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Stimulation of Erythrocyte Cell Membrane Scrambling by Amiodarone

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Key Words
Annexin V • Apoptosis • Ceramide • Calcium • Channels

Abstract
Side effects of amiodarone, an effective antiarrhythmic drug, include anemia, which may be caused by decreased formation or accelerated death of erythrocytes. Suicidal erythrocyte death (eryptosis) is characterized by cell shrinkage and cell membrane scrambling leading to phosphatidylserine exposure at the cell surface. Stimulators of erythrocyte membrane scrambling include increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) following activation of Ca^{2+}-permeable cation channels. Moreover, eryptosis is triggered by ceramide. The present study has been performed to test for an effect of amiodarone on eryptosis. Erythrocytes from healthy volunteers were exposed to amiodarone and phosphatidylserine exposure (annexin V binding), cell volume (forward scatter), $[Ca^{2+}]i$ (Fluo3-dependent fluorescence), and ceramide formation (anti-ceramide-FITC antibody and radioactive labelling) determined by flow cytometry. Exposure of erythrocytes to amiodarone (1 μM) increased $[Ca^{2+}]i$ and triggered annexin V binding, but did not significantly decrease forward scatter and did not significantly influence ceramide formation. Amiodarone augmented the increase of annexin binding following hypertonic shock (addition of 550 mM sucrose) but did not significantly alter the enhanced annexin binding following Cl^- removal (replacement with gluconate). Amiodarone did not significantly modify the decrease of forward scatter following hypertonic shock or Cl^- removal. The present observations disclose a novel action of amiodarone which may contribute to the side effects of the drug.

Introduction

Suicidal erythrocyte death or eryptosis [1] may be triggered by activation of Ca^{2+}-permeable cation channels [2-4] with subsequent entry of Ca^{2+}. Ca^{2+} then activates Ca^{2+}-sensitive K^+ channels leading to exit of K^+, Cl^- and osmotically obliged water and thus to cell shrinkage [5, 6]. Ca^{2+} further triggers Ca^{2+}-sensitive scrambling of the cell membrane [7], i.e. translocation of plasma membrane phospholipids and exposure of phosphatidylserine at the erythrocyte surface [2]. Cell membrane scrambling is further triggered by ceramide (acylsphingosine) [8]. Phosphatidylserine exposing erythrocytes are engulfed

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and degraded by macrophages [9]. Accordingly, phosphatidylserine exposing erythrocytes are rapidly eliminated from circulating blood [10].

Several diseases are associated with accelerated eryptosis including sickle cell disease, thalassemia and glucose-phosphate dehydrogenase deficiency [11], phosphate depletion [12], iron deficiency [10], Hemolytic Uremic Syndrome [13], sepsis [14], malaria [15], Wilson disease [16], accumulation of methylglyoxal [17] or amyloid [18], treatment with paclitaxel [19], chlorpromazine [20] or cyclosporine [21], as well as intoxication with lead [22] or mercury [23] lead to enhanced phosphatidylserine exposure and thus accelerated clearance of peripheral red blood cells. Eryptosis is similar but not necessarily identical to erythrocyte senescence [24-26] and neocytolysis [27].

Drugs leading to anemia include amiodarone, an ion channel blocker effective in the treatment of cardiac arrhythmias [28-32], which may decrease the number of circulating erythrocytes by impaired erythropoiesis and/or accelerated erythrocyte death [33, 34]. Amiodarone has been shown to inhibit cardiac Na^+, Ca^{2+} and K^+ currents [35-37]. Amiodarone has been shown to trigger apoptosis of diverse nucleated cells, an effect presumably independent from channel regulation [38-41]. Beyond that amiodarone has been shown to stimulate the formation of nitric oxide [42].

The present experiments have been performed to elucidate the effect of amiodarone on triggering and course of eryptosis. To this end, the effect of amiodarone was tested on phosphatidylserine exposure and forward scatter of erythrocytes from healthy volunteers.

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 $MgSO_4$, 1 $CaCl_2$, 5 glucose, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.4). Where indicated, chloride was replaced by gluconate or osmolarity increased by addition of 550 mM sucrose.

