Inversion of the Intracellular Na\(^+\)/K\(^+\) Ratio Blocks Apoptosis in Vascular Smooth Muscle at a Site Upstream of Caspase-3*  
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Long term elevation of the intracellular Na\(^+\)/K\(^+\) ratio inhibits macromolecule synthesis and proliferation in the majority of cell types studied so far, including vascular smooth muscle cells (VSMC). We report here that inhibition of the Na\(^+\),K\(^+\) pump in VSMC by ouabain or a 1-h preincubation in K\(^-\)-depleted medium attenuated apoptosis triggered by serum withdrawal, staurosporine, or okadaic acid. In the absence of ouabain, both DNA degradation and Caspase-3 activation in VSMC undergoing apoptosis were insensitive to modification of the extracellular Na\(^+\)/K\(^+\) ratio as well as to hyperosmotic cell shrinkage. In contrast, protection of VSMC from apoptosis by ouabain was abolished under equimolar substitution of Na\(^+\) with K\(^+\), showing that the antiapoptotic action of Na\(^+\),K\(^+\) pump inhibition was caused by inversion of the intracellular Na\(^+\)/K\(^+\) ratio. Unlike VSMC, the same level of increment of the [Na\(^+\)]/[K\(^+\)] ratio caused by a 2-h preincubation of Jurkat cells with ouabain did not affect chromatin cleavage and Caspase-3 activity triggered by treatment with Fas ligand, staurosporine, or hyperosmotic shrinkage. Thus, our results show for the first time that similar to cell proliferation, maintenance of a physiologically low intracellular Na\(^+\)/K\(^+\) ratio is required for progression of VSMC apoptosis.

The maintenance of the transmembrane gradient of monovalent cation (high [K\(^+\)], and low [Na\(^+\)]) is a universal property of all nucleated cells, and its dissipation is viewed as a hallmark of necrotic-type cell death (1, 2). It was shown that a transient and moderate rise of intracellular Na\(^+\) concentration in mitogen-treated cells is involved in rejoining DNA strand breaks preceding DNA synthesis (3), whereas long term inversion of the intracellular Na\(^+\)/K\(^+\) ratio blocks macromolecule synthesis and cell cycle progression in the majority of eukaryotic cells studied so far (4–7), including vascular smooth muscle cells (VSMC)\(^{1}\).Much less is known about the role of the transmembrane gradient of monovalent ions in the triggering and progression of programmed cell death (apoptosis).

Cell shrinkage is one of the initial morphological markers of apoptosis in all types of cells, particularly in VSMC (10). In immune system cells, apoptotic shrinkage is so impressive that the term “shrinkage-mediated necrosis” was originally proposed to describe this type of cell death (11), and the striking increase in the density of shrunken cells was used to separate intact from apoptotic cells (12, 13). In lymphocytes, the apoptotic cell volume decrease is caused by the loss of potassium chloride (14) and a major organic osmolyte, taurine (15), because of the CD95 receptor-mediated activation of Cl\(^-\) and K\(^+\) channels and the taurine outward transporter (for a recent review, see Ref. 16). However, the involvement of perturbation of intracellular ion composition and the ionic strength of the cytoplasm in the triggering and development of the apoptotic machinery remains unclear. Recently, it was shown that equimolar substitution of extracellular Na\(^+\) by K\(^+\) protects Jurkat cells from apoptosis induced by Fas ligand receptors (14), suggesting that dissipation of K\(^+\) gradients plays a role in the triggering of apoptosis in immune system cells. Here, we report that in contrast to Jurkat cells, inversion of the [Na\(^+\)]/[K\(^+\)] ratio blocks apoptosis of VSMC at a site upstream of Caspase-3, independently of the transmembrane gradient of monovalent cations and cell volume.

