Cell volume and monovalent ion transporters: their role in cell death machinery triggering and progression

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Submitted 4 February 2013; accepted in final form 18 April 2013

Orlov SN, Platonova AA, Hamet P, Grygorczyk R. Cell volume and monovalent ion transporters: their role in cell death machinery triggering and progression. Am J Physiol Cell Physiol 305: C361–C372, 2013. First published April 24, 2013; doi:10.1152/ajpcell.00040.2013.—Cell death is accompanied by the dissipation of electrochemical gradients of monovalent ions across the plasma membrane that, in turn, affects cell volume via modulation of intracellular osmolyte content. In numerous cell types, apoptotic and necrotic stimuli caused cell shrinkage and swelling, respectively. Thermodynamics predicts a cell type-specific rather than an ubiquitous impact of monovalent ion transporters on volume perturbations in dying cells, suggesting their diverse roles in the cell death machinery. Indeed, recent data showed that apoptotic collapse may occur in the absence of cell volume changes and even follow cell swelling rather than shrinkage. Moreover, side-by-side with cell volume adjustment, monovalent ion transporters contribute to cell death machinery engagement independently of volume regulation via cell type-specific signaling pathways. Thus, inhibition of Na+–K+-ATPase by cardiotonic steroids (CTS) rescues rat vascular smooth muscle cells from apoptosis via a novel Na+-K+-mediated, Ca2+-independent mechanism of excitation-transcription coupling. In contrast, CTS kill renal epithelial cells independently of Na+-K+-ATPase inhibition and increased [Na+]/[K+], ratio. The molecular origin of [Na+]/[K+], sensors involved in the inhibition of apoptosis as well as upstream intermediates of Na+/K+-independent death signaling triggered by CTS remain unknown.

apoptosis; necrosis; cell volume; intracellular Na+, intracellular K+

introduced more than a hundred years ago by Rudolf Virchow. Necrotic stimuli evoke cell swelling, plasma membrane disruption, lysosomal enzyme release, inflammation and digestion of damaged cells by macrophages (38, 39, 59). In contrast to necrosis, the death of single cells without inflammation is termed apoptosis. This mode of cell death is accompanied by cell and nuclei condensation, membrane segment evolution (so-called membrane blebbing), nucleus fragmentation, and the formation of apoptotic bodies. In the initial stages, apoptosis occurs without plasma membrane rupture and leakage of intracellular constituents, thus without triggering an inflammatory response. Apoptosis can be evoked by either intrinsic or extrinsic stimuli. Intrinsic stimuli, such as staurosporine (a non-selective inhibitor of protein kinase C), thapsigargin (a sarco-plasmic reticulum Ca2+ pump inhibitor), etoposide (a DNA topoisomerase II inhibitor), dexamethasone, and radiation, interact with intracellular targets. They perturb mitochondrial cytochrome c release that, together with apoptotic protease-activating factor, stimulates procaspase-9, a cysteine protease family member with aspartate substrate specificity. Extrinsic apoptosis is elicited by extracellular stimuli, such as Fas-ligand and other members of the tumor necrosis factor (TNF) family, via their interaction with CD95 receptor, which, in turn, recruits procaspase-8 to its

CELL DEATH IS ACCOMPANIED by the dissipation of electrochemical gradients of monovalent ions across the plasma membrane that, in turn, may cause cell volume perturbations by altering the content of intracellular osmolytes and osmotically obliged water (51, 54, 70, 71). Our mini-review focuses on three major questions. First, should we consider shrinkage and swelling as obligatory hallmarks of apoptosis and necrosis, i.e., two morphologically distinct modes of cell death? Second, are volume perturbations an obligatory step triggering the cell death machinery? Third, do intracellular monovalent cations contribute to the cell death machinery independently of their impact on cell volume adjustment?

Cell Volume Behavior as an Approach to Cell Death Classification

Two major morphologically distinct modes of cell death have been documented in morphological investigations. The demise of clustering, neighboring cells under complement attack, severe hypoxia and hyperthermia, lytic viral infection, or exposure to toxins and poisons is known as necrosis, a term introduced more than a hundred years ago by Rudolf Virchow. Necrotic stimuli evoke cell swelling, plasma membrane disruption, lysosomal enzyme release, inflammation and digestion of damaged cells by macrophages (38, 39, 59). In contrast to necrosis, the death of single cells without inflammation is termed apoptosis. This mode of cell death is accompanied by cell and nuclei condensation, membrane segment evolution (so-called membrane blebbing), nucleus fragmentation, and the formation of apoptotic bodies. In the initial stages, apoptosis occurs without plasma membrane rupture and leakage of intracellular constituents, thus without triggering an inflammatory response. Apoptosis can be evoked by either intrinsic or extrinsic stimuli. Intrinsic stimuli, such as staurosporine (a non-selective inhibitor of protein kinase C), thapsigargin (a sarco-plasmic reticulum Ca2+ pump inhibitor), etoposide (a DNA topoisomerase II inhibitor), dexamethasone, and radiation, interact with intracellular targets. They perturb mitochondrial cytochrome c release that, together with apoptotic protease-activating factor, stimulates procaspase-9, a cysteine protease family member with aspartate substrate specificity. Extrinsic apoptosis is elicited by extracellular stimuli, such as Fas-ligand and other members of the tumor necrosis factor (TNF) family, via their interaction with CD95 receptor, which, in turn, recruits procaspase-8 to its
death effector domain. Both extrinsic and intrinsic apoptosis is executed by caspase-3 activation (23, 39).