Amiodarone was used at concentrations ranging from 0.01 to 3 μM and ionomycin at a concentration of 1 μM. The final concentration of the solvent dimethyl sulfoxide (DMSO) was < 0.1 %. Ionomycin and amiodarone were purchased from Sigma (Taufkirchen, Germany), 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 [43]. After incubation in the presence or absence of amiodarone, 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+}

Intracellular Ca^{2+} ($[Ca^{2+}]i$) measurements were performed as described previously [44]. 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 (0.16 % hematocrit) containing different concentrations of amiodarone, 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 $[Ca^{2+}]i$, Fluo-3-labelled erythrocytes were incubated for 5 min in Ringer containing 1 μM of ionomycin.

Determination of ceramide formation

For determination of ceramide, cells were incubated with different concentrations of amiodarone for 24 h in Ringer solution. Then the erythrocytes were stained for 1 hour at 4°C with an anti-ceramide antibody (Alexis) at a concentration of 40 μg/ml in phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS) as described recently [8] and then stained with a polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific antibody (Pharmingen, Hamburg, Germany) in PBS/1 % FCS at a dilution of 1:50 for 30 min. Unbound secondary antibody was removed by washing the cells two times with PBS/1% FCS and samples were analyzed by flow cytometric analysis on a FACS-Calibur. FITCfluorescence intensity was measured in FL-1. As a positive control, 1 unit/ml of purified *Streptomyces sp*. Sphingomyelinase (Sigma) was added.

In a separate series of experiments, ceramide formation was determined utilizing a radioactive method. To this end, erythrocyte concentrates (3% hematocrit) were incubated for 24 hours in the presence or absence of amiodarone (1 μM) in

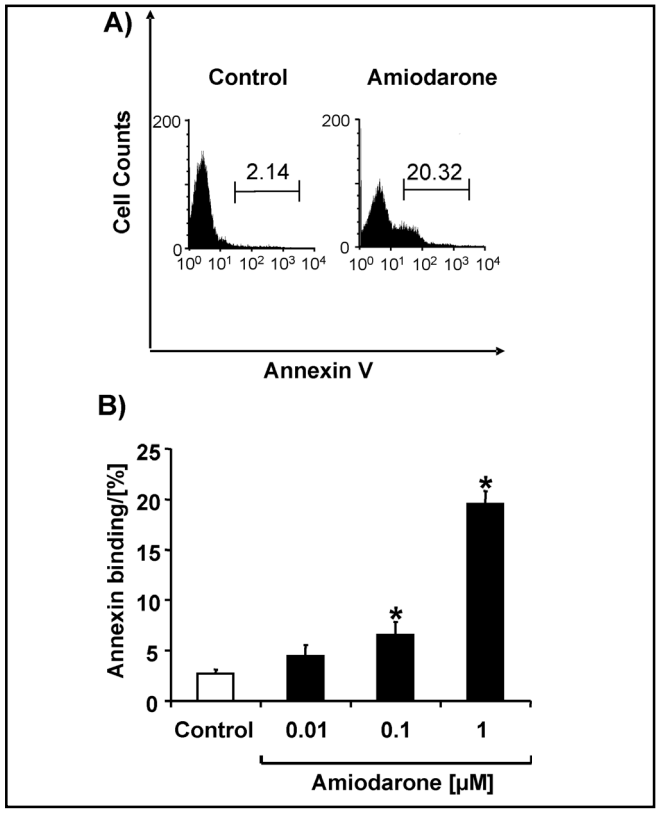

Fig. 1. Stimulation of phosphatidylserine exposure by amiodarone: A. Histograms of annexin V binding in a representative experiment of erythrocytes from healthy volunteers exposed for 24 hours to Ringer solution without (left panel) or with (right panel) 1 µM amiodarone. B. Arithmetic means ± SEM (n = 4) of the percentage annexin V binding erythrocytes exposed for 24 hours to Ringer solution without (white column) or with (black columns) amiodarone at concentrations ranging from 0.01 to 1 µM. * indicates significant difference from control values (ANOVA; $P < 0.05$).

