Depolarisation of the plasma membrane in the arsenic trioxide (As_2O_3)- and anti-CD95-induced apoptosis in myeloid cells

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Abstract Depolarisation of the plasma membrane has been shown to be actively regulated during lymphocyte-apoptosis. Here, we present data about anti-Fas and As_2O_3 induced depolarisation of myeloid U-937 cells. Anti-Fas but not As_2O_3-induced depolarisation was significantly dependent on caspase-activation. Na^+-fluxes contributed to the depolarisation in early stages of As_2O_3-induced apoptosis, whereas the membrane potential in late stages depended on Cl^--fluxes. Cl^--channels also played an important role in the induction of cell shrinkage in As_2O_3-induced apoptosis. However, none of these ions contributed significantly to anti-Fas induced depolarisation. This indicates the existence of different mechanisms for apoptotic plasma membrane depolarisation within one cell type.

Keywords: Arsenic trioxide; Fas; Membrane potential; Apoptosis; Chloride channel

1. Introduction

Arsenic trioxide (As_2O_3) is highly effective in the treatment of acute promyelocytic leukaemia and has therefore experienced a renaissance as an antineoplastic agent [1,2]. It is known to induce differentiation and apoptosis by different mechanisms, such as through direct opening of the permeability transition pore or production of reactive oxygen species [3].

In lymphocyte-apoptosis, actively induced plasma membrane depolarisation has been previously investigated [4,5] as the maintenance of the plasma membrane potential (E_m) is of vital importance for cells. E_m contributes to the driving force of ions like Ca^{2+} across the plasma membrane and is therefore essential for different signal transduction pathways and the regulation of uptake and excretion of metabolites. The mechanisms of the breakdown of E_m and the regulation of ion channels during apoptosis are under debate. These processes located at the plasma membrane are of great importance due to their key role in the regulation of apoptotic volume decrease (AVD) [6–8], a feature that distinguishes apoptosis from necrosis. To date, breakdown of E_m has been described for a few apoptosis inducing agents like inducing Fas-antibodies, Ca^{2+}-ionophores, thapsigargin and dexamethasone [6,5].

In this study, we investigated E_m during As_2O_3 and anti-Fas induced apoptosis in myeloid cells to gain more insights into the mechanisms of action of As_2O_3 and Fas-ligand on acute myeloid leukaemia cells, which have become the main target in the clinical use of As_2O_3. Because the promyelocytic cell lines HL-60 and NB-4 do not express functional CD95 [9], we have chosen monocytic U-937 cells as preparation in this investigation for their capacity to be induced to apoptosis by the inducing antibody-clone 7C11.

We tested the following hypotheses: (1) As_2O_3 and anti-Fas induce depolarisation in myeloid cells as it has been described in lymphocytic cells. (2) Apoptotic depolarisation of the plasmamembrane (AD) is driven by a Na^+-influx. The facts that (i) U-937 cells express an ENaC (epithelial Na^+-channel) like Na^+-channel [10] and (ii) saxitoxin prevents apoptotic Jurkat cells from an increase in intracellular Na^+ ($[Na^+]_{in}$) [6] support this hypothesis.

2. Materials and methods

2.1. Cell culture-procedures and acquisition of fluorescence-data by flow cytometry (FACS)

U-937 cells were cultured as previously described [11].

Acquisitions were made by Becton Dickenson flow cytometers (FACScan or FACSCalibur, Heidelberg, Germany). As_2O_3 or anti-Fas (clone 7C11, Immunotech, Marseille, France) was added to the cell culture to induce apoptosis. Specifity was tested with an inhibitory Fas-antibody, which blocked 7C11 induced effects (not shown).

Analysis of fluorescence referred to the non-shrunken cells as determined by scatter-light [12].

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined as previously described [12,13].

E_m was determined by bis-(1,3- dibutylbarbituric acid) trimethine oxonol ($DiBAC_3(4)$, Molecular Probes, Leiden Netherlands, 150 nM, incubation time $\geqslant 10$ min, the dye was not washed off).