Numerous biochemical changes, such as cytochrome c release from mitochondria, activation of caspases, phosphatidylserine exposure on plasma membrane outer surfaces, chromatin cleavage, and DNA degradation with the appearance of multiple 180- to 200-base pair fragments (so-called DNA laddering), have been discerned in morphologically defined apoptotic cells (39). It should be underlined, however, that cytochrome c release is also observed in Jurkat cells undergoing morphologically defined oncosis (52), whereas caspase inhibitors delay cell death caused by plasma membrane disruption triggered by chemically induced hypoxia or toxins (113). Moreover, caspase-3 activation in viable lymphocytes is an obligatory step of T-cell receptor activation (6). In several cell types, morphologically defined apoptosis occurs in the absence of internucleosomal DNA cleavage (35), whereas both chromatin cleavage and DNA laddering are seen in cells during oncosis (35). Phosphatidylserine exposure results from scramblase activation by intracellular Ca

Thermodynamics of Cell Volume Changes

Thermodynamic models predict that the final outcome of cell death stimuli on cell volume depends on diverse parameters, including electrical membrane potential ($E_m$), relative permeability to monovalent cations ($P_{Na}$, $P_{K}$, $P_{Cl}$) and driving force predicted by their electrochemical gradients. Thus, in electrically excitable cells with $E_m> -E_K$, $[K^+]_i=[K^+]_N$ and $[Cl^-]>[Cl^-]_N$, activation of K⁺ channels does not affect cell volume. Here, $[K^+]_N$ and $[Cl^-]_N$ stand for intracellular concentrations of K⁺ and Cl⁻ predicted by Nernst equilibrium potentials. In this cell type, AVD can be triggered by stimulation of Cl⁻ channels that, in turn, lead to membrane depolarization and K⁺ efflux (Fig. 1A). In mammalian erythrocytes and other cells with $E_m> -E_K$, $[K^+]_i=[K^+]_N$, $[Cl^-]_i< [Cl^-]_N$ and $P_{Cl} >> P_{Na}$ activation of K⁺ channels elicits hyperpolarization and efflux of Cl⁻ and osmotically obliged water (Fig. 1B). This is a case of suicidal death of erythrocytes or eryptosis mediated by elevation of $[Ca^{2+}]_i$, and activation of intermediate-conductance, voltage-, and Ca²⁺-gated K⁺ channels (IKCa) (74, 76, 112). In cells with $E_K - E_m< 0$, activation of K⁺ or Cl⁻ channels exerts opposite effects on Cl⁻ and K⁺ fluxes, minimizing or even opposing their impact on cell volume. Indeed, in Jurkat cells undergoing apoptosis in the presence of Fas-L or ceramides, the activity of voltage-gated K⁺ channels (Kv1.3) is decreased rather than increased (47, 119).

Unlike AVD, NVI could be mediated by activation of Na⁺/H⁺ exchanger and other inwardly directed, Na⁺-coupled transporters, including Na⁺-K⁺-2Cl⁻, Na⁺-HCO₃⁻, and Na⁺-Pc cotransporters, or by sympot of Na⁺ and low-molecular-weight organic osmolytes. Several research groups have reported that progression of apoptosis is accompanied by inhibition of Na⁺/H⁺ exchanger (for review, see Ref. 73).

Fig. 1. Thermodynamic model of the ion transport pathways underlying dying cell volume decrease (DVD) and increase (DVI). A and B: K⁺ and Cl⁻ channel cross talk triggering DVD in cells with different membrane potential ($E_m$) and intracellular K⁺ ([K⁺]$_i$) and Cl⁻ ([Cl⁻]$_i$) concentrations. C: ion transporters involved in DVI. 1, Na⁺-K⁺-ATPase; 2, Na⁺-coupled transport of organic osmolytes (OROS). For other abbreviations, see text.
However, to the best of our knowledge, no one has demonstrated the cell volume-dependent implication of this carrier in the necrotic mode of cell death. An alternative model suggests that NVI is triggered by Na\(^+\) accumulation via the activation of nonselective cation channels (88) (Fig. 1C). Increased permeability for Na\(^+\), in turn, leads to the dissipation of Gibbs-Donnan equilibrium that is terminated by membrane depolarization and accumulation of Cl\(^-\) and osmotically obliged water (32). Dissipation of Gibbs-Donnan equilibrium is also accelerated by energy depletion caused by Na\(^+\)/K\(^-\)-ATPase activation in an attempt to normalize [Na\(^+\)], at the expense of high ATP consumption. The data supporting this model were mainly obtained from ischemic hepatocytes (33).

Side-by-side with elevated Na\(^+\) uptake, NVI can be triggered by sustained Na\(^+\)/K\(^+\)-ATPase inhibition (Fig. 1C). It should be underlined that this model can be applied to cells with \(P_K > P_{\text{Na}}, [\text{Cl}^-] < [\text{Cl}^-]_{i\text{N}}\) and \([\text{Na}^+] > [\text{Na}^+]_{i\text{N}}\), and its time course depends on the rate of Na\(^+\) influx (79). Indeed, sustained Na\(^+\)/K\(^+\)-ATPase inhibition did not affect the volume of \([^{14}\text{C}]\text{urea} available space in Jurkat cells (89) and vascular smooth muscle cells (VSMCs) from the rat aorta (121), decreasing rather than increasing the volume of ouabain-treated cardiomyocytes (116). The latter phenomenon is probably caused by K\(^-\) efflux via Ca\(^{2+}\)-activated channels and the lack of Na\(^+\) accumulation due to an extremely active Na\(^+\)/Ca\(^{2+}\) exchanger in these cells (16). Indeed, shrinkage of ouabain-treated cardiomyocytes turns to swelling in Ca\(^{2+}\)-free medium (116).

**Cell Volume Changes Triggered by Death Stimuli**

Analysis of volume perturbations in cells exposed to apoptotic stimuli has documented 2 stages of AVD. The primary stage is observed in the absence of morphological markers of apoptosis and is associated with 10–30% attenuation of cell volume. The secondary stage constitutes 50–70% decreased cell volume and develops in parallel with plasma membrane blebbing as well as accumulation of apoptotic bodies (12, 23). These data, however, contradict several reports on the absence of primary AVD in cells undergoing apoptosis (56).