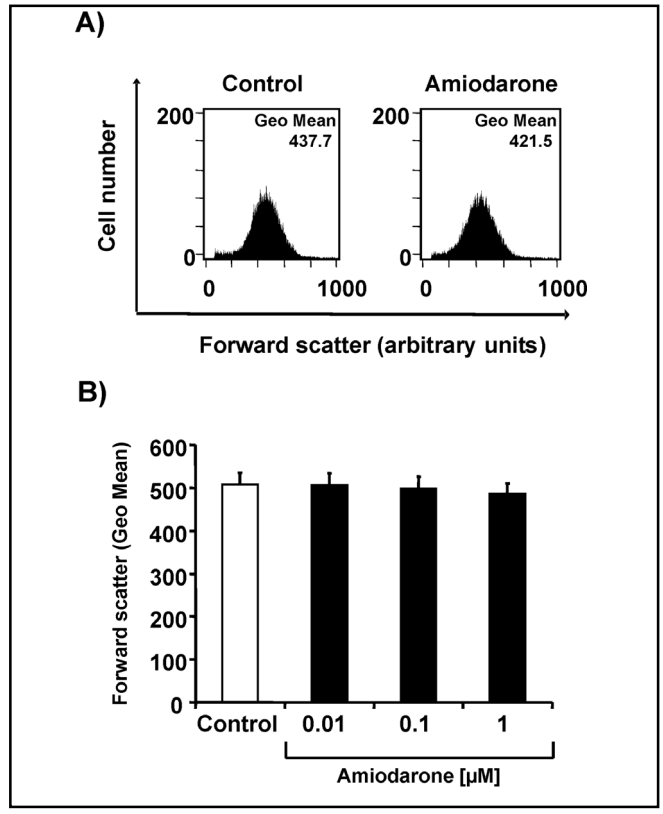

Fig. 2. Effects of amiodarone on erythrocyte forward scatter: A. Histograms of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 24 hours to Ringer solution without (left panel) or with (right panel) 1 µM amiodarone. B. Arithmetic means ± SEM (n = 4) of the forward scatter from erythrocytes exposed for 24 hours to Ringer solution without (open column) or with (closed columns) amiodarone at concentrations ranging from 0.01 to 1 µM.

Ringer solution. Cells were then washed twice with Ringer, and the samples were extracted in chloroform:methanol:1N HCl (100:100:1), the lower phase was collected and dried. Diacylglycerol was then degraded by alkaline hydrolysis of the samples in 100 µl of 0.1 N methanolic KOH at 37°C for 60 min. The samples were re-extracted, dried and resuspended in 20 µl of detergent solution (7.5% (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC)). The samples were then sonicated for 10 min. in a bath sonicator and the kinase reaction was initiated by addition of 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 min at room temperature. The reaction was terminated by addition of 1 ml of chloroform: methanol: 1 N HCl (100:100:1). 170 µl 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, scraped from the plate and quantified by liquid scintillation counting. The amount of ceramide was determined by comparison with a ceramide standard curve using C_{16}-ceramide (Biozol; Eching, Germany).

Statistics

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

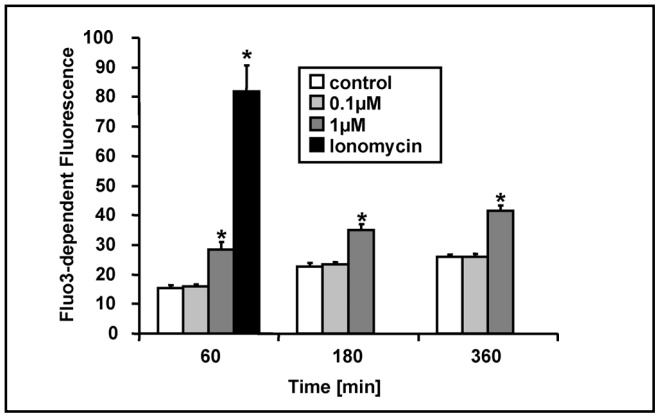

Fig. 3. Effects of amiodarone on [Ca^{2+}]i: Arithmetic means ± SEM (n = 4) of Fluo3-dependent fluorescence in erythrocytes incubated for 60 – 360 minutes in Ringer solution without amiodarone (white columns), with 0.1 µM (light grey columns) or with 1 µM (dark grey columns) amiodarone, or with 1 µM ionomycin (black column) as a positive control. * indicates significant difference from the respective Ringer-treated controls (ANOVA; $P < 0.05$).

Results

In the absence of amiodarone, spontaneous eryptosis affected only a small percentage of the erythrocytes. The percentage of annexin V binding cells approached in average 2.7 ± 0.4% (n = 4) following a 24 hour exposure to drug-free Ringer solution. The addition of amiodarone concentration-dependently increased the percentage of annexin V binding cells (Fig. 1). The effect of amiodarone reached statistical significance at 0.1 µM of the drug.