Calibration of $DiBAC_4(3)$ was performed using solutions with different NaCl concentrations (in mM: 2.5, 5, 25, 50, 100, and 145) and a KCl concentration of 3.7 mM, HEPES 10 mM, and CaCl 1 mM, pH

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Abbreviations: AD, apoptotic depolarisation of the plasma membrane; AVD, apoptotic volume decrease; E_m, plasma membrane potential; $\Delta\Psi_m$, mitochondrial membrane potential; $[X]_{in}$, intracellular concentration of ion X; STS, staurosporine; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate

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7.4, with the assumption that $[Na^+]_{in} = 5$ mM and $[K^+]_{in} = 150$ mM. To preserve osmolarity, NaCl was substituted by choline-chloride. 10 nM gramicidine was added to the solution to keep sure that E_m mainly and equally depends on the distribution of Na^+ and K^+. E_m was then calculated using the Goldmann–Hodgkin–Katz-equation:

$$E_m = \frac{RT}{F} \ln \frac{P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex} + P_{Cl}[Cl^-]_{in}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{ex}} \quad (1)$$

where P represents the permeability-ratio, R the Boltzmann-constant, T the temperature and F is the Faraday-constant. This equation allows the calculation of E_m for given ion-concentrations and relative permeabilities and is the most commonly used formalism for this purpose. The equation is based on the *constant field theory* that assumes that the electrical field in the membrane is constant. We suggest that this theory is a good approach for verifying hypotheses on the generation of AD. In the case of gramicidine-treatment, it is $P_{Na} = P_K = 1$ and $P_{Cl} = 0$.

$[Na^+]_{in}$ was indicated by sodium green-AM (Molecular Probes, 5 μM, incubation time 20 min, cells washed once).

Disruption of membrane integrity was determined using 7-amino-actinomycin D (7-AAD, Sigma, 200 μg/ml, 20 min incubation time, at 4 °C and washed twice). In the case of double-staining with Di-$BAC_4(3)$, 7-AAD staining was performed first.

CCCP, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), quinidine and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma (Deisshofen, Germany).

2.2. Confocal laser scanning microscopy and 4-ANEPPS stain

4-ANEPPS (2 μM, Molecular Probes) was loaded for at least 10 minutes, the loading was supported by pluoronics (Molecular Probes). 4-ANNEPS was exited at 488 nm and measured with a highpass filter of 565 nm, a decrease in fluorescence intensity indicates depolarisation. Cells were induced 24 h by As_2O_3 or anti-Fas and allowed to adhere on a poly-L-lysin covered slide (30–60 min) in RPMI-medium without serum. Imaging was performed with an Olympus Fluoview 300 confocal system with an IX71 microscope using a 60× oil immersion objective, the zoom factor was set to 10. The resolution of each image was 256 × 256 pixels. Time series were recorded with a frequency of ca. 2.3 Hz (200 images in total) and the integrated fluorescence of single cells was displayed. Data were normalised to the first fluorescence value ($F/F_0 \times 100$) and corrected for photobleaching. We measured fluctuations of E_m by calculating the standard deviation of normalised fluorescence values in a time series, the unit is % [14].

2.3. Data analysis

Statistical significance was tested for $n > 5$ with a Wilcoxon-matched pairs test or a Mann–Whitney–*U*-test and stated if $P \leqslant 0.05$, "*" denotes $P \leqslant 0.05$, "**" denotes $P \leqslant 0.01$ and "***" denotes $P \leqslant 0.001$. Data are given as means ± standard error (S.E.M.).

3. Results

As_2O_3 and anti-Fas significantly induced AD and increased $[Na^+]_{in}$ in non-shrunken U-937 cells after 24 h of incubation (Fig. 1). The resting E_m of U-937 cells was found to be −75 mV and was determined from a calibration curve (see inset of Fig. 1C). AD-values were then calculated from the parameters