Analysis of this controversy should consider that cell volume measurement methods have several limitations. Thus, light scattering, refractometry, and Coulter electronic size techniques are applicable to suspended cells only and work best with cells of simple, e.g., spherical, shape. Analysis of volume alterations in cells of more complex shape might be prone to artifacts, since these methods do not distinguish between shape changes at constant cell volume and those resulting from genuine volume modulation. Moreover, preparation of substrate-attached cell suspensions will inevitably perturb resting cell shape and volume during trypsinization. In addition to shape transition, detachment of several types of cells causes massive cell death (so-called anoikis) that per se is sufficient to trigger AVD (4). Measurement of intracellular water volume with membrane-permeable nonmetabolized compounds, such as \([^{14}\text{C}]\text{urea} and methyl-\([^{14}\text{C}]\text{glucose, needs prolonged incubation for the steady-state distribution of radioisotopes as well as extensive washing, which complicates precise kinetics studies. Moreover, data obtained by this method might be also impacted by changes in the hydrophobic properties of intracellular milieu detected in apoptotic cells (55). Three-dimensional (3D) reconstruction of single-cell images by laser interference or holographic microscopy (109) cannot be employed for volume measurement because the average refractive index of the cytoplasm is affected by cell shrinkage (131). The calcein-quenching technique assumes its homogenous distribution within intracellular milieu (117). Complete 3D information on cell shape and volume behavior is possible with confocal laser scanning microscopy, scanning ion conductance microscopy, and atomic force microscopy (65). However, temporal resolution of the former techniques is limited and does not permit monitoring of rapid volume changes, often fully completed within 2–3 min. Further limitations include photodynamic damage to cells during longer exposure to laser light (69).

Keeping the above listed limitations in mind, we developed the dual-image surface reconstruction (DISUR) technique based on phase-contrast digital video microscopy, which allows us to simultaneously measure cell height, total surface area, and volume of unperturbed, substrate-attached cells with temporal resolution of \(\sim 100\) ms (27). Taking this approach, we estimated, for the first time, the size of plasma membrane reserves (45), the role of cytoplasm hydrogel in extracellular pH-sensing and osmo-sensing (42), and renal epithelial cell volume regulation by P2Y receptor agonists (63). More recently, we employed DISUR to assess the volume of single VSMCs transfected with E1A-adenoviral protein (E1A-VSMCs) (107). In the absence of growth factors, these cells undergo rapid death, presenting biochemical markers of “classic” apoptosis, including chromatin cleavage, DNA laddering, phosphatidylserine exposure, and caspase-3 activation (13). After a 30- to 60-min lag-phase, the volume of serum-deprived E1A-VSMCs increases by \(\sim 40\%\) (Fig. 2), preceding maximal increments of caspase-3 activity and chromatin cleavage. Swollen cells undergo rapid apoptotic collapse, documented by plasma membrane budding, with termination occurring in 10–15 min by the formation of numerous apoptotic bodies. In contrast to this experimental model of extrinsic apoptosis, the collapse of E1A-VSMCs undergoing intrinsic apoptosis in the presence of staurosporine follows \(\sim 30\%\) attenuation of their volume (Fig. 2). Complex volume behavior was observed in...
Ehrlich ascites tumor cells exposed to cisplatin by Poulsen and coworkers (109). In the initial phase, cell volume was decreased by 6–8%, then restored and reduced by ~30% during the executive phase of apoptosis (Fig. 2). Differential volume behavior of VSMCs undergoing apoptosis in serum-deprived medium and in the presence of staurosporine is consistent with the findings of Bortner and Cidlowski (20), who reported that, in the absence of extracellular Na⁺, anti-Fas-treated Jurkat cells swell in conjunction with canonical biochemical markers of apoptosis, such as chromatin cleavage and caspase activation (for more details, see Ref. 23).

The data presented in this section disclose that cell shrinkage cannot be considered as a universal marker of apoptosis. Alternatively, apoptosis should be further subclassified in accordance with distinct cell volume behaviors. However, such classification seems premature. Indeed, considering the absence of any definitive quantitative biochemical markers of apoptosis, Kroemer and coworkers (67) wrote that “classification of types of cell death is more important for the forensic department of the police than for the cell death research community” (67). In other words, is classification of cell death modes in any way helpful? Keeping this uncertainty in mind, the Nomenclature Committee of Cell Death suggested replacement of the attractive Greek words apoptosis and necrosis by expressions, such as “cell death induced by osmotic shock,” “cell death triggered by serum withdrawal,” etc. (67). We have adopted the terminology throughout our mini-review, leaving “cell death triggered by serum withdrawal” for more details, see Refs. 18, 22, 46, 72, and 73.)

Does Cell Shrinkage Contribute to Apoptosis?

Several independent approaches have been taken to answer this question. These data are briefly summarized below. (For more details, see Refs. 18, 22, 46, 72, and 73.)

**DVD kinetics.** Cross talk in DVD kinetics and the appearance of biochemical and morphological markers of cell death seem to be both cell type and apoptotic stimuli specific. Thus, in staurosporine-treated U937, KB, NG108-15, HeLa, and human T cells, DVD was observed 2–5 h before apoptotic nuclei were evident and was resistant to the broad-spectrum caspase inhibitors zVAD-fmk and zD-dcb (128). In contrast, decreased Fas-ligand-treated Jurkat cell volume was correlated with cell populations presenting DNA laddering, loss of mitochondrial potential, and caspase-3 activation (17, 19, 24, 26, 124) and was partially abolished by zVAD-fmk (89). These observations disclose that DVD contributes to cell death machinery development at a step downstream of caspase activation. In contrast, primary DVD was not detected in UV-irradiated HeLa cells and X-irradiated rat thymocytes. In these cells, the kinetics of cell volume attenuation and the appearance of morphological markers of apoptosis were identical, suggesting that AVD is a consequence rather than a trigger of apoptosis (91).