The effect of amiodarone on annexin V binding was not paralleled by a significant change of forward scatter reflecting cell volume. As shown in Fig. 2, the erythrocyte forward scatter remained almost constant up to 1 µM amiodarone. Thus, amiodarone stimulated phosphatidylserine exposure but did not elicit appreciable cell shrinkage.

Additional experiments have been performed to elucidate the underlying mechanisms. According to Fluo3-dependent fluorescence, 1 µM amiodarone increased significantly [Ca^{2+}]i (Fig. 3). For comparison, the effect of the Ca^{2+} ionophore ionomycin (1 µM) on the Fluo3-dependent fluorescence was tested. The increase of [Ca^{2+}]i could contribute to or even account for the stimulation of phosphatidylserine exposure following amiodarone treatment.

In contrast to its effect on intracellular calcium amiodarone did not increase the formation of ceramide (Fig. 4). In those experiments, purified bacterial sphingomyelinase served as a positive control. As illustrated in Fig. 4, the exposure of erythrocytes to 1 U/ml sphingomyelinase resulted in the expected increase of ceramide formation.

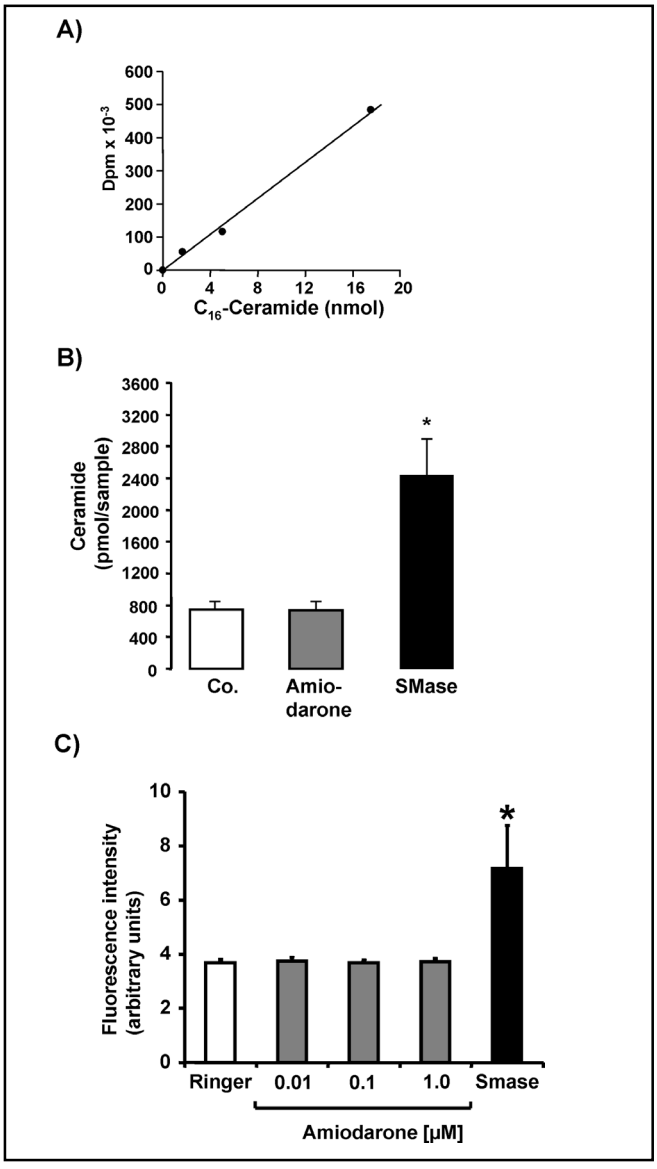

Fig. 4. Effects of amiodarone on ceramide formation: A. Calibration curve of radioative determination of ceramide formation. B. Arithmetic means ± SEM (n = 3) of (radioactively determined) ceramide formation in the absence (Co) and presence (Amiodarone) of amiodarone or sphingomyelinase (SMase). C. Arithmetic means ± SEM (n = 4) of anti-ceramide-FITC fluorescence (reflecting ceramide formation) in erythrocytes incubated for 24 hours in Ringer solution without amiodarone (white column), or with 0.01 µM, 0.1 µM and 1 µM amiodarone (grey columns), or for 10 minutes with 1 U/ml purified bacterial sphingomyelinase (SMase) (black column) as a positive control. * indicates significant difference from Ringer-treated controls (ANOVA; $P < 0.05$)