EXPERIMENTAL PROCEDURES

Cells—VSMC were obtained by explant methods from the aortas of 10–13-week-old male rats as described previously (17), cultured in DMEM with 10% calf serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin, and used between 10 and 16 passages. Cells transfected with b-myct (VSMC-MYC) and E1A adenovirus (VSMC-E1A) were obtained in accordance with a previously described protocol (18, 19) and were cultured in the same medium with the addition of 500 \(\mu\)g/ml of Genetin. Jurkat cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium supplied with 10% calf serum, antibiotics, sodium pyruvate, glutamate, and \(p\)-mercaptoethanol.

Chromatin Cleavage Assay—Chromatin cleavage in VSMC was estimated by a technique described previously in detail (10). Briefly, VSMC grown in 24-well plates were labeled for 24 h in serum-supplied DMEM with \(^3\)H(thymidine (1 \(\mu\)Ci/ml), washed with 2 \(\times\) 2 ml of DMEM, and incubated in serum-supplied DMEM. For 48 h, the cells were pretreated with ouabain or K\(^-\)-depleted medium as indicated in Fig. 2 and Table III, washed twice with serum-containing medium, and incubated with 0.5 ml of medium with or without ouabain and containing different inducers of apoptosis. To measure the content of chromatin fragments, the cells were transferred on ice, and 1 ml of ice-cold lysis buffer (10 \(\times\) EDTA, 10 \(\times\) Tris-HCl, 0.5% Triton X-100 (pH 8.0)) was added. In 15 min, the cell lysate was transferred to Eppendorf tubes and sedimented (12,000 rpm, 10 min), and 1 ml of supernatant was transferred for the measurement of radioactivity (A\(_{\text{tr}}\)). The remaining radioactivity from pellets and wells was extracted with 0.5 ml of a mixture of 1% SDS and ster ovar; AMC, 7-amino-4-methylcoumarin; YVAD, N-acetyl-Tyr-Val-Ala-Asp; DEVD, N-acetyl-Asp-Glu-Val-Asp.

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‡ The abbreviations used are: VSMC, vascular smooth muscle cell(s); DMEM, Dulbecco’s modified Eagle’s medium; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CHO, Chinese hamster ovary; AMC, 7-amino-4-methylcoumarin; YVAD, N-acetyl-Tyr-Val-Ala-Asp; DEVD, N-acetyl-Asp-Glu-Val-Asp.
4 mM EDTA, combined, and counted (Aν). Chromatin cleavage was
quantified as the content of chromatin fragments normalized by total
content of 3H-labeled DNA in accordance with the equation (1.5 × (Aν
− Aν) [Aν + Aν] × 100%, where Aν is the value of Aν before
induction of apoptosis. To measure chromatin fragmentation in 3Hthy-}
midine-labeled Jurkat cells, the cells were resuspended at a density of
106 cells/ml in medium containing 10% calf serum; the additions are
indicated in Fig. 4 and Table IV. At the time intervals shown in Fig. 4,
100 μl of cell suspension was mixed with 100 μl of phosphate-buffered
saline containing 0.5% Triton X-100, and the content of chromatin
fragments was measured as described previously in detail (20).

Caspase Activity—VSMC-E1A seeded in 20-cm2 flasks and treated as
indicated in Fig. 5 were scratched, transferred to centrifuge tubes,
washed twice with phosphate-buffered saline, and lysed in 0.5 ml of
Buffer A containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mg/ml
EDTA, and 0.1% CHAPS. Then, 50–100 μl of cell lysate was mixed with 600 μl of
Buffer A containing 1 mM dithiothreitol and 40 μl of YVAD-AMC or
DEVD-AMC, with or without 1 μM Caspase-1 and Caspase-3 inhibitors
(Ac-YVAD-CHO and Ac-DEVD-CHO, respectively), incubated for 2 h at
37 °C, and diluted 15-fold with 80 mM glycine-NaOH buffer (pH 10).
Fluorescence of the samples was measured as steady-state distribution of
isotopes (22Na and 86Rb), as described previously in detail (22). Briefly, to establish the stable-state distribution of isotopes,
VSMC growing in 12 (22Na)- or 24 (86Rb)-well plates or
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ouabain-treated VSMC-E1A was insensitive to hyperosmotic shrinkage caused by the addition of 350 mM mannitol (Fig. 2b). This is consistent with previous results obtained in nontransfected VSMC (10).