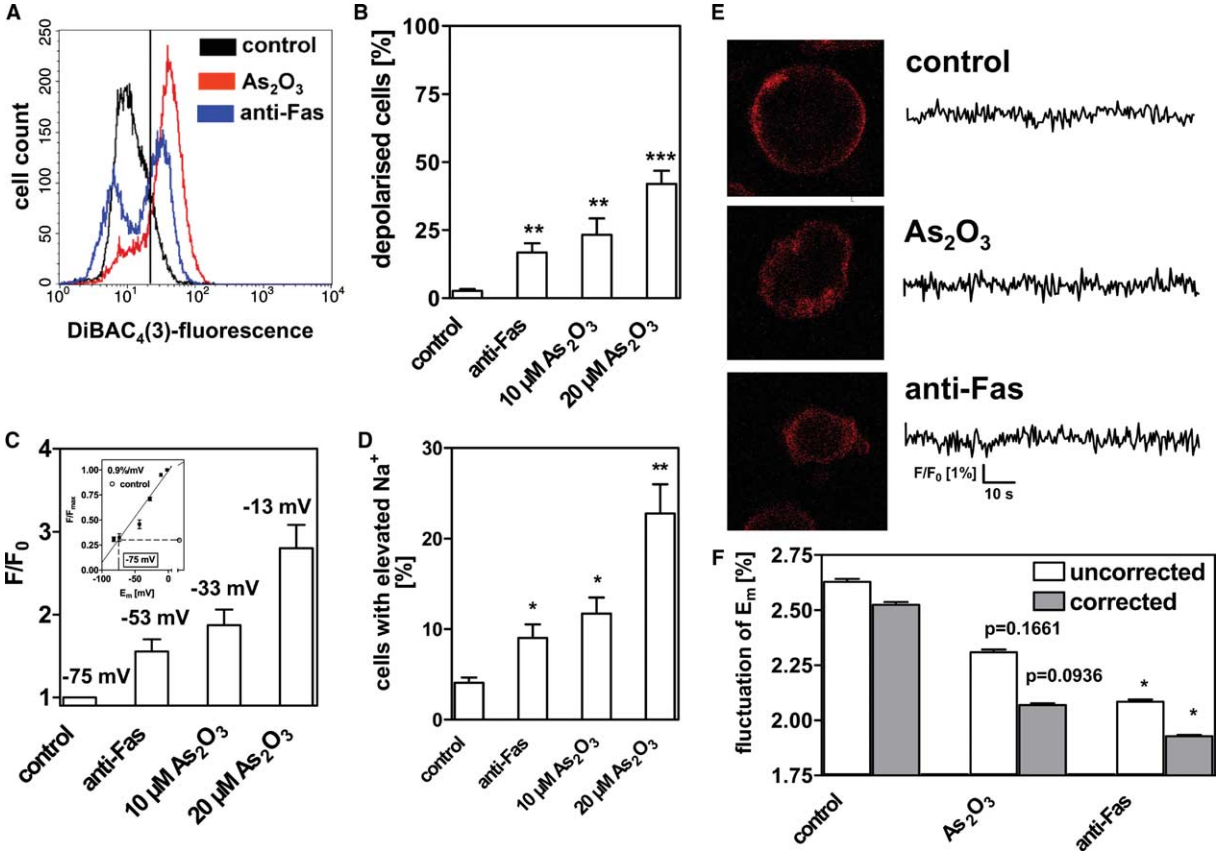

Fig. 1. (A) Pattern of $DiBAC_4(3)$-fluorescence after incubation with anti-Fas or As_2O_3 (20 μM). (B) AD with 10 and 20 μM As_2O_3 and anti-Fas ($n > 9$). (C) Changes of mean fluorescence and the corresponding E_m were calculated from the calibration curve (inset, $n = 5$). $DiBAC_4(3)$-fluorescence increases 0.9% per mV (inset in D). Apoptosis inducers increased $[Na^+]_{in}$ ($n = 6$). (E) Confocal images of cells after 24 h of incubation. Decrease in fluorescence of the dye 4-ANEPPS (red) indicates depolarisation. Trace shows averages of integrated fluorescence intensities, control $n = 24$, 20 μM As_2O_3: $n = 18$, anti-Fas: $n = 19$ (error bars not shown). (F) The standard deviation of the normalised integrated fluorescence value during the time course is a measure for potential fluctuation. Standard deviations of 200 normalised integrated fluorescence values of each trace were calculated for values corrected (grey bars) and not corrected for photobleaching (white bars).

of the calibration curve in combination with the change of mean fluorescence (mean fluorescence of control, F_0, divided by mean fluorescence of apoptotic cells, F). The calculated values were in the range of −53 to −13 mV (Fig. 1C).

AD might be followed by short enduring repolarisations that might induce rapid fluctuations of E_m. To address the question if E_m shows increased fluctuations after apoptosis induction, we performed experiments using a fast responding potentiometric dye (4-ANEPPS) in combination with confocal microscopy. Time series were obtained from cells that were incubated with anti-Fas or 20 μM As_2O_3 for 24 h. Fig. 1E shows averaged traces of time courses of integrated fluorescence of control and induced cells. Further analysis showed that fluctuations of E_m were smaller in apoptotic cells than in control cells (Fig. 1F).