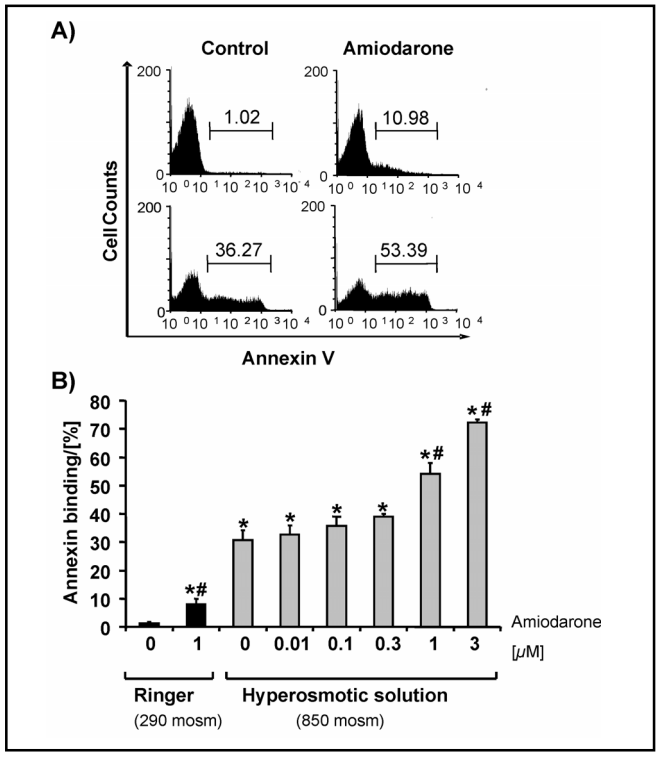

Fig. 5. Effects on annexin V binding of hyperosmotic shock in the presence or absence of amiodarone: A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed for 6 hours to either isotonic (290 mOsm) Ringer solution (upper panels) or hypertonic (850 mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 1 μM amiodarone.B. Arithmetic means ± SEM (n = 4) of the percentage annexin V binding erythrocytes following incubation for 6 hours in isotonic (290 mOsm; black columns) or hypertonic (850 mOsm; grey columns) Ringer solution either in the absence or presence of amiodarone at concentrations from 0.01 to 3 μM. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from respective value in the absence of amiodarone (ANOVA; $P < 0.05$).

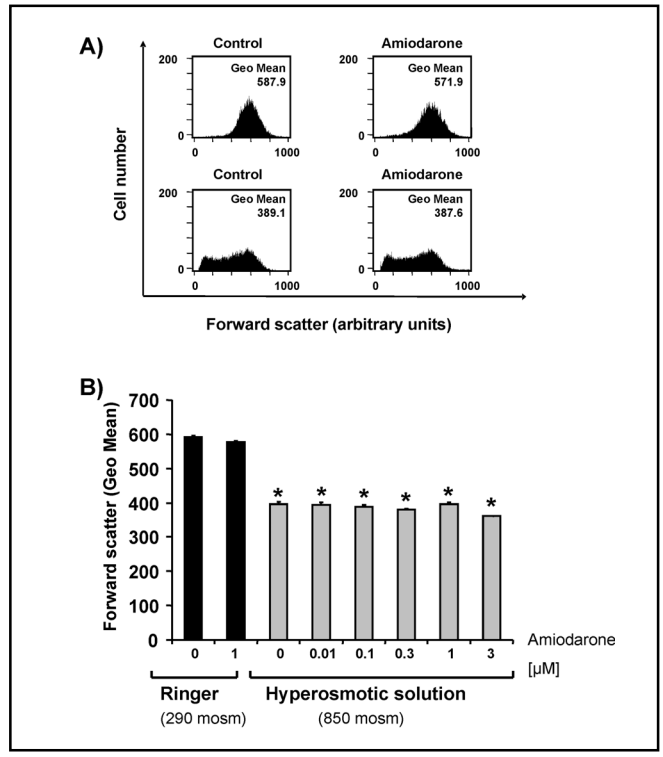

Fig. 6. Effects on forward scatter of hyperosmotic shock in the presence or absence of amiodarone: A. Histograms of forward scatter in a representative experiment of erythrocytes exposed for 6 hours to either isotonic (290 mOsm) Ringer solution (upper panels) or hypertonic (850 mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 1 μM amiodarone. B. Arithmetic means ± SEM (n = 4) of forward scatter of erythrocytes incubated for 6 hours in isotonic (290 mOsm; black columns) or hypertonic (850 mOsm; grey columns) Ringer solution either in the absence or presence of amiodarone at concentrations from 0.01 to 3 μM. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$).

