarol (ANOVA).

Fig. 6. Effect of Ca^{2+} withdrawal on dicoumarol induced phosphatidylserine exposure. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer's solution without (white bar) or with (black bars) 30 μM dicoumarol in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of calcium. *** ($p < 0.001$) indicate significant difference from the absence of dicoumarol (ANOVA), ### indicates significant difference ($p < 0.001$) from the respective values in the presence of Ca^{2+} .

Exposure to dicoumarol was further followed by hemolysis, reflected by hemoglobin release. As illustrated in Fig. 5B, the exposure to dicoumarol was followed by an increase of hemoglobin release, an effect reaching statistical significance at 1 μM dicoumarol concentration. The percentage of hemolysed erythrocytes remained, however, almost one magnitude smaller than the percentage of phosphatidylserine exposing erythrocytes.

Further experiments were done in the absence of extracellular Ca^{2+} to clarify, whether cell membrane scrambling depended on Ca^{2+} entry. To this end the percentage of annexin V binding erythrocytes was determined following a 48 hours treatment with dicoumarol in the presence and nominal absence of extracellular Ca^{2+} . As shown in Fig. 6, the effect of dicoumarol on annexin V-binding was virtually abolished in the nominal absence of Ca^{2+} .

Discussion

The present study reveals a novel side effect of dicoumarol, i.e. activation of the erythrocyte cation channel with subsequent triggering of Ca^{2+} entry into and eryptosis of human erythrocytes. The dicoumarol concentrations required for those effects are in the range of those approached *in vivo*. Plasma concentrations of dicoumarol have been reported to approach $7.7 \pm 1.1 \mu\text{g}/\text{ml}$ or approximately 20 μM [41].

The eryptosis is apparently the result of Ca^{2+} entry with subsequent increase of the cytosolic Ca^{2+} concentration. At higher dicoumarol concentrations activation of current and Ca^{2+} entry are observed within a few hours and could thus account for the triggering of cell membrane scrambling within 48 hours. Accordingly, the phosphatidylserine exposure is abrogated in the absence of extracellular Ca^{2+} . The Ca^{2+} entry into erythrocytes involves TRPC6 [24]. Dicoumarol may stimulate the channels indirectly. For instance, dicoumarol may induce oxidative stress [8, 15, 42], which in turn activates the erythrocyte cation channels [22]. On the other hand, dicoumarol may counteract oxidation [43, 44]. Thus, the mechanism of channel activation remains elusive. Whatever mechanism involved in channel activation, the increase of cytosolic Ca^{2+} concentration triggers erythrocyte membrane scrambling with subsequent phosphatidylserine exposure at the cell surface [33, 35]. At higher concentrations (30 μM), dicoumarol may, in addition, stimulate the formation of

ceramide (data not shown), which sensitizes the cell membrane for the cell membrane scrambling effect of cytosolic Ca^{2+} [36, 37].

Cytosolic Ca^{2+} further activates Ca^{2+} sensitive K^+ channels [30, 31] with subsequent exit of K^+ , hyperpolarisation of the cell membrane, exit of Cl^- and thus cellular loss of KCl with osmotically obliged water [32]. Along those lines, exposure of erythrocytes to dicoumarol decreased the erythrocyte forward scatter, an observation pointing to cell shrinkage.

Eryptosis may be triggered in the course of several clinical conditions [20] including iron deficiency [45], phosphate depletion [46], Hemolytic Uremic Syndrome [47], sepsis [48], sickle cell disease [49], malaria [49-53], Wilson's disease [54] and possibly metabolic syndrome [55]. Eryptosis is further stimulated by a wide variety of small molecules [56-63]. The proeryptotic effect of dicoumarol could be compounded in those clinical conditions and in the presence of those chemicals.

Erythrocyte phospholipid scrambling fosters the clearance of affected erythrocytes from circulating blood [20]. To the extent that the loss of erythrocytes cannot be compensated by release of erythropoietin and subsequent hormonal stimulation of erythrocyte formation [25, 64] the loss of erythrocytes may lead to overt anemia.

Moreover, erythrocyte phospholipid scrambling may foster adherence of phosphatidylserine-exposing erythrocytes to the vascular wall and thus impede microcirculation [65-69]. Eryptotic erythrocytes may further stimulate blood clotting [65, 70, 71].

At least in theory, dicoumarol may similarly stimulate Ca^{2+} entry into nucleated cells, which may trigger of apoptosis [72-78]. Moreover, apoptosis may be triggered by ceramide [54, 79-85]. Thus, the ability of dicoumarol to stimulate Ca^{2+} entry and generate ceramide formation may result in apoptosis.

In conclusion, the present observations disclose that dicoumarol exposure leads to cell shrinkage and cell membrane scrambling, effects largely due to Ca^{2+} entry.

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