We have previously shown that the pan-caspase inhibitor Boc.D(OMe)-Fmk can significantly inhibit As_2O_3 induced mitochondrial depolarisations in U-937 cells [13]. But co-incubation of Boc.D(OMe)-Fmk had only a minor effect on the prevention of AD, which was not statistically significant (Fig. 2A). As expected, CD95-mediated AD was significantly blocked by Boc.D(OMe)-Fmk. Depolarisation of mitochondria by CCCP for 24 h was not sufficient to cause plasma membrane depolarisation, but induced significant lowering of depolarised cells which can be interpreted as induction of hyperpolarisation (Fig. 2B).

In order to correlate AD with other important signs of apoptosis, we performed experiments with a $DiBAC_4(3)$/7-AAD double-stain. A sample from the same experiment was also stained by JC-1. Fig. 2C shows that AD went along with other apoptotic processes like mitochondrial depolarisation or loss of plasma membrane integrity.

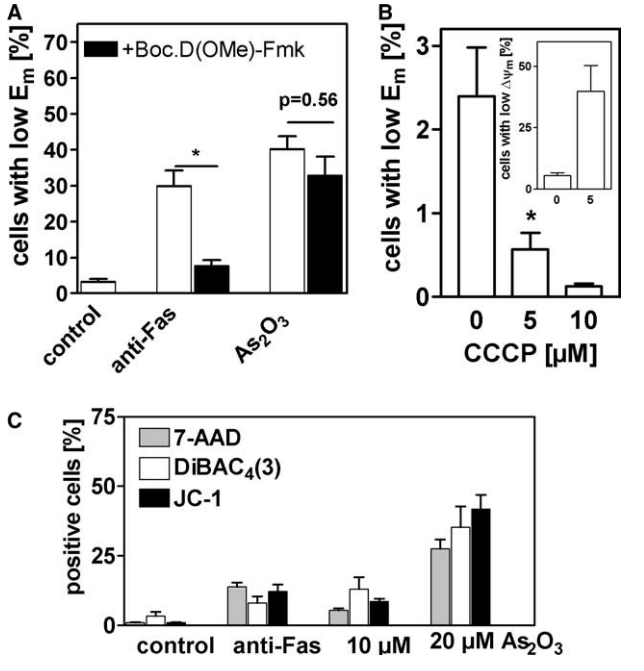

Fig. 2. (A) Incubation with 50 μM Boc.D(OMe)-Fmk decreased the amount of depolarised cells ($n \geqslant 6$). (B) Mitochondrial depolarisation by different concentrations of CCCP (inset) induces lowering of cells with depolarised plasmamembrane ($n = 6$ for 5 μM, $n = 3$ for 10 μM). (C) Depolarisation of plasmamembrane went along with other apoptotic signs like mitochondrial depolarisation determined by JC-1 or loss of plasmamembrane integrity measured by 7-AAD ($n = 6$).

The role of Na^+-influx in AD was tested by comparing apoptotic cells which were suspended in Na^+-free, choline-substituted solution with cells that were suspended in normal ringer solution (Fig. 3). As_2O_3 induced AD was clearly independent from a Na^+-influx after 24 h but showed significant dependence on Na^+-influx after 3 h at a concentration of 20 μM. The dependence on Na^+ in anti-Fas induced AD was only small and not significant. Amiloride decreased steroid induced Na^+-currents in U-937 cells [10], but it failed to induce repolarisation in As_2O_3 treated cells (Fig. 4C). This result strongly suggests the involvement of another Na^+-conducting channel than the ENaC-like channel that has been found in U-937 [10] cells in As_2O_3 induced AD.