Additional experiments have been performed to explore, whether amiodarone modified the well known eryptotic effect of isotonic or hypertonic cell shrinkage. As illustrated in Fig. 5, hyperosmotic shock markedly increased the percentage of annexin V binding erythrocytes. The effect of hyperosmotic shock on annexin V binding was significantly enhanced by addition of amiodarone. Osmotic shock resulted further in a marked decrease of erythrocyte forward scatter (Fig. 6), an effect, however, not significantly augmented in the presence of amiodarone.

Isotonic cell shrinkage by Cl^- removal again markedly increased the percentage of annexin V binding erythrocytes. In contrast to hypertonic shock, amiodarone did not significantly enhance annexin V binding in chloride-free solution (Fig. 7). The effect of Cl^- removal on erythrocyte forward scatter was again not augmented in the presence of amiodarone. Instead, the presence of amiodarone tended to increase forward scatter during Cl^- depletion, an effect, however, not reaching statistical significance (Fig. 8).

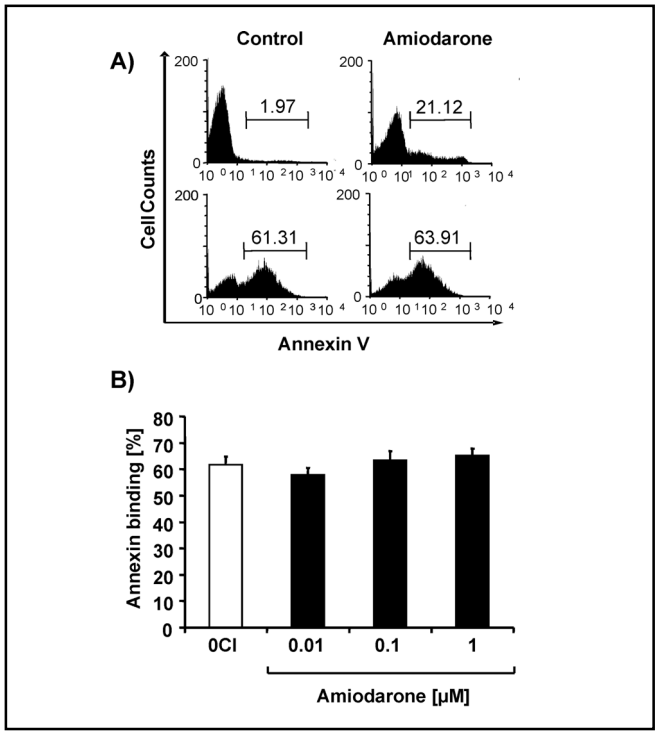

Fig. 7. Effects on annexin V binding of Cl^- removal in the presence or absence of amiodarone: A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed 24 hours to either Ringer solution (upper panels) or to Cl^--free solution (lower panels) either in the absence (left panels) or presence (right panels) of 1 µM amiodarone. B. Arithmetic means ± SEM (n = 4) of annexin V binding in % of the total population of erythrocytes incubated for 24 hours in Cl^--free solution (0 mM Cl^-) either in the absence (white column) or presence (black columns) of amiodarone at concentrations from 0.01 to 1 µM.

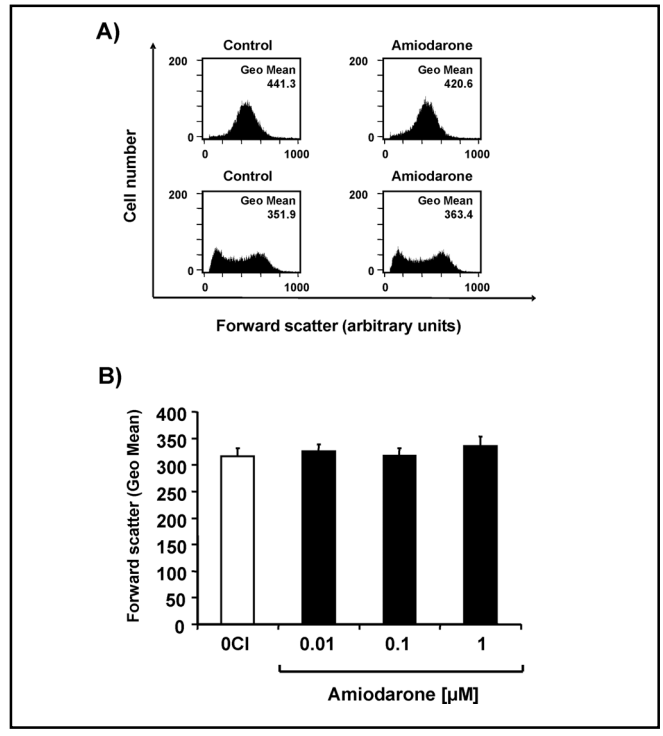

Fig. 8. Effects on forward scatter of Cl^- removal in the presence or absence of amiodarone: A. Histograms of forward scatter in a representative experiment of erythrocytes exposed 24 hours to either Ringer solution (upper panels) or Cl^--free solution (lower panels) either in the absence (left panels) or presence (right panels) of 1 µM amiodarone. B. Arithmetic means ± SEM (n = 4) of erythrocyte forward scatter following incubation for 24 hours in Cl^- -free solution (0 mM Cl^-) either in the absence (white column) or presence (black columns) of amiodarone at concentrations from 0.01 to 1 µM.

Discussion

The present experiments disclose that the antiarrhythmic drug amiodarone triggers phosphatidylserine exposure, one of the hallmarks of suicidal erythrocyte death or eryptosis [1]. The amiodarone concentration required to trigger phosphatidylserine exposure is in the range of concentrations encountered during chronic therapy [45]. However, considering the strong protein binding of amiodarone [46, 47], the free plasma concentration may be lower during therapeutic dosages. Thus, the present observations may not be relevant for the side effects of the drug at subtoxic concentrations.

The effect of amiodarone is at least partially due to increase of erythrocyte $[Ca^{2+}]i$, a known trigger of eryptosis [1]. Amiodarone does apparently not stimulate ceramide formation, another important trigger of eryptosis [8]. The stimulating effect of amiodarone may be surprising in view of its inhibitory effect on voltage gated Ca^{2+} channels [28, 29, 48]. However, the mechanism of Ca^{2+} entry into erythrocytes is clearly different from voltage gated Ca^{2+} channels.

Interestingly, amiodarone does not decrease the forward scatter and does not significantly augment the shrinking effect of hyperosmotic shock or Cl^- removal despite its effect on $[Ca^{2+}]i$. Ca^{2+} is expected to activate Ca^{2+}-sensitive K^+ channels. The subsequent exit of K^+ should hyperpolarize the cell membrane, which should in turn drive Cl^- exit. The exit of K^+, Cl^- and osmotically obliged water should lead to cell shrinkage and should thus decrease forward scatter [5, 6]. Possibly, amiodarone inhibits the K^+ channels and/or the Cl^- channels and thus impairs the exit of KCl. In cardiac myocytes, amiodarone

has indeed been shown to inhibit Na^+, Ca^{2+} and K^+ currents [35-37]. However, the decrease of forward scatter following Cl^- removal is only slightly blunted in the presence of amiodarone. Thus, the cellular loss of Cl^- and accompanying cations is apparently not abrogated in the presence of amiodarone. The decrease of forward scatter following osmotic shock does not require exit of KCl and is thus expected to be insensitive to inhibition of ion channels.

Despite a similar degree of cell shrinkage, the effects of amiodarone during hyperosmotic shock and Cl^- removal on phosphatidylserine exposure are different. Amiodarone augments the scrambling effect of hyperosmotic shock but not of Cl^- removal. The present observations do not allow any safe interpretation of this striking difference.

The exposure of phosphatidylserine at the cell surface favors the binding to phosphatidylserine receptors on macrophages [49]. The phosphatidylserine exposing erythrocytes are subsequently engulfed and degraded [9]. Thus, phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. At least in theory, the proeryptotic effect of amiodarone could thus decrease the life span of erythrocytes. Amiodarone has been reported to cause pancytopenia, hemolytic anemia and aplastic anemia [34].

In conclusion, exposure of erythrocytes to the antiarrhythmic drug amiodarone increases $[Ca^{2+}]i$ and thus stimulates phosphatidylserine exposure, a hallmark of eryptosis. Our study thus reveals a novel effect of amiodarone which may favor the development of anemia at toxic concentrations of the drug.

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