J. Membrane Biol. 209, 43–58 (2006)
DOI: 10.1007/s00232-005-0837-5

The Journal of
Membrane
Biology

Potential Roles of Electrogenic Ion Transport and Plasma Membrane Depolarization in Apoptosis

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Received: 28 December 2005/Online publication: 17 April 2006

Abstract. Apoptosis is characterized by the programmed activation of specific biochemical pathways leading to the organized demise of cells. To date, aspects of the intracellular signaling machinery involved in this phenomenon have been extensively dissected and characterized. However, recent studies have elucidated a novel role for changes in the intracellular milieu of the cells as important modulators of the cell death program. Specially, intracellular ionic homeostasis has been reported to be a determinant in both the activation and progression of the apoptotic cascade. Several apoptotic insults trigger specific changes in ionic gradients across the plasma membrane leading to depolarization of the plasma membrane potential (PMP). These changes lead to ionic imbalance early during apoptosis. Several studies have also suggested the activation and/or modulation of specific ionic transport mechanisms including ion channels, transporters and ATPases, as mediators of altered intracellular ionic homeostasis leading to PMP depolarization during apoptosis. However, the role of PMP depolarization and of the changes in ionic homeostasis during the progression of apoptosis are still unclear. This review summarizes the current knowledge regarding the causes and consequences of PMP depolarization during apoptosis. We also review the potential electrogenic ion transport mechanisms associated with this event, including the net influx/efflux of cations and anions. An understanding of these mechamisms could lead to the generation of new therapeutic approaches for a variety of diseases involving apoptosis.

Key words: Apoptotic volume decrease — ATPases — Cell death — Cell signaling — Cell volume — Electrogenic transport — Ion channels — Ion flux — Ion transporters — Ionic gradients — Ionic homeostasis — Plasma membrane potential

Introduction

Programmed cell death or apoptosis, is a genetically encoded pathway that enables cells to undergo a highly regulated and organized death in response to specific signals. Apoptosis is involved in several physiological and pathophysiological states of the organism. For example, apoptosis is critical for the maintenance of normal tissue homeostasis (cell number) and is involved in the removal of cells during tissue development and remodeling, as well as during cell senescence and organism aging. Moreover, apoptosis occurs as a consequence of distinct pathologies, and its deregulation can lead to autoimmune, cancer and neurodegenerative diseases. This process is a ubiquitous, evolutionary highly conserved, cell death program that requires the specific activation of several signaling cascades, which ultimately lead to distinct biochemical and morphological alterations in the cell. These changes occur in different and sequentially organized stages. Early stages of apoptosis are characterized by the activation of caspases and endonucleases, phosphatidylserine externalization, cell shrinkage and nuclei condensation. Advanced stages of the cell death program are typified by plasma membrane blebbing or apoptotic body formation, DNA degradation, and finally cell fragmentation (Bortner & Cidlowski, 2002; Green, 2003).

The signaling machinery involved in apoptosis has been extensively studied and characterized. Recent studies have demonstrated a role for changes in

Abbreviations: AVD, apoptotic volume decrease; PMP, plasma membrane potential; RVD, regulatory volume decrease; VGNC, voltage-gated Na^+ channel; VGCC, voltage-gated Ca^{2+} channels.

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the intracellular milieu of cells as important modulators and/or regulators of the cell death program. Changes in the intracellular ionic homeostasis of apoptotic cells have now been reported to occur in a variety of experimental paradigms. Moreover, it has been demonstrated that changes in the intracellular ionic homeostasis are important regulators for the progression of apoptosis (Yu, Canzoniero & Choi, 2001; Rizzuto et al., 2003; Bortner & Cidlowski, 2004). Recently, plasma membrane potential (PMP) depolarization has been reported to occur during early stages of apoptosis, but its consequences on the progression of the cell death program are less understood.

Ionic Homeostasis and Generation of Plasma Membrane Potential (PMP) in Cells

Ionic homeostasis is probably the most ancient of all the homeostatic mechanisms implicated in the maintenance, not only of cell physiology, but also of normal organ and body functions. In biological systems ions are not uniformly distributed, thus, concentrations in one compartment differ from those in other compartments. Major ionic gradients across the plasma membrane include sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-). In both prokaryotic and eukaryotic cells, lipid membranes function as permeability barriers selective to ions, providing a mechanism for the steady-state maintenance of highly asymmetric concentrations of the major cations and anions across both plasma membranes and intracellular organelles. Thus, if there were no maintenance mechanisms, the concentrations of these ions inside and outside the cells would equilibrate, with deleterious effects on the overall cell homeostasis.