The antiapoptotic action of ouabain in VSMC was further confirmed by phase contrast microscopy and by analysis of DNA laddering. Fig. 3A shows that pretreatment with ouabain sharply decreased the number of apoptotic cells after 6 h of incubation of VSMC-E1A in serum-deprived medium. Treatment of VSMC-E1A with ouabain prevented DNA laddering and reduced the accumulation of low molecular weight 3'-end-labeled DNA oligonucleosomal fragments triggered by serum deprivation (Fig. 3B). Similar results were obtained with cells that underwent apoptosis in the presence of staurosporine and okadaic acid (data not shown).

Recently, we reported that heat stress triggers necrosis but does not affect apoptosis in VSMC (24). Indeed, as can be seen from Table II, in contrast to serum-deprived cells, heat stress (46 °C, 30 min) did not modulate chromatin cleavage in VSMC but led to a 5-fold increase of lactate dehydrogenase release, a marker of necrosis. In contrast to apoptosis, we did not observe modulation by ouabain of heat-stress-induced lactate dehydrogenase release. Viewed collectively, these data demonstrate that inhibition of the Na$^+$,K$^+$ pump protects VSMC against apoptosis but does not affect the necrotic type of cell death.

**Ouabain Does Not Affect the Induction of Apoptosis in Jurkat Cells**—Fig. 4a shows that during a 7-h incubation of Jurkat cells in control medium, the content of chromatin fragments was increased monotonously up to ~5%. After a 1.5-h incubation in the presence of Fas ligand (anti-human Fas, mouse monoclonal IgM), the content of chromatin fragments was increased by 10-fold and did not change much during the next 5.5 h of incubation (Fig. 4a, curve 3). These kinetics are consistent with previously reported data (20, 25). Apart from Fas receptor-induced apoptosis playing a key role in the functioning of activated T-lymphocytes, the apoptotic machinery in immune system cells can be triggered by other stimuli, including inhibitors of protein kinase C (14, 26) and hyperosmotic shrinkage (27). After a 3-h incubation of Jurkat cells in the presence of 0.25 μM staurosporine or in hyperosmotic medium (addition of 350 mM mannitol), the content of chromatin fragments was increased from 2.6 ± 1.2% to 19.7 ± 4.3% and 15.6 ± 2.9%, respectively (Fig. 4b).

In the absence of triggers of apoptosis, a 2-h pretreatment of Jurkat cells with ouabain did not significantly modify chromatin cleavage after a subsequent 3 h of incubation. Prolongation of incubation with ouabain for up to 7 h increased the content of chromatin fragments from 4.9 ± 2.1 to 16.0 ± 4.6% (Fig. 4a, curve 2 versus 1). We did not observe any effect of ouabain on the increment of chromatin cleavage triggered by a 3-h treatment of Jurkat cells with Fas ligand, staurosporine, or mannitol (Fig. 4b). After a 7-h incubation in the presence of Fas ligand, chromatin cleavage was significantly higher in ouabain-treated cells (Fig. 4a, curve 4 versus 3). However, increment of
the content of chromatin fragments triggered by Fas ligand in control and ouabain-treated Jurkat cells was not different (23.6 ± 3.9 and 26.2 ± 4.7%, respectively).