**Effect of hyper- and isosmotic shrinkage.** Hyperosmotic shrinkage, triggered by increment of osmolarity from 300 to 600–700 mosM, results in the death of immune system cells (17, 101), vascular endothelial cells (7, 61, 83), mIMCD3 renal epithelial cells (86), HeLa cells (15), and D54-MG human glioma cells (41). In all of the studies cited, dying cells exhibited markers of classic apoptosis, such as DNA laddering, chromatin cleavage, phosphatidylserine appearance, and caspase activation. It should be noted, however, that similar elevation of medium osmolarity only slightly augmented apoptosis in SH-SY5Y neuroblastoma cells (84) and had no effect in MDCK (94), Cos-7, GH3, and HeLa cells (17). Moreover, Lang and coworkers observed that 20–30% augmentation of osmolarity inhibited rather than triggered apoptosis in Fas-L-treated Jurkat cells (48).

The differential sensitivity of mammalian cells to hypertonic environments was further confirmed by comparative analysis of the dose-dependent action of elevated osmolarity on cell survival (97). These experiments demonstrated that the addition of 300 mM mannitol increased chromatin fragmentation in Jurkat and porcine aorta endothelial cells (PAECs) by ~10- and 2-fold, respectively, but did not affect chromatin cleavage in MDCK cells. The lowest sensitivity to medium osmolarity was displayed by VSMCs. These cells, chromatin cleavage did not exceed 10% of total [³H]-labeled DNA, even in the presence of 500 mM mannitol (Fig. 3A). High resistance to apoptosis was also detected in chondrocytes subjected to hyperosmotic treatment (77). Independently of cell origin, the addition of 400 mM mannitol decreased the volume of [¹⁴C]Su rea available space by ~40% (Fig. 3B), which was twice higher than primary DVD in an overwhelming number of cell types studied so far (see above).

Shrunken cells normalize their volume via uptake of osmoles [so-called regulatory volume increase (RVI) (70)]. Thus, it may be assumed that resistance to apoptosis in hyperosmotic medium is caused by highly active RVI (17, 82). To explore the hypothesis, Bortner and coworkers (25) subjected Jurkat cells to multiple rounds of hyperosmotic stress, followed by isosmotic recovery. This approach resulted in selection of the OS4-15 cell line with augmented RVI. Significantly, OS4-15 cells were resistant to modest hypertonic stress as well as to staurosporine, dexamethasone, and thapsigargin, while remaining sensitive to Fas-ligand (25). These data suggest that RVI is involved in volume adjustment of cells exposed to intrinsic rather than extrinsic apoptotic stimuli.

In an analysis of the role of RVI in DVD and apoptosis, it should be underlined that dissipation of electrochemical gradients of monovalent ions, occurring in dying cells, sharply decreases the driving force for RVI. Indeed, Subramanyam et al. (118) reported that canonical apoptotic stimuli, such as staurosporine and TNF-α, almost completely abolished RVI in
HeLa cells. DISUR discerned negligible RVI in MDCK cells and VSMCs (62, 107), exhibiting high resistance to apoptosis in hypertonic medium (Fig. 3A). Yurinskaya and coworkers (130) detected apoptosis in hyperosmotically shrunken U937 cells capable of RVI. Viewed collectively, the data indicate that RVI does not determine the resistance of these cells to apoptosis evoked by hyperosmotic stress.

The physiological consequences of hyperosmotic and isosmotic shrinkage are different. Indeed, hyperosmotic shrinkage increases intracellular ionic strength whereas isosmotic shrinkage is mediated by loss of intracellular osmolytes (62). Several independent approaches have been employed to examine the role of cell volume modulation in death signaling. It is known that the majority of cells exposed to hypotonic solution undergo rapid regulatory volume decrease (RVD) caused by efflux of intracellular osmolytes, and normalization of medium osmolality leads to their shrinkage (70). We observed that this protocol reduced the volume of E1A-VSMCs by ~2-fold but did not affect their survival (97). In contrast, ~20–30% attenuation of cell volume, triggered by isosmotic substitution of Cl\(^-\) by gluconate or aspartate or Na\(^+\) by N-methyl-D-glucamine, resulted in the death of HeLa and U937 cells bearing canonical markers of apoptosis (81, 90). It should be noted, however, that side-by-side with cell shrinkage, Na\(^+\) and Cl\(^-\) depleted media sharply affect the intracellular concentration of monovalent ions. The role of intracellular monovalent cations distinct from volume regulation in apoptosis triggering and progression is considered in the last section of our mini-review.

**Effect of high-K\(^+\) medium.** In numerous cell types, DVD is mediated by loss of K\(^+\), a major intracellular inorganic osmolyte. Thus, in dexamethasone-treated thymocytes, K\(^+\) concentration, measured by inductively coupled plasma/mass spectrometry and \(^{86}\)Rb as a radioactive analog of K\(^+\), was decreased by 2- to 3-fold (43, 58). Activation of apoptosis in dexamethasone-treated CEM-C7A cells was accompanied by ~40% loss of total K\(^+\) concentration, measured by flame photometry (14). Similar K\(^+\) reduction was observed in mouse L cells that underwent complete apoptosis in the presence of cell cycle progression inhibitors (10). Two phases in changes of intracellular monovalent ion composition were demonstrated in staurosporine-treated U937 cells. The early phase decreased K\(^+\) and Cl\(^-\) content concomitant with cell shrinkage and preceded caspase-3 activation, whereas the later phase started with caspase-3 activation and was characterized by Na\(^+\) accumulation (9). In contrast, in Fas-L-treated Jurkat cells, 2-fold increment of \(^{86}\)Rb efflux was completely blocked by the pan-caspase inhibitor zVAD-fmk (89), suggesting that these events are a consequence rather than an executive mechanism of apoptosis. For more details, see Ref. 73.