The maintenance of ionic concentration gradients involves both active and passive transport processes across the plasma membrane (Figure 1). Active transport of ions is mediated by proteins in the plasma membrane capable of pumping ions from one side of the membrane to the other against their concentration gradient. These mechanisms require energy from the hydrolysis of ATP (for the case of ATPases), or can use the driving force of another ion (coupled transporters). The Na^+-K^+ pump (or ATPase) is the primary ion transport mechanism involved in the maintenance of both Na^+ and K^+ concentration gradients across the plasma membrane. This pump is electrogenic and extrudes three Na^+ for every two K^+ pumped into the cell. Ion concentration gradients can also be maintained by their passive flux across the plasma membrane determined by the selective and distinct permeability of the membrane to various ions. The distinct permeability of the plasma membrane to different ions gives an electrical polarity across the lipid bilayer or membrane potential at rest that is present in all cell types. Most cell types have high resting permeability to K^+, which together with the outwardly directed K^+ gradient makes the interior of the cell electrically negative to the external solution. Thus in most cases PMP values are close to those of the actual Nernstian K^+ potential. These permeability pathways are mediated by ion channels, and the movement of ions through these membrane pores does not require energy consumption but depends on the electrochemical gradient of each ion species (Wright, 2004).

The movement of ions across the plasma membrane results in changes in electrical potential across the membrane. Such voltage changes have been reported to be primary signals that convey biological messages within the cell. Changes in the PMP occur during the normal physiology of the cells. Ion concentration gradients, ion transport activity and PMP reflect a triad whose regulation is critical for most homeostatic cellular functions. For example, the electrochemical gradient energy across the plasma membrane influences the transport of a vast array of nutrients in the cells and is the driving force in the movement of salt and water across cell membranes and between organ-based compartments. It is also essential in the signaling processes associated with coordinated movements of cells and organisms and is the basis of cognitive processes. In contrast, deregulated ionic imbalances are associated with pathological consequences, since ionic disturbances occur during apoptosis, ischemia and several channelopathies (Ronquist & Waldenstrom, 2003).

Changes in Cell Ionic Homeostasis and PMP During Apoptosis

Changes in the PMP of the cells reflect movement of ions across the plasma membrane. Thus, the PMP is a consequence of the alteration in the distribution of ions. Alterations in the transmembrane gradients of several ions have been reported to influence the cell death program. Plasma membrane depolarization has been reported to occur in response to different apoptotic stimuli including receptor-induced, stress-induced and drug-induced apoptosis (Dallaporta et al., 1999; Detre et al., 2005; Bortner, Gomez-Angelats & Cidlowski, 2001; Mann et al., 2001; Mann & Cidlowski, 2001; Borzi et al., 2002; Dussmann et al., 2003b; Nolte et al., 2004; Scoltock & Cidlowski, 2004; Esteves et al., 2005). For example, increasing concentrations of Fas ligand result in concurrent increases in the number of cells that have a depolarized PMP (Fig. 2). Interestingly, a PMP hyperpolarization phenotype has been reported as one of the mechanisms by which Bcl-2 promotes apoptosis resistance (Gilbert et al., 1996; Williams et al., 2000);