Effect of Equimolar Substitution of Extracellular Na\(^{+}\) by K\(^{-}\)—To examine whether or not protection of VSMC cells against apoptosis by inhibition of Na\(^{+}\), K\(^{-}\)-ATPase is mediated by the alteration of the intracellular Na\(^{+}\)/K\(^{-}\) ratio, we compared the modulation of apoptosis and the intracellular content of Na\(^{+}\) and K\(^{-}\) by ouabain in control ([Na\(^{+}\)] = 137 mM; [K\(^{-}\)] = 5 mM) and K\(^{-}\)-enriched, Na\(^{+}\)-depleted medium ([Na\(^{+}\)] = 14 mM; [K\(^{-}\)] = 128 mM). Table III shows that in contrast to control medium, neither the intracellular content of Na\(^{+}\) and K\(^{-}\) in VSMC-E1A nor base-line apoptosis or apoptosis triggered by serum withdrawal was affected by ouabain in K\(^{-}\)-enriched, Na\(^{+}\)-depleted medium. Similar to VSMC-E1A, ouabain did not significantly affect the [Na\(^{+}\)]/[K\(^{-}\)] ratio in Jurkat cells incubated in K\(^{-}\)-enriched, Na\(^{+}\)-depleted medium (Table IV). In Jurkat cells, substitution of Na\(^{+}\) by K\(^{-}\) in incubation medium attenuated apoptosis induced by a 7-h treatment with Fas ligand (20.0 ± 2.2 versus 28.5 ± 3.3% in K\(^{-}\)-enriched and control medium, respectively) and completely blocked the effect of long term ouabain administration on base-line chromatin cleavage (5.7 ± 2.0 versus 16.0 ± 4.6% in K\(^{-}\)-enriched and control medium, respectively; Table IV).

\[\text{TABLE II}\]

| Chromatin fragments | LDH release |
|---------------------|-------------|
| Control cells       | Ouabain-treated cells | Control cells | Ouabain-treated cells |
| 1. Serum-supplied cells | 2.3 ± 0.4 | 1.3 ± 0.4\(^{a}\) | 1.1 ± 0.4 | 1.2 ± 0.3 |
| 2. Serum-deprived cells | 4.5 ± 0.7 | 1.8 ± 0.3\(^{b}\) | 2.2 ± 0.5 | 1.8 ± 0.4 |
| 3. Serum-supplied, heat-treated cells | 2.3 ± 0.6 | 1.6 ± 0.4 | 10.2 ± 2.4\(^{c}\) | 11.0 ± 2.8\(^{c}\) |

\(^{a}\) p < 0.05 compared with ouabain-untreated cells.

\(^{b}\) p < 0.001 compared with ouabain-untreated cells.

\(^{c}\) p < 0.001 compared with serum-supplied, heat-untreated cells.
To estimate chromatin cleavage, cells were incubated for 1 h in the presence of 10% calf serum (CS) in the control solution (DMEM, [Na\(^+\) = 137 mM; [K\(^+\) = 5 mM; line 1) or under equimolar substitution of NaCl with KCl ([Na\(^+\) = 14 mM; [K\(^+\) = 128 mM; line 2) with or without 1 mM ouabain and then for an additional 6 h in the same media with or without CS. Intracellular Na\(^+\) and K\(^+\) content was measured as steady-state distribution of isotopes after a 24-h preincubation of cells in control Na\(^+\)-enriched solution containing 0.5 μCi/ml \(^{86}\)Rb or 2 μCi/ml \(^{22}\)Na and an additional 6-h incubation in Na\(^+\) (line 1)- or K\(^+\) (line 2)-enriched medium, containing 10% CS and isotopes with the same specific activity with or without 1 mM ouabain. For more details, see “Experimental Procedures.” Mean values ± S.E. obtained in three experiments performed in triplicate (ionic content) or quadruplicate (chromatin cleavage) are given.