Bortner and Cidlowski tested the fluorescence of K\(^+\) and Na\(^+\) chelators (PBFI and SBFI, respectively) to study rapid perturbations of the [Na\(^+\)]/[K\(^+\)] ratio in apoptotic cells. They determined that PBFI and SBFI fluorescence was decreased ~15-fold in shrunken dexamethasone-treated thymocytes and Fas-L-treated Jurkat cells compared with cells with normal volume (24, 58). On the basis of these findings, the authors proposed that Na\(^+\) loss also contributes to DVD of immune system cells. It should be noted, however, that this conclusion contradicts the data obtained in cell-free systems and in cells exposed to the nonselective Na\(^+\)/K\(^+\) ionophore gramicidin. These studies demonstrated that [K\(^+\)], reduction from 135 to 5 mM, i.e., a condition mimicking complete K\(^+\) transmembrane gradient dissipation, and [Na\(^+\)], attenuation from ~10 mM to zero decreased PBFI and SBFI fluorescence by only 2- and 3-fold (10, 87). Thus the sharp decline of fluorescence, documented in the above cited studies, was mainly attributed to nonspecific leakage of fluorescent indicators across the plasma membrane in apoptotic cells rather than by alteration of transmembrane K\(^+\) and Na\(^+\) fluxes mediated by ion pumps, channels, and carriers.
The thermodynamic model predicts that dissipation of the transmembrane K\(^+\) gradient in high-K\(^+\) medium will suppress DVD-mediated cell death (Fig. 1). Indeed, Cidlowski and co-workers observed that extracellular K\(^+\) concentration ([K\(^+\)\(_o\)]\(_o\)) elevation from 5 to 100 mM rescued Fas-L-treated Jurkat cells (24, 43). Modest inhibition of caspase-3 activity by high-[K\(^+\)\(_o\)]\(_o\) medium was also documented in dexamethasone-, thapsigargin-, and staurosporine-treated thymocytes (58). VSMCs exposed to the protonophore FCCP (66), and in amyloid-treated neuronal cells (34). Addition of 200 mM KCl completely blocked the death of HeLa cells triggered by hyperosmotic medium (400 mM sorbitol) (115). However, similar protection was also witnessed under equimolar substitution of KCl by NaCl. These data suggest that, at least in several cell types, the antiapoptotic activity of high-K\(^+\) medium is mediated by elevation of ionic strength rather than by inhibition of K\(^+\)-mediated DVD. It should also be noted that, independently of suppression of K\(^+\) loss and DVD, [K\(^+\)\(_o\)]\(_o\) increment has profound electrical effects on the activity of membrane-bound proteins. Moreover, long-term dissipation of Gibbs-Donnan equilibrium in high-K\(^+\) medium can affect numerous cellular functions, including cell survival through differential expression of [Na\(^+\)]\(_o\)-[K\(^+\)]\(_o\)-sensitive genes (see below). Keeping this in mind, we studied E1A-VSMCs undergoing rapid apoptosis in the absence of growth factors. We did not see any protection of serum-deprived E1A-VSMCs against apoptosis by [K\(^+\)\(_o\)]\(_o\) elevation from 5 to 126 mM (97).

**Effect of inhibitors of ion transporters involved in DVD.**

Apoptosis alters the activity of diverse ion transporters involved in DVD. The list includes voltage-gated K\(^+\) channels (K\(_v\), K\(_v\)-1.3, K\(_v\)-1.1, K\(_v\)-4), Ca\(^{2+}\)-activated K\(^+\) channels, K\(_{ATP}\), nonselective cation channels, voltage-regulated anion channels, and voltage-dependent anion channels (for comprehensive reviews, see Refs. 53, 73, and 110). These findings and thermodynamic models (Fig. 1) predict that modulators of ion transport pathways will affect DVD-dependent cell death. Indeed, Maeno and coworkers (80) reported that, in HeLa, U937, PC12, and NG108–15 cell lines, nonselective inhibitors of anion channels [4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and phloretin] and K\(^+\) channels (5 mM Ba\(^{2+}\)) completely blocked DVD triggered by two distinct inducers of apoptosis and restored cell survival. Transfection of mutationally inactivated cystic fibrosis transmembrane regulator anion channels suppressed the death of etoposide-treated epithelial cells (44), whereas disruption of voltage-gated chloride channels CIC-3 decreased apoptosis in the myocardium of mice subjected to ischemia-reperfusion (29). Wei et al. (127) reported that DIDS and NPPB inhibited DVD but did not affect caspase-3 activation and DNA fragmentation in staurosporine-treated cortical neurons. The IK\(_{CA}\) inhibitor clotrimazole blocked DVD and the death of staurosporine-treated glioblastoma cells, but not cells exposed to TRAIL, a trigger of the extrinsic apoptosis pathway (85). Very modest inhibition or the absence of any protective action of K\(^+\) channel blockers was documented in other studies (12, 34, 41, 44, 57, 66, 68, 120, 125).