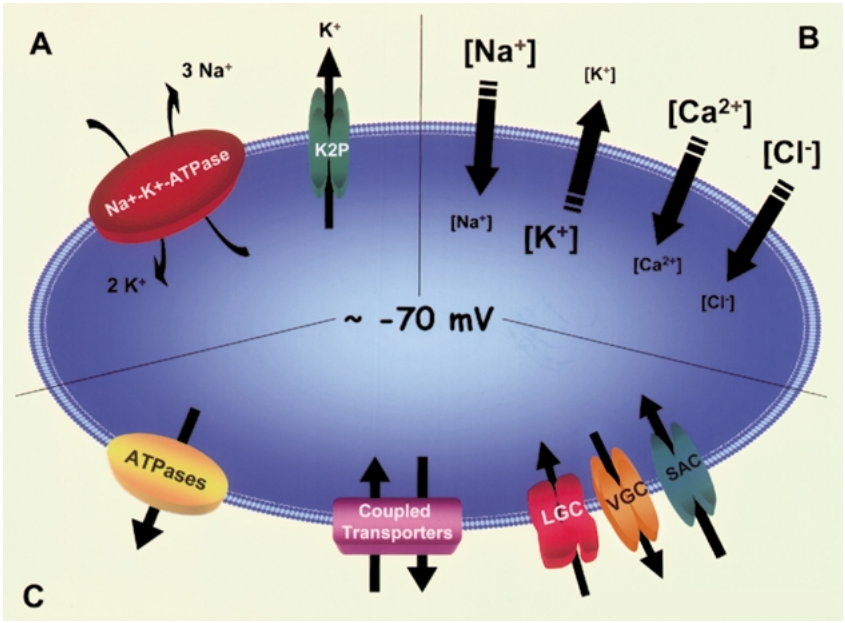

Fig. 1. Ionic homeostasis and generation of plasma membrane potential (PMP) in the cells. The electrical gradient in the cells or PMP at rest arises mainly from two physiological parameters: 1) the presence of large gradients for K^+ and Na^+ across the plasma membrane; and 2) the relative permeability of the plasma membrane to those ions. The maintenance of the gradient distribution of ions in the cells, and thus of the PMP, involves both active and passive transport mechanisms across the plasma membrane. From this, the Na^+–K^+ pump (or ATPase), which is the main ion transport mechanism involved in the maintenance of both Na^+ and K^+ concentration gradients, and the high permeability to K^+ driven by the presence of background or leak conductances, mediated by the recently described K2P K^+-channels (Kim, 2005), are the major determinants of PMP at rest (***A***). In a typical mammalian cell, major ionic gradients across the plasma membrane (***B***) are those of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-). Most biological membranes are, to varying degrees, permeable to K^+ and Cl^- ions. On the other hand, there is a reduced Na^+ and Ca^{2+} permeability of most plasma membranes. The distinct permeability of the plasma membrane to different ions gives an electric polarity across the lipid bilayer. (***C***) Other transport mechanisms including ATPases (like the Ca^{2+}-pump PMCA), transporters coupled to the driving force of another ion (like the Na^+/Ca^{2+} exchanger NCX), the HCO_3–Cl^- exchanger, and the Na^+–K^+–Cl^- and K^+–Cl^- cotransporters) and ion channels (voltage-gated, ligan-gated or stress-activated ion channels) can also contribute to PMP at rest (by their tonic activity) or modulate it during different processes by their activation upon different stimuli. Due to the outwardly directed K^+ and inwardly directed Na^+ gradients maintained by the activity of the Na^+–K^+ ATPase, and high resting permeability to K^+, the interior of the cell is electrically negative in relation to the external solution, thus in most cases PMP values are close to those of the actual Nernstian K^+ potential (around ~ -70 mV).

however, the role of PMP depolarization during apoptosis remains elusive. It is still unclear whether it is just an epiphenomenon associated with the ionic imbalance that occurs during apoptosis, or if there is a specific signaling role for the ionic changes associated with PMP depolarization during apoptosis. According to the electrochemical gradients across the plasma membrane of cells (Fig. 1), PMP depolarization can only arise from the net electrogenic inflow of Na^+ and Ca^{2+} cations and / or the outflow of intracellular anions like Cl^- or other intracellular organic anions (OA^-). Plasma membrane potential depolarization during apoptosis has been associated with intracellular cation overload (Bortner et al., 2001; Mann et al., 2001; Nowak, 2002; Dussmann et al., 2003b; Nolte et al., 2004; Waring, 2005). This overload reflects an early rise in Na^+ and Ca^{2+} that may account for the observed cellular depolarization (Fig. 3). Additionally, anion efflux has also been reported to mediate PMP depolarization during apoptosis (Nolte et al., 2004). Several reports have suggested the participation of a wide variety of ion transport proteins on the net intracellular cation overload and/or anion efflux associated with PMP depolarization during apoptosis, and are reviewed in the following section.

Ion Transport Pathways that Mediate Cation Overload During Apoptosis

Plasma Membrane ATPases

The P-type ATPases comprise a nearly ubiquitous ion pump family with catalytic activities involved in diverse cellular homeostatic processes including the maintenance of osmotic balance and intracellular ionic composition (Apell, 2003). Two members of this group have been reported to be involved in the changes in intracellular cation overload that might be associated with PMP depolarization during apoptosis.