U-937 cells express three different types of Cl^--channels [15]. We therefore evaluated the effect of Cl^--channel blockers. After an incubation time of 24 h with apoptosis inducers, the cells were split, one part was co-incubated with the Cl^--channel inhibitors NPPB or DIDS for at least 10 min, while the other was not. NPPB as well as DIDS induced depolarisation of untreated cells, this indicates that a Cl^--flux contributes to the maintenance of the resting E_m in U-937 cells. NPPB significantly reduced the amount of depolarised cells after incubation with As_2O_3 but not with anti-Fas (Fig. 4A). DIDS seemed to significantly induce repolarisation after As_2O_3 treatment (Fig. 4A). But by analysing the cells in the scatter light, we detected an increase of depolarised cells in the shrunken population (not shown). In contrast, addition of NPPB induced a much slighter shrinkage of cells of only a few percent (not shown). So, we conclude that the reduction of depolarised cells by DIDS might be overestimated due to a rapid shrinkage of some of the depolarised cells. The difference between the two Cl^--channel blockers in inducing cell shrinkage might be due to differences in their selectivity for different Cl^--channels. The significant induction of cell shrinkage by DIDS after As_2O_3-treatment is shown in Fig. 4B. It is surprising as it has been shown that co-incubation of DIDS with TNF-α or staurosporine (STS) inhibited AVD of U-937 cells [8]. In contrast, we detected a small but significant induction of cell shrinkage by DIDS after incubation with 4 μM STS (Fig. 4B). However, DIDS did not significantly re-

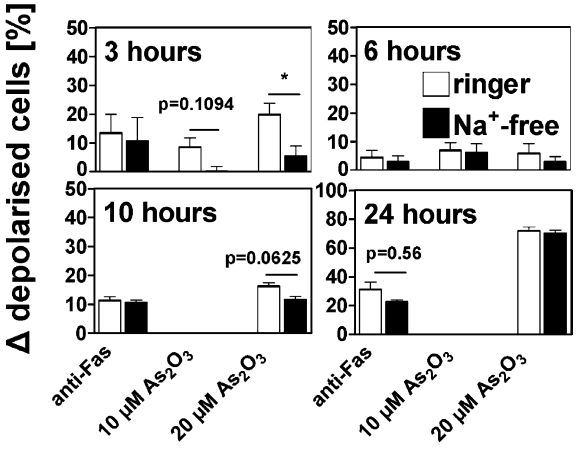

Fig. 3. Differences in the increase of depolarised cells after times of 3–24 h measured in choline-substituted (black bars) and ringer solution (white bars). For anti-Fas after 3 h of incubation $n = 4$. For 6 h $n = 5$, all others $n > 5$. Note the different y-axis of the graph of 24 h.

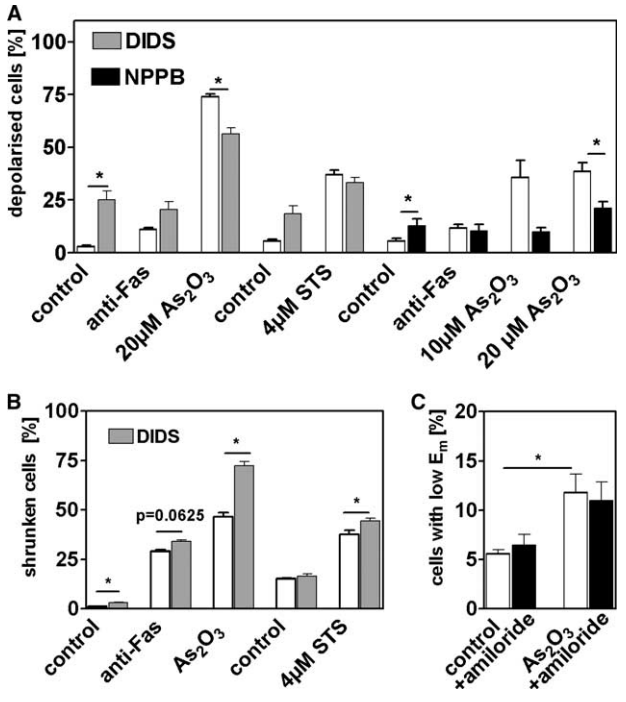

Fig. 4. (A and B) Effects of the Cl^--channel blockers DIDS and NPPB on E_m of cells incubated for 24 h with STS, anti-Fas or As_2O_3. (C) 500 μM amiloride was added for at least 10 min after an incubation time of 3 h and could not prevent AD induced by 20 μM As_2O_3 ($n > 5$ for all values, with exception of 10 μM As_2O_3 in (A), $n = 4$).

duce the amount of depolarised cells (Fig. 4A). Quinidine, a blocker of volume-activated Cl^--currents in endothelial cells [16], reduced also the amount of depolarised cells after treatment with 20 μM As_2O_3 (from $36.6 \pm 6.7\%$ to $13.3 \pm 6.0\%$, $n = 6$, $P = 0.0313$, data not shown).