It should be mentioned that the channel blockers employed in the above cited investigations had diverse side effects. Thus the K\(^+\) channel blocker Ba\(^{2+}\) suppressed Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport activity (103), and phloretin sharply decreased intracellular ATP content (114), whereas the blocker of volume-sensitive anion channels, 4-(2-butyl-6,7-dichloro-2cyclopentyl-inden-1-on-5-yl) oxybutyric acid, potently inhibited connexin hemichannels and the glutamate transporter GLT-1 (28). Using charybdotoxin, a more selective inhibitor of IK\(_{CA}\) and large-conductance Ca\(^{2+}\)-activated K\(^+\)-channels (BK\(_{Ca}\)), Elliot and Higgins (40) demonstrated attenuation of DVD and apoptosis in thymocytes exposed to Ca\(^{2+}\) ionophore. In contrast, neither charybdotoxin nor apamin, an inhibitor of small-conductance Ca\(^{2+}\)-activated K\(^+\)-channels (SK\(_{Ca}\)), affected apoptosis in Fas-L-treated Jurkat cells (89).

**Does Cell Swelling Contribute to Necrosis?**

Several research teams have proposed that the necrotic mode of cell death is caused by DVI-mediated plasma membrane rapture (11, 32, 92). Data supporting this hypothesis were mainly obtained in hepatocytes subjected to ATP depletion. It was shown that swelling and death of ATP-depleted hepatocytes were abolished in Na\(^+\)-free medium and were augmented when K\(^+\) efflux was inhibited by BaCl\(_2\) (32, 33, 106). The same research team also reported that the volume of ATP-depleted hepatocytes was compensated by RVD (11, 32). The former observation is surprising because Na\(^+\)-K\(^+\)-ATPase inhibition, depolarization, and dissipation of the electrochemical K\(^+\) gradient in ATP-depleted cells sharply diminish RVD efficiency.

The action of hypotonic medium on cell survival was investigated to verify the chemiosmotic model of necrosis. Malek and coworkers (83) demonstrated that attenuation of medium osmolarity by 2-fold only slightly diminished the survival of bovine aortic endothelial cells. Decreasing medium osmolarity from 290 to 150 mosM led to similar elevation of laser beam forward angle scatter, as detected in ATP-depleted hepatocytes, but did not reduce cell survival (32). In this study, hypotonic swelling slightly increased the number of dead cells after their exposure to KCN, whereas modest swelling at 200 mosM promoted hepatocyte resistance to hypoxia via swelling-induced ATP-release and P2Y activation (31).

Groulx and coworkers (45) undertook DISUR to evaluate the surface area and volume of single substrate-attached A549, 16HBE14o- CHO, and NIH 3T3 cells subjected to severe (6 mosM) hypotonic stress. This study disclosed that all cell types exhibited extremely large membrane reserves and increased their surface area as well as their volume by 4- and 10-fold, respectively, mainly by shape transition and by drawing extra membrane from preexisting surfaces and intracellular membrane reserves. Large membrane reserves underline the maintenance of membrane tension below lytic levels during various hyper- and isosmotic cell volume perturbations and argue against rupture of plasma membrane integrity by DVI. Indeed, even after plasma membrane permeabilization and complete collapse of Gibbs-Donnan equilibrium, the volume of all nucleated cells studied so far increased by less than 2-fold (42). This value is ~5-fold less than the threshold of cell volume increment, resulting in plasma membrane rupture in nucleated cells (45).

**Intracellular Monovalent Cations Influence the Death Machinery Independently of Cell Volume Regulation**

In an analysis of the complex volume behavior of dying cells, Bortner and Cidlowski (21) proposed that altered handling of intracellular ions rather than cell volume modulation...
was associated with apoptosis and necrosis. At that time, it was shown that elevation of KCl concentration from 10 to 200 mM completely blocked DNA degradation of isolated nuclei, nuclelease activity in extracts from dexamethasone-treated thymocytes (58), and (dATP + cytochrome c)-dependent procaspase-3 activity in thymocytes and Jurkat cell lysates (58, 124). These data prompted researchers to hypothesize that K⁺ loss rather than DVD contributes to apoptosis progression (21). It should be noted, however, that similar inhibition of cytochrome c-dependent apoptosis formation (30) and procaspase-3 activity (unpublished observations) occurs in the presence of equimolar amounts of Na⁺. These results strongly suggest that suppression of the above listed apoptotic pathways is caused by elevated ionic strength rather than by K⁺ and question the relevance of findings on regulation in vivo.

To further explore the cell volume-independent involvement of intracellular monovalent cations in the cell death machinery, we inhibited Na⁺/K⁺-ATPase with ouabain, i.e., hydrophilic CTS, in a large number of in vitro experiments. Surprisingly, we found that these compounds protected E1A-VSMCs against apoptosis triggered by growth factor withdrawal, staurosporine, and inhibitors of serine-threonine phosphatases (101), blocking the swelling of serum-deprived E1A-VSMCs (107). E1A-VSMC death was also suppressed by Na⁺/K⁺-ATPase inhibition in K⁺-free medium, whereas dissipation of the transmembrane gradient of monovalent cations in high-K⁺ medium completely abolished the antiapoptotic action of ouabain (101). Because the protective action of ouabain was absent in K⁺-free, low-Na⁺ medium, we concluded that the antiapoptotic signal was mediated by [Na⁺⁺], elevation rather than by [K⁺⁺], loss (96, 102).

In additional experiments, we found that inhibitors of macromolecular synthesis, such as actinomycin D and cycloheximide, abolished the protective effect of ouabain (99). Deploying a rat multiprobe template set, we failed to detect differential expression of mRNA species encoding major pro- and antiapoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-xS, Bax, and caspases-1–3, in ouabain-treated E1A-VSMCs (95). Keeping these negative data in mind, we adopted a proteomics approach to characterize a set of [Na⁺⁺]-sensitive genes. Twelve soluble proteins, including mortalin, whose expression is triggered by ouabain, were identified by mass spectrometry (123). Previous studies showed the pancytolsomal and mitochondrial/juxtanuclear localization of mortalin in mortal and immortal cells, respectively (126). Northern and Western blotting confirmed the induction of mortalin expression in ouabain-treated VSMCs and documented its mitochondrial localization. We established that, similarly to ouabain, transfection with mortalin delayed apoptosis in serum-deprived E1A-VSMCs. We also found that transfection with mortalin inhibited p53 translocation into the nucleus (123). These data demonstrate that [Na⁺⁺], elevation suppresses programmed cell death via augmented mortalin expression that, in turn, blocks p53 nuclear translocation triggered by apoptotic stimuli.