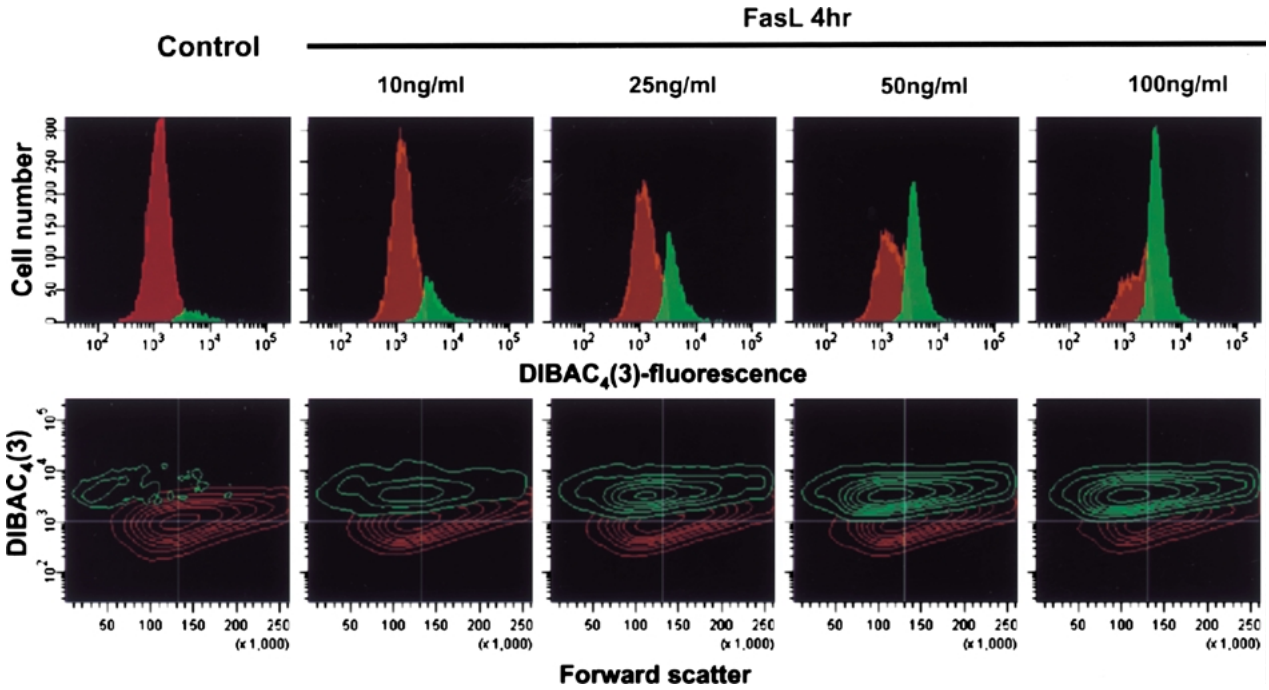

Fig. 2. Plasma membrane potential (PMP) depolarization is an early hallmark during apoptosis. Changes in the PMP were measured by flow cytometry using $DiBAC_4(3)$. $DiBAC_4(3)$ is a negatively charged oxonal dye that freely crosses the plasma membrane. As the cell depolarizes more of this dye can enter the cell resulting in an increase in fluorescence. Fas ligand (FasL)-induced PMP depolarization was analyzed by fluorescence-activated cell sorting analysis, FACS, using a BD LSR II flow cytometer and BD FacsDiva Software (Becton Dickinson, San Diego, CA) for data analysis. Thirty minutes prior to each time of examination, Jurkat cells were preloaded with $DiBAC_4(3)$ at a final concentration of 150 nM, and the incubation was continued at 37°C, 7 % CO_2 atmosphere. Immediately prior to flow cytometry examination, propidium iodide (PI) was added, and cells with increased PI fluorescence (i.e., loss of plasma membrane integrity) were discarded. Cells were analyzed at a cell density of 5×10^5 cells/ml, and in all cases, ten thousand cells were analyzed. $DiBAC_4(3)$ was excited using an Argon 488 laser and the fluorescence was detected with a 530/30 detector. For PI, cells were excited with an Argon 488 nm laser and emission was acquired with a 695/40 detector. A depolarized PMP is reflected by the appearance of a different population of cells with increased $DiBAC_4(3)$ (*green*) compared to control cells' fluorescence (*red*). Frequency histograms of $DiBAC_4(3)$ fluorescence (*upper panels*) show that FasL induced the appearance of a population of cells with a depolarized PMP respective to control cells, in a concentration-dependent manner. We also analyzed the changes in $DiBAC_4(3)$ fluorescence in contour plots (*lower panels*) against changes in cell size. Cell size was determined as changes in the forward scatter pattern by exiting the cells with an Argon 488 nm laser. The forward-angle light scatter relates to cell diameter, i.e., cell shrinkage is reflected as a decrease in the amount of forward scatter light. We observed that the cells with a depolarized PMP (*green*) had a slight decrease in cell size reflected as a decrease in their forward scatter properties. The center of the quadrants is used as a reference for the mean $DiBAC_4(3)$ fluorescence and forward scatter signal of control cells.