| Concentration of Na\(^+\) and K\(^+\) in incubation medium | Intracellular cations | Chromatin fragments |
|-------------------------------------------------------------|-----------------------|---------------------|
| Control cells | Ouabain-treated cells | Control cells | Ouabain-treated cells |
| Na\(^+\)\(^i\) | K\(^+\)\(^i\) | Na\(^+\)\(^i\) | K\(^+\)\(^i\) | 10% CS | CS free | 10% CS | CS free |
| mM | nmol/mg protein | % | % | % | % | % | % |
| 1. [Na\(^+\)]\(_i\) = 137; [K\(^+\)]\(_i\) = 5 | 42 ± 14 | 297 ± 36 | 312 ± 47 | 99 ± 21 | 5.2 ± 0.6 | 32.8 ± 4.8 | 1.9 ± 0.5 | 6.3 ± 1.4 |
| 2. [Na\(^+\)]\(_i\) = 14; [K\(^+\)]\(_i\) = 128 | 38 ± 11 | 311 ± 40 | 51 ± 18 | 283 ± 36 | 5.9 ± 1.0 | 34.6 ± 5.0 | 5.0 ± 0.8 | 32.0 ± 5.5 |

\(^{a}p < 0.001\) compared with ouabain-untreated cells.

To estimate chromatin cleavage, cells were incubated for 2 h in the presence of 10% calf serum in the control solution (DMEM, [Na\(^+\) = 137 mM; [K\(^+\) = 5 mM; line 1) or under equimolar substitution of NaCl with KCl ([Na\(^+\) = 14 mM; [K\(^+\) = 128 mM; line 2) with or without 1 mM ouabain and then for an additional 7 h in the same media with or without 100 ng/ml Fas ligand (Fas-L). Intracellular Na\(^+\) and K\(^+\) content was measured as steady-state distribution of isotopes after a 24-h preincubation of cells in control Na\(^+\)-enriched solution containing 0.5 μCi/ml \(^{86}\)Rb or 2 μCi/ml \(^{22}\)Na and an additional 6-h incubation in Na\(^+\) (line 1)- or K\(^+\) (line 2)-enriched medium containing 10% calf serum and isotopes with the same specific activity with or without 1 mM ouabain. For more details, see “Experimental Procedures.” Mean values ± S.E. obtained in experiments performed in triplicate are given.

| Concentration of Na\(^+\) and K\(^+\) in incubation medium | Intracellular cations | Chromatin fragments |
|-------------------------------------------------------------|-----------------------|---------------------|
| Control cells | Ouabain-treated cells | Control cells | Ouabain-treated cells |
| Na\(^+\)\(_i\) | K\(^+\)\(_i\) | Na\(^+\)\(_i\) | K\(^+\)\(_i\) | Control | +Fas-L | Control | +Fas-L |
| mM | nmol/mg protein | % | % | % | % | % | % |
| 1. [Na\(^+\)]\(_i\) = 137; [K\(^+\)]\(_i\) = 5 | 183 ± 33 | 1545 ± 96 | 1137 ± 10 | 107 ± 21 | 4.9 ± 2.1 | 28.5 ± 3.3 | 16.0 ± 4.6 | 42.7 ± 5.0 |
| 2. [Na\(^+\)]\(_i\) = 14; [K\(^+\)]\(_i\) = 128 | 162 ± 33 | 1584 ± 99 | 204 ± 30 | 498 ± 47 | 4.0 ± 0.6 | 20.0 ± 2.2 | 5.7 ± 2.0 | 22.7 ± 3.3 |

\(^{a}p < 0.001\) compared with ouabain-untreated cells.

Modulation of Caspase Activity—Activation of the Caspase superfamily protease cascade is involved in apoptotic DNA degradation in the majority of cells studied so far (28, 29). However, to the best of our knowledge, there are no data on the measurement of Caspase activity in VSMC undergoing apoptosis. Using YVAD-AMC and DEVD-AMC as substrates for the Caspase-1 and Caspase-3 subfamilies (30), we observed that base-line activities of YVAD-ase and DEVD-ase in VSMC-E1A were 1571 ± 256 and 673 ± 235 pmol (mg protein)\(^{-1}\) h\(^{-1}\). In these cells, activity of the Caspase-1 and Caspase-3 measured as YVAD-CHO and DEVD-CHO-sensitive components of YVAD-ase and DEVD-ase, respectively, was 56 ± 47 and 547 ± 111 pmol (mg protein)\(^{-1}\) h\(^{-1}\) (n = 9). In Jurkat cells, total YVAD-ase and DEVD-ase activity was 1830 ± 201 and 831 ± 95 pmol (mg protein)\(^{-1}\) h\(^{-1}\), whereas Caspase-1 and Caspase-3 activity levels were in the range of 121 ± 67 and 305 ± 41 pmol (mg protein)\(^{-1}\) h\(^{-1}\), respectively (n = 12), which is in accordance with previously reported data (31).

Neither Jurkat cells nor VSMC-E1A undergoing apoptosis showed any modulation of Caspase-1 activity (data not present-
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**DISCUSSION**

The data obtained in the present study show for the first time that independently of the origin of apoptotic signals and transfection with c-myc or its functional analogue E1A-adenoviral protein, inhibition of the Na\(^+\),K\(^+\) pump blocks the development of apoptosis in VSMC (Figs. 2 and 3) without any modulation of VSMC necrosis triggered by severe heat stress (Table II). In contrast to VSMC, a 2-h preincubation with ouabain in the absence of triggers of apoptosis following a 3-h incubation in the presence of three different apoptotic stimuli (Fas ligand, staurosporine, and cell shrinkage with mannitol) did not modulate apoptosis in Jurkat cells (Fig. 4). Prolongation of incubation with ouabain for up to 7 h resulted in the activation of base-line chromatim cleavage in Jurkat cells but did not affect the increment of accumulation of chromatin fragments triggered by activation of the Fas receptor (Fig. 4a). Our results on the activation of base-line apoptosis under a 7-h inhibition of the Na\(^+\),K\(^+\) pump in Jurkat cells are consistent with recent data from Olej et al. (32) on the induction of apoptotic DNA degradation in human peripheral lymphocytes treated with ouabain for 48 h. The potentiation of base-line apoptosis after long term treatment of immune system cells with ouabain may be caused by the rise of intracellular Ca\(^{2+}\) concentration due to activation of the Na\(^+\)/Ca\(^{2+}\) exchange, which is highly active in human T-lymphocytes (33) and in Jurkat cells (34). This hypothesis is supported by prevention of the induction of base-line apoptosis in Jurkat cells treated with ouabain in Na\(^+\)-depleted medium (Table IV), i.e. under inhibition of the Na\(^+\)/Ca\(^{2+}\) mode of operation of the Na\(^+\)/Ca\(^{2+}\) exchanger, and is consistent with numerous data on the implication of sustained elevation of [Ca\(^{2+}\)], in triggering apoptosis in immune system cells (35, 36). In contrast to immune system cells, we did not observe any effect of moderate elevation of [Ca\(^{2+}\)], by thapsigargin and ionomycin on VSMC apoptosis (37).

Our results show that inhibition of apoptosis by ouabain in VSMC is caused by inversion of the [Na\(^+\)]/[K\(^+\)] ratio rather than by Na\(^+\)/K\(^+\)-independent modulation of ion current and membrane potential mediated by electrogenic Na\(^+\),K\(^+\) pump. Indeed, suppression of VSMC apoptosis by ouabain was abolished in K\(^+\)-enriched Na\(^+\)-depleted medium, i.e. when inhibition of the Na\(^+\)/K\(^+\) pump did not affect intracellular Na\(^+\) and K\(^+\) content (Table III). As with ouabain, VSMC apoptosis was blocked by inhibition of the Na\(^+\)/K\(^+\) pump in K\(^+\)-depleted medium (Table I). We did not observe any significant effect of an 8-h incubation of VSMC with ouabain on lactate dehydrogenase release (Table II), ATP content, and protein synthesis (data not shown). DNA synthesis in VSMC, measured as serum-induced \(^{3}H\)thymidine incorporation, was also insensitive to a 5-h preincubation with ouabain and was decreased by ~60% only after 48 h of the Na\(^+\)/K\(^+\) pump inhibition (data not presented). Viewed collectively, these results rule out the possible side effects of ouabain on VSMC as well as the toxic effect of inversion of the [Na\(^+\)]/[K\(^+\)] ratio.

It may be assumed that inversion of the [Na\(^+\)]/[K\(^+\)] ratio blocks VSMC apoptosis via inhibition of the net K\(^+\) efflux involved in cell shrinkage revealed in most of the cells undergoing programmed cell death, including the VSMC (10). This hypothesis is based on the induction of apoptotic DNA degradation in hyperosmotically shrunken mouse lymphoma cells, rat thymocytes (27), and Jurkat cells (14). Similar results were obtained in the present study by analysis of the effect of mannitol-induced shrinkage on chromatin cleavage in Jurkat cells.

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**Fig. 5. Effect of ouabain on Caspase-3 activity in VSMC-E1A (A) and Jurkat cells (B).** VSMC-E1A and Jurkat cells were pretreated with 1 mM ouabain for 1 and 2 h, respectively, and the additions indicated below the bars were delivered in media with 10% calf serum (CS) and with the base-line (137/5) or inverse (14/128) [Na\(^+\)]/[K\(^+\)] ratio for 6 (VSMC-E1A) or 3 h (Jurkat cells). In experiments with VSMC-E1A, calf serum was omitted where indicated (−), staur., staurosporine (0.25 μM); ok. ac., okadaic acid (1 μM); mann., mannitol (350 mM); Fas-L, Fas ligand (100 ng/ml). Mean values ± S.E. obtained in experiments performed in quadruplicate (A) or triplicate (B) are given.*p < 0.001 compared with ouabain-untreated cells.

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ed). In contrast to Caspase-1, Caspase-3 activity in VSMC-E1A was increased after a 6-h incubation in serum-deprived medium or in the presence of staurosporine and okadaic acid by 6-, 8-, and 10-fold, respectively, but was insensitive to mannitol-induced cell shrinkage (Fig. 5A). Pretreatment of VSMC-E1A for 1 h with ouabain decreased the base-line activity of Caspase-3 in Jurkat cells (Fig. 5B), and 20-, and 10-fold after 3 h of treatment with Fas ligand, staurosporine, and cell shrinkage with mannitol, respectively. Pretreatment of Jurkat cells for 2 h with ouabain increased base-line Caspase-3 activity from 296 ± 38 to 497 ± 67 pmol (mg protein)\(^{-1}\) h\(^{-1}\). Neither ouabain nor equimolar substitution of Na\(^+\) by K\(^+\) in the incubation medium affected the increment of Caspase-3 activity in Jurkat cells triggered by Fas ligand, staurosporine, and hyperosmotic shrinkage (Fig. 5B).
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Mal vessels (39, 40) or in E1A-transfected VSMC. In our study, equimolar substitution of Na

IgM (14). In our study, equimolar substitution of Na

observed in Jurkat cells treated with 10 ng/ml anti-human Fas

membrane-bound proteins involved in the triggering of apoptotic machinery. In this case, it can be predicted that similar to ouabain-treated VSMC, apoptosis can also be blocked by a rise of extracellular K+ concentration. Indeed, suppression of apoptosis by equimolar substitution of Na+i with K+o was observed in Jurkat cells treated with 10 ng/ml anti-human Fas IgM (14). In our study, equimolar substitution of Na+i with K+o led to a 30–40% inhibition of the increment of chromatin cleavage triggered by treatment of Jurkat cells with 100 ng/ml anti-human Fas IgM (Table IV). However, in contrast to Jurkat cells, equimolar substitution of Na+i by K+o did not protect ouabain-untreated VSMC-E1A from apoptosis triggered by serum withdrawal (Table III). These results strongly suggest that inversion of the [Na+/K+]i/o ratio blocks VSMC apoptosis independently of modulation of the Na+ and K+ electrochemical gradient across the plasma membrane.

We report here that suppression of apoptosis in ouabain-treated VSMC-E1A is accompanied by inhibition of Caspase-3 activity (Fig. 5A). Thus, it may be assumed that the increased [Na+]i/[K+]i ratio blocks VSMC apoptosis via direct inhibition of the activity of this enzyme. To examine this hypothesis, we measured the activity of Caspases in lysates of VSMC-E1A subjected to 6 h of serum withdrawal. Both Caspase-3 and Caspase-1 activity was decreased by 10–20% by the addition of 100 mM KCl. However, the same level of inhibition was also observed with the addition of 100 mM NaCl or choline chloride (data not shown). Thus, it may be concluded that the rise in ionic strength slightly inhibits Caspases, whereas the Na+/K+ ratio does not affect enzyme activity.

It is well documented that activation of Caspase-3 by Fas ligand is caused by cleavage of pro-Caspase-3 triggered by Fas-activated death domain-mediated activation of Caspase-8 (41) and is independent of cytochrome c release from mitochondria (42). However, as mentioned above, the Fas ligand signaling pathway is quenched in VSMC and VSMC-E1A. In several cell types, Fas-independent cleavage of pro-Caspase-3 is triggered by Caspase-9, which in turn is activated by the Apaf-1/cytochrome c complex (43). At least in HeLa cells, cytochrome c release is caused by Caspase-8-mediated proteolysis of Bid, a BH3 domain-containing protein that interacts with both Bak and Bel-2 (43) and is independent of dissociation of the mitochondrial transmembrane potential (44). Recently, it was proposed that pro-Caspase-3 activity is controlled by the Na+/K+ ratio (31). This hypothesis was based on the selective inhibition by KCl of Caspase-3 activity in rat thymocyte lysates treated with 10 μg/ml cytochrome c and 1 μm dATP. However, we failed to detect any effect of these compounds at the same concentration on Caspase-3 activity in VSMC-E1A lysates in the absence of monovalent cations or in the presence of 100 mM KCl or NaCl (data not shown). Viewed collectively, these results demonstrate that the [Na+]i/[K+]i ratio blocks VSMC apoptosis at a site upstream of Caspase-3.

In conclusion, our results show that inversion of the [Na+]i/[K+]i ratio blocks apoptosis in VSMC via the inhibition of trigger-independent steps of the programmed death machinery at a site upstream of the Caspase-3-triggering cascade. Comparison of the requirements for Caspase-9 and Caspase-3 in fibroblasts and immune system cells treated with UV and γ-irradiation, staurosporine, or anti-CD95 indicates the existence of at least four different types of apoptosis (45). Data obtained in the present study show that Caspase-3-dependent apoptosis may be further subclassified based on its sensitivity to the [Na+]i/[K+]i ratio and cell volume. In VSMC, Caspase-3-dependent apoptosis can be blocked by inversion of the [Na+]i/[K+]i ratio, but is insensitive to cell shrinkage. In contrast, in Jurkat cells, cell shrinkage activates Caspase-3-dependent apoptosis, whereas the [Na+]i/[K+]i ratio does not affect apoptosis triggered by Fas ligand, staurosporine, or cell shrinkage. Is this mechanism limited to VSMC, or is it also expressed in other electrical excitable tissue subjected to modulation of the [Na+]i/[K+]i ratio under sustained depolarization? Which molecules are involved in the sensing of intracellular Na+(K+) concentration(s) and transduction of the antiapoptotic signal? These questions will be addressed in future studies.

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