Sustained Na/K pump inhibition affects the expression of dozens of genes (122). These data, and augmented RNA synthesis seen in ouabain-treated VSMCs (100), prompted us to propose that transcriptomic changes are at least partially mediated by the augmented expression of immediate-response genes (IRG). Indeed, in VSMCs, 2- and 12-h treatment with ouabain resulted in 10- and 4-fold elevations of immunoreactive c-Fos and c-Jun, respectively (121). Fourfold increment of c-fos mRNA concentration was detected within 30 min of ouabain addition. At this time point, [Na⁺⁺], was increased by ~5-fold whereas [K⁺⁺], was decreased by only 10–15%. This observation indicates that [Na⁺⁺], elevation rather than [K⁺⁺], attenuation generates a signal that evokes c-fos expression.

These findings motivated us to search for mechanisms of regulation of gene expression by increased [Na⁺⁺],. A priori, this mechanism can be mediated by [Ca²⁺], elevation evoked by Ca²⁺ influx via inwardly directed electroneutral 1-[Ca²⁺⁺]/2[Na⁺⁺], or electrogenic 1-[Ca²⁺⁺]/3[Na⁺⁺], exchange. This hypothesis is also consistent with the presence of [Ca²⁺⁺] and cAMP response element within the c-fos promoter. Indeed, we have documented that [K⁺⁺]-induced depolarization evokes c-Fos accumulation that is completely abolished by the selective L-type Ca²⁺ channel blocker nicardipine (121). However, the data listed below strongly indicate that c-fos expression in ouabain-treated VSMCs is a Ca²⁺⁺-independent phenomenon. First, c-fos expression in ouabain-treated cells is not sensitive to nicardipine. Second, neither [Ca²⁺⁺], nor total exchangeable Ca concentration in VSMCs is affected by ouabain. This observation is consistent with low Na⁺⁺/Ca²⁺⁺ exchanger activity detected in cultured VSMCs (98) compared with other electrically excitable cells (16). Third, neither extracellular (EGTA) nor intracellular (BAPTA-AM) Ca²⁺⁺ chelators abolish ouabain-induced c-fos expression (121).

In more recent experiments, we identified ubiquitous and tissue-specific [Na⁺⁺]/[K⁺⁺]-sensitive transcriptomes by comparative analysis of differentially expressed genes in VSMCs from the rat aorta, the human adenocarcinoma cell line HeLa, and human umbilical vein endothelial cells (HUVECs). To increase the [Na⁺⁺]/[K⁺⁺] ratio, cells were treated for 3 h with the Na⁺⁺-K⁺⁺-ATPase inhibitor ouabain or placed for the same time period in K⁺⁺-free medium. Employing Affymetrix-based technology, we detected changes in the expression levels of 684, 737, and 1,839 transcripts in HeLa, HUVECs, and VSMCs, respectively, that were highly correlated between two treatments. Among these [Na⁺⁺⁺]-[K⁺⁺⁺]-sensitive genes, 80 transcripts were common for all 3 cell types (64). Surprisingly, the presence of extrarn- and intracellular Ca²⁺⁺ chelators increased rather than decreased the number of ubiquitous and cell-type-specific [Na⁺⁺⁺]-[K⁺⁺⁺]-sensitive genes. Among the ubiquitous [Na⁺⁺⁺]-[K⁺⁺⁺]-sensitive genes whose expression was regulated independently of Ca²⁺⁺ chelators by more than 3-fold, we found several transcription factors (Fos, Jun, Hes1, Nkiba) as well as genes involved in the regulation of cell cycle progression and death (cycin L1, dual-specificity phosphatase-1, −8 and −10, DNA-damage inducible transcript-3, Bcl6) (64). Overall, our results indicate that [Ca²⁺⁺]-independent signals, triggered by increased [Na⁺⁺⁺]/[K⁺⁺⁺] ratio, contribute to cell death machinery regulation (Fig. 4).

In contrast to massive c-fos expression seen in ouabain-treated cells, we failed to discern any effect of ouabain on luciferase accumulation driven by c-fos 5'-untranslated region (UTR) from −650 to +103, containing all known response elements activated by growth factors and [Ca²⁺⁺]-elevating compounds (50). These results reveal that c-fos expression triggered by [Na⁺⁺⁺]/[K⁺⁺⁺] ratio elevation is not mediated by 5'-UTR-containing transcriptional elements activated by [Ca²⁺⁺], and other canonical signaling pathways. Further investigation is

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needed to elucidate this novel mechanism of excitation-transcription coupling.

**[Na\(^+\)]/[K\(^+\)]\_Independent Death Signaling Triggered by CTS**

Data obtained during the past decade show that the action of CTS on cell survival and death is tissue and species dependent. Indeed, ouabain and other CTS, at concentrations that elicit full-scale inhibition of Na\(^+\)/K\(^+\)-ATPase and inversion of the [Na\(^+\)]/[K\(^+\)] ratio, did not affect the survival of rat VSMCs (100, 101), Jurkat cells (104), NIH 3T3 mice fibroblasts (93), rat astrocytes (2), and rat aorta endothelial cells (unpublished observations). In contrast, prolonged exposure to ouabain evokes massive death of MDCK cells (37), porcine and human endothelial cells (102, 111) as well as human astrocytes (Dr. A. Mongin, personal communication). The death of ouabain-treated MDCK cells is represented by combined markers of "classic" necrosis (cell swelling, negligible labeling with nucleotides in the presence of terminal transferase, nuclei staining with cell-impermeable dyes, such as propidium iodide), and apoptosis (nuclear condensation seen in cells stained with cell-permeable dyes, such as Hoechst 33342, chromatin cleavage, caspase-3 activation) (37, 105). We noted that the death of ouabain-treated MDCK cells was completely abolished by acidification of medium from pH 7.4 to 7.0, i.e., under conditions when pH was decreased from ~7.2 to 6.9 (3). This observation contrasted with the lack of any impact of acidification on the appearance of necrotic and apoptotic markers in atrial cardiomyocytes subjected to ischemia-reperfusion (8). Surprisingly, almost complete Na/K pump inhibition and full-scale increase of the [Na\(^+\)]/[K\(^+\)] ratio evoked by K\(^+\)-free medium did not affect the survival of MDCK cells (36, 105). Significantly, a similar left-hand shift was noted in comparison to the dose-dependent action of ouabain on Na/K pump activity and death of MDCK cells and PAECs incubated in K\(^+\)-free

![Stimuli leading to elevation of the [Na\(^+\)]/[K\(^+\)], ratio](image)

**Fig. 4.** Working hypothesis of the mechanisms of apoptosis suppression by increase of [Na\(^+\)]/[K\(^+\)], ratio. Activation of Na\(^+\)-permeable channels (1), Na\(^+\)/H\(^+\) exchanger (2), voltage-gated K\(^+\) channels (5), and Na\(^+\)-K\(^+\)-ATPase inhibition (4) leads to [Na\(^+\)]/[K\(^+\)], ratio elevation. In cells with abundant Na\(^+\)/Ca\(^{2+}\) exchanger (3), dissipation of transmembrane gradients of monovalent cations is accompanied by [Ca\(^{2+}\)], increment. [Na\(^+\)]/[K\(^+\)], ratio elevation affects the expression of immediate response genes (IRG) via unknown Na/K sensor(s) and Na/K response elements (Na/KRE). Expression of the subset of [Na\(^+\)]-[K\(^+\)]-sensitive genes is also subjected to regulation by increased [Ca\(^{2+}\)], via calmodulin (CaM) and other [Ca\(^{2+}\)], sensors as well as diverse Ca\(^{2+}\)-response elements (CaRE). Accumulation of IRG, in turn, heightens mortalin expression, which suppresses apoptosis via inhibition of p53 translocation to the nucleus. For more details, see text.

![Fig. 5.](image)

**Fig. 5.** Working hypothesis of the mechanism of cardiotonic steroid (CTS) cytotoxic action in cells expressing \(\alpha_1S\) Na\(^+\)-K\(^+\)-ATPase. AP and API, unknown adaptor proteins whose interaction with the \(\alpha_1S\) subunit is respectively abolished and triggered by CTS. The different sizes of [Na\(^+\)] and [K\(^+\)] symbols reflect modulation of their intracellular concentrations by CTS. For more details, see text.
independently of inhibition of Na\(^{+}/\)H\(^{+}\)-ATPase-\(\alpha\)-subunit rather than with other potential \([K^+]_o\)-insensitive receptors. However, in contrast to suppression of apoptosis in VSMCs (101), this death signaling pathway is not mediated by the inhibition of Na\(^{+}/\)K\(^{-}\)-ATPase-evoked ion fluxes and \([Na^+]_i/[K^+]_i\) ratio elevation.

Mechanisms underlying the tissue- and species-specific impact of CTS on cell survival and death remain poorly understood. Numerous studies have demonstrated that in rodents, ouabain inhibits ubiquitous \(\alpha_1\)-Na\(^{+}/\)K\(^{-}\)-ATPase at concentrations up to 5 orders of magnitude higher than in other mammals. Lingrel and coauthors (78) were the first to report that ATPase, with Arg and Asp, respectively. To examine the role of cell volume regulation. Significantly, these signaling pathways is not sufficient to evoke plasma membrane rupture. Recent cells, the 50 –70% volume increase triggered by necrotic stimuli is not sufficient to evoke plasma membrane rupture. Recent findings are consistent with thermodynamic over, apoptotic collapse may occur in the absence of cell volume regulation. Significantly, these signaling pathways is not sufficient to evoke plasma membrane rupture. Recent studies have demonstrated that ion transporters are involved in cell death machinery triggering and progression independently of cell volume regulation. Significantly, these signaling pathways are also cell type specific. Thus, ouabain and other CTS rescue rat VSMCs from apoptosis via \([Na^+]_i/[K^+]_i\)-mediated, \([Ca^{2+}]_i\)-independent signaling pathways but kill epithelial cells from the canine kidney independently of \([Na^+]_i/[K^+]_i\) ratio elevation. Why does long-term exposure to CTS not affect survival and even inhibit apoptosis in rodent cells but kill cells from other mammals? What is the mechanism of \([Na^+]_i/[K^+]_i\)-independent cell death signaling triggered by CTS? What is the molecular origin of \([Na^+]_i/[K^+]_i\), sensors involved in \([Ca^{2+}]_i\)-independent transcription of antiapoptotic genes? Further studies are needed to address these questions.

**Acknowledgments**

Manuscript editing by Ovid Da Silva and logistical services provided by the Research Support Office, CRCHUM, are appreciated.

**Grants**

This work was supported by grants from the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada, the Kidney Foundation of Canada, the Ministry of Education and Science of the Russian Federation, and the Russian Foundation for Fundamental Research.

**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**Author Contributions**

S.N.O., P.H., and R.G. interpretation of the results of the experiments; S.N.O. and P.H., and R.G. edited and revised the manuscript; S.N.O., P.H., and R.G. performed the experiments; A.A.P. analyzed the data.

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