Plasma membrane Ca^{2+}–ATPase (PMCA)

Plasma membrane Ca^{2+}-ATPases are a subfamily of P-type ATPases, that extrude ionic Ca^{2+} across the plasma membrane against its electrochemical gradient (Lehotsky et al., 2002). Recent reports have shown a downregulation of the PMCA expression and activity during different pathological situations leading to apoptosis (Garcia et al., 2001). Furthermore, antisense-knockdown of the PMCA induces apoptosis in muscle cells (Sasamura et al., 2002). Overexpression of PMCA or its stimulation by growth factors has also been reported to protect cells from apoptosis (Garcia et al., 2001; Peluso, 2003). Interestingly, the PMCA is cleaved by caspase 3 during apoptosis, which impairs intracellular Ca^{2+} homeostasis and results in a further Ca^{2+} overload (Paszty et al., 2002; Schwab et al, 2002; Chami et al., 2003).

Sodium-Potassium Pump (Na^+–K^+ ATPase)

Alterations in the ionic gradient distribution of Na^+ and K^+ are early hallmarks of initial stages of the apoptotic program. Several reports have associated these phenomena with a reduction in the Na^+–K^+ ATPase activity during apoptosis (Tang, Cheng & Lin, 1996; Nobel et al., 2000; Bortner et al., 2001; Mann et al., 2001; Nowak, 2002; Orlov et al., 2003; Wang et al., 2003a, 2003b; Yu, 2003a; Arrebola et al., 2005). Accordingly, inhibition of the Na^+/K^+ ATPase with cardiac glycosides has been widely reported to induce PMP depolarization paralleled by cell toxicity and cell death (Chatterjee & Roy, 1965; Mason

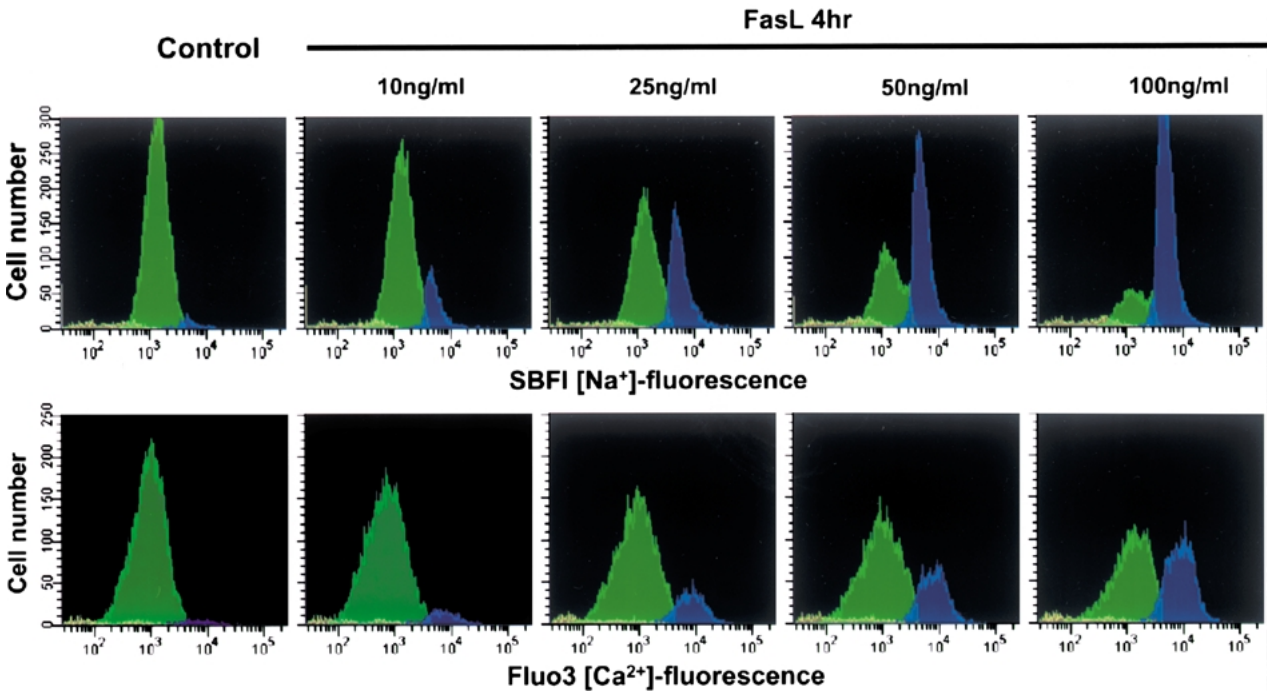

Fig. 3. Fas ligand (FasL)-induced apoptosis increases the intracellular concentration of sodium (Na^+) and calcium (Ca^{2+}) of cells. For the intracellular Na^+ and Ca^{2+} measurements, cells were preloaded at 37°C, 7 % CO_2 atmosphere with 5 μM (1 h) of SBFI-AM (Na^+ fluorophore), or 2 μM (30 min) Fluo3-AM (Ca^{2+} fluorophore) prior to the time of examination. Fas ligand-induced changes in the intracellular concentration of these cations were analyzed by FACS. Immediately prior to flow cytometry examination, PI was added. For SBFI, cells were excited with a UV 350/360 laser and emission was acquired with a 440/40 detector. For Fluo3, cells were excited with an Argon 488 nm laser and emission was acquired with a 530/30 detector. Changes in the concentration of these cations are reflected by the appearance of different populations of cells with differences in SBFI or Fluo3 fluorescence, reflecting changes in the intracellular concentration of this ion. Frequency histograms of the fluorescence of these dyes show that an initial stage of FasL-induced apoptosis is characterized by an increase in the concentration of both Na^+ and Ca^{2+} in the cells. This is reflected by the appearance of a population of cells with an increase in the concentration of these cations (*blue population*) compared to control cells (*green population*). A second population of cells (*yellow*) with a dramatic loss of intracellular cations, consists of cells in a late stage of the apoptotic program.

et al., 1971; Watabe et al., 1996; Olej et al., 1998; Kawazoe et al., 1999; Stelmashook et al., 1999; McConkey et al., 2000; Omar, Senatorov & Hu, 2000; Chueh et al., 2001; Kurosawa et al., 2001; Hennion et al, 2002; Schmiedt et al., 2002; Xiao et al., 2002a, 2002b; Huang et al., 2004; Esteves et al., 2005; Lang, Schulte & Schmiedt, 2005), or to enhance apoptosis induced by different stimuli in various model systems (Thevenod & Friedmann, 1999; Nobel et al., 2000; Penning et al., 2000; Verheye-Dua & Bohm, 2000; Bortner et al., 2001; Xiao et al., 2002a; Orlov et al., 2003; Esteves et al., 2005). Alterations in the expression and activity of the Na^+-K^+ ATPase have also been widely reported to occur during the pathogenesis of cardiovascular, neurological, renal and metabolic diseases, as well as during heavy metal-induced toxicity (Patrick & Hilton, 1979; Lees, 1991; Rose & Valdes, 1994; Chauhan, Lee & Siegel, 1997; Mishra & Delivoria-Papadopoulos, 1999; Thevenod & Friedmann, 1999; Ziegelhoffer et al., 2000; Kumar & Kurup, 2002; Rodrigo et al., 2002; Yu, 2003a; Cimen et al., 2004).

Recent reports have shown that degradation of the Na^+-K^+ ATPase occurs during apoptosis induced by death receptor activation (Bortner et al., 2001), glucocorticoids (Mann et al., 2001), and staurosporine (Dussmann et al., 2003a). Interestingly, apoptotic-resistant phenotypes of bcl-2–overexpressing cells (Gilbert et al., 1996; Gilbert & Knox, 1997), and of progesterone receptor-(Gonzalez Deniselle et al., 2002) and angiotensin receptor-mediated neuroprotection (Grammatopoulos et al., 2004), have been associated with an elevated Na^+-K^+ ATPase activity. The mechanisms and signals by which apoptosis modulates ATPase activity are still unclear. Since apoptosis is generally recognized to require intracellular ATP for its progression, increases in the intracellular ATP concentration occur under these circumstances (Eguchi et al., 1999; Nicotera & Melino, 2004; Zamaraeva et al., 2005). Thus, energy failure can likely be discarded as one of the mechanisms. Recent reports suggest the role of caspases (Mann et al., 2001; Dussmann et al., 2003a), protein kinases (Nowak, 2002; Wang & Yu, 2005), and

reactive oxygen/nitrogen species (Chakraborti et al., 1998; Cimen et al., 2004; Rodrigo et al., 2002; Sen et al., 2004; Thevenod & Friedmann, 1999; Wang et al., 2003a) in the modulation of Na^+-K^+ ATPase expression and activity during apoptosis. But functional studies are needed to characterize the precise signaling pathways.

Ion Channels

Ion channels are integral membrane proteins that provide pores for the passive diffusion of ions across biological membranes that result in trans-membrane currents, thus being the main source of electrogenic ion flux pathways. They are normally classified depending on the ion species involved or according to their regulation of the gating or activation process. Ion channels participate in membrane potential maintenance, transduction of chemical signals to electric stimuli, and generation, coordination and propagation of electrical currents in excitable tissues. Other physiological functions ascribed to ion channels include the regulation of cell volume and pH, regulation of transepithelial transport of salt and water, hormone secretion and cell proliferation (Wehner et al., 2003; Wang, 2004). Recent studies have demonstrated the participation of several ion channels in apoptotic cell death (Lang et al., 2003a; Storey et al., 2003; Yu, 2003b; Okada et al., 2004; Wang, 2004; Remillard & Yuan, 2004). Additionally, deregulation of ion channel expression and function leads to diverse pathological conditions or channelopathies (Jentsch, Hubner & Fuhrmann, 2004; Waxman, 2001).

Voltage-gated Ion Channels

Voltage-gated Na^+ channels (VGNC) have been widely studied in nerve and muscle cells where they mediate regenerative cell membrane depolarization and conduction of electrical signaling, and its deregulation has been associated with the appearance of hyperexcitability-derived diseases. They are also expressed in non-excitable cells including fibroblasts, osteoblasts, lymphocytes, glia, and metastatic cancer cells of epithelial origin, although their functions are less understood (Diss, Fraser & Djamgoz, 2004). The role of VGNC in apoptosis was suggested first by the observation that the VGNC activator, veratridine, induced apoptosis in neurons independently of the activation of voltage-gated Ca^{2+} channels (VGCC) (Dargent et al., 1996; Ulbricht, 1998; Koike & Ninomiya, 2000; Koike et al., 2000). These data implied a primary role of Na^+ overload in the induction of apoptosis by PMP depolarization, although the mechanisms by which veratridine-induced Na^+ overload induces apoptosis are still unclear. Other studies have also suggested a synergistic action of Ca^{2+} influx, reactive oxygen species generation, and p53 activation on veratridine-induced apoptosis that might further regulate the activation of the mitochondrial pathway of apoptosis and execution caspases (Callaway et al., 2001; Jordan et al., 2003; Banasiak, Burenkova & Haddad, 2004; Gomez-Lazaro et al., 2005). Several VGNC blockers have been reported to reduce brain damage and cell death during ischemia, hypoxia and traumatic brain injury (Taylor & Meldrum, 1995; Carter, 1998; Small, Morley & Buchan, 1999; Goldin, 2001; Banasiak et al., 2004) as well as to protect against apoptosis induced by ouabain (Xiao et al., 2002b) and death-receptor activation (Bortner & Cidlowski, 2003).

The voltage-gated family of Ca^{2+} channels (VGCC) is comprised of a large group of structurally related heterooligomers that couple cell excitability to intracellular signaling by permitting Ca^{2+} ions to enter thus producing transient intracellular Ca^{2+} signals (Miller, 2001). Thus, VGCC have also been implicated in initiating intracellular Ca^{2+}-dependent events, such as contraction, secretion, synaptic transmission and gene expression. Voltage-gated Ca^{2+}-channels have been shown to be activated during neurodegenerative disease- or aging- induced apoptosis (Mason et al., 1999; MacManus et al., 2000; Ho, Ortiz & Shea, 2001; Yagami et al., 2002, 2004; Otori et al., 2003; Ma et al., 2005), as well as during excitotoxicity- and ischemia-induced cell death (Kobayashi & Mori, 1998; Read, McCall & Gregg, 2002). Voltage-gated Ca^{2+} channel antagonists have been widely reported to prevent Ca^{2+} overload and apoptosis. Additionally, other types of apoptotic stimuli including cytokine withdrawal (Wang et al., 1999), lipid oxidation (Ares et al., 1997), cytotoxic agents (Kim et al., 2000a; Barone et al., 2004; Tanaka et al., 2004; Barone, Aguanno & D'Agostino, 2005) and phospholipase activation (Yagami et al., 2003b, 2003c, 2005) have also been shown to activate several types of VGCC whose inhibition protects from the progression of cell death. Apoptosis has also been correlated with the overexpression of the L-type VGCC alpha(1) subunits (Ba, Pang & Benishin, 2004; Grassi et al., 2004). The activation of VGCC during apoptosis has been associated with either changes in PMP, protein kinase activation or the generation of reactive oxygen species (MacManus et al., 2000; Cano-Abad et al., 2001; Yagami et al., 2003a; Ba et al., 2004; Grassi et al., 2004; Waring, 2005).