4. Discussion

As_2O_3 and anti-Fas induced AD in non-shrunken U-937 cells as it has been reported for lymphocytes. We have shown that AD varied in strength over hours (Fig. 3) and was not followed by a repolarisation but was equilibrated regarding shorter time scales as apoptotic cells showed fewer fluctuations in E_m. AD was dependent on caspase-activation if the CD95 pathway was activated but caspases played a minor role in As_2O_3 induced AD. We have previously shown that in As_2O_3 induced apoptosis, caspases are particularly involved in strong mitochondrial depolarisations in U-937 cells [13]. This suggests that E_m and $\Delta\Psi_m$ are affected through different pathways by As_2O_3. The lowering of $DiBAC_4(3)$-positive cells by CCCP depolarised mitochondria excludes the possibility that AD is just the result of ATP-depletion due to an impairment of oxidative phosphorylation. As we have previously shown, the mitochondrial depolarisation by CCCP induces an increase in $[Ca^{2+}]_{in}$ in hemopoietic cells [12] and the effect of CCCP on E_m might be the result of an increase in $[K^+]_{in}$ after 24 h due to an increased open-probability of an inward-rectifying Ca^{2+}-activated K^+-channel that is expressed on U-937 cells [15,17].

Apoptotic features after As_2O_3-treatment can be detected early at a concentration of 50 μM in U-937 cells [18], which are relatively insensitive to As_2O_3 [11]. At clinically achievable concentrations, apoptotic signs have been detected much later (12–24 h) [13,11,9,19]. AD resulting from Na^+-influx is an early sign of apoptosis induced by relatively low [11] concentrations of As_2O_3. As it has been shown that prevention of an increase in $[Na^+]_{in}$ can inhibit apoptosis induction [6], we speculate that the Na^+-influx might be an important step in the initiation of As_2O_3-induced apoptosis.

In the case of anti-Fas treated cells, $[Na^+]_{in}$ might be increased due to an impairment of the Na^+/K^+-ATPase. Such a mechanism has been described in CD95 mediated lymphocyte apoptosis [4]. Bortner et al. also suggested that this impairment leads to an increase of $[Na^+]_{in}$ as a reason of AD [4]. A limitation of this model is that it is not in accordance with the constant field theory, since an isolated increase in $[Na^+]_{in}$ would induce hyperpolarisation (Eq. (1)) and can therefore not explain AD. We have clearly shown that early AD induced by As_2O_3 is driven by a Na^+-influx, whereas the depolarisation after 24 h is clearly mediated by another mechanism. As Cl^--channel blockers repolarised the As_2O_3 treated cells and promoted AVD in our experimental approach, we suggest that Cl^--channels are involved in As_2O_3 induced late stage depolarisation. This is in accordance to a previously described activation of Cl^--channels in apoptosis [20] and the findings that Cl^--channel blockers inhibit STS induced AVD in U-937 cells [8]. At first glance our findings seem contradictory, since we showed promotion of AVD by DIDS. However, our experimental procedures differ from those used by Maeno et al. [8] since they co-incubated the apoptosis inducers together with the blocker, whereas we added the blocker after apoptosis-induction. This indicates that Cl^--channels might have opposite roles in AVD: promoting it in early stages when the ion milieu is still physiologic and preventing it in stages where the ion concentrations might have changed. Anti-Fas induced AD was not significantly dependent on Na^+ or Cl^--fluxes. This result might indicate the existence of additional mechanisms for AD in U-937 cells, such as K^+-depletion [7,5], due to Na^+/K^+-ATPase-impairment [4] or a decline of K^+-conductance [21,22]. The difference in the mechanism and strength of AD between As_2O_3 and anti-Fas might be the result of fundamental differences in the apoptotic pathways affected by these agents [9].

In accordance to the constant field theory, Na^+ and Cl^--fluxes, K^+-depletion and a decline of K^+-conductance might variably contribute to the generation of membrane potentials that we have found in As_2O_3 (−33 and −13 mV) and anti-Fas (−53 mV) induced apoptosis.

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