Non-selective Cation Channels

Non-selective cation channels are a diverse group of ion channels characterized by their relatively low selectivity between cation species. Their activity is

modulated by various extracellular and intracellular signals. These nonselective cation channels are gated by diverse mechanisms, which can include voltage, cyclic nucleotides, ligands, reactive oxygen species and stretch. They contribute to depolarization of the membrane and in most cases to an increase in the intracellular Ca^{2+} concentration. The activation of NSCC in apoptosis has been widely reported in different cell types under various apoptotic stimuli (Gutierrez et al., 1999; Kim et al., 1999, 2006; Manion et al., 2000; Tapia-Vieyra & Mas-Oliva, 2001; Estacion & Schilling, 2002; Lang et al., 2003a,b,c; Jeulin, Dazy & Marano, 2002; Sook Han et al., 2003; Mukherjee et al., 2002; Sudhandiran & Shaha, 2003; Lang et al., 2004a,b; Lee, 2004; Mahta & Shaha, 2004). However, the molecular identities of the channels involved seem to vary in each case and usually according to the cell type studied.

The transient receptor potential (TRP) channels regulate the plasma membrane permeability of cells to a variety of ions in response to a wide diversity of stimuli. With the exception of the Ca^{2+}-selective melastatin TRP channels (TRPM) and two members of the vanilloid receptor family of calcium-permeable channels (TRPV5 and TRPV6), which show a high selectivity for Ca^{2+}, TRP channels are non-selective cation influx channels. TRP channels are activated by a variety of stimuli including intra- and extracellular ligands or signaling molecules, Ca^{2+}-store depletion and mechanical or thermal stress. Thus, their activity has recently been shown to be involved in different physiological processes such as capacitative or store-operated Ca^{2+} entry (SOCE), sensory- and mechanotransduction (Voets & Nilius, 2003; Putney, 2005). Recent reports have also underlined the role of TRP channel activation in the induction of apoptosis. Activation of TRP channels in *Drosophila* leads to massive photoreceptor cell death (Yoon et al., 2000; Hong et al., 2002). Additionally, redox stress-induced and nicotinamide adenine dinucleotide-induced apoptosis have been suggested to depend on TRPM2 mediated Ca^{2+} influx (Sano et al., 2001; Hara et al., 2002; Zhang et al., 2003). Moreover, overexpression of TRPM7 in HEK293 has been reported to induce cell death (Yoon et al., 2000; Hong et al., 2002), and its activation by free radicals mediates neuronal death induced by oxygen-glucose deprivation (Aarts et al., 2003; Aarts & Tymianski, 2005). Apoptosis can also be induced by TRPV1 channel activation by particulate matter or capsaicin (Agopyan et al., 2003, 2004; Bodo et al., 2005).

Store-operated Ca^{2+} entry (SOCE) is a widespread phenomenon that represents the major mechanism of regulation of Ca^{2+} influx in non-excitable cells. Store-operated Ca^{2+} entry has been widely reported to induce apoptosis and has also been suggested to mediate glucocorticoid-induced cell death (Nam et al., 2003; Parekh & Putney, 2005), It has also been suggested that antiapoptotic effects of Bcl-2 are associated with the downregulation of SOCE channels, suggesting a pivotal role of SOCE on the progression of apoptosis (Vanden Abeele et al., 2002). A large number of studies have suggested the possibility that TRPC channels function as a source for SOCE (Parekh & Putney, 2005); however, this proposal has not been clearly established. SOCE associated with TRPC2 channel activation induces apoptosis triggered by the growth arrest and DNA damage-inducible gene (GADD153) overexpression, and acute K^+ loss (Pigozzi et al., 2004). A recent study has shown that several genes involved in apoptosis are upregulated in cells with high levels of SOCE currents (Zagranichnaya et al., 2005).

Ion Transport Pathways that Mediate Anion Efflux During Apoptosis

Anion channels in the plasma membrane are permeable to anions such as iodide, bromide, nitrates, phosphates, and negatively charged amino acids. However, they are usually referred to as chloride (Cl^-) channels since it is the most abundant anion and the predominant specie in all organisms. Chloride channel activity contributes to cell membrane potential, and maintains intracellular pH and cell volume. Chloride channels also play important roles in different physiological processes including epithelial transport and blood pressure regulation, muscle tone, cellular excitability, cell cycle and proliferation, and apoptosis (Okada et al., 2004). There are multiple families of chloride channels described by their electrophysiological and pharmacological properties (Nilius & Droogmans, 2003; Jentsch et al., 2005). Diverse apoptotic stimuli have been reported to activate Cl^--channels (Meng, Carruth & Weinman, 1997; Okada & Maeno, 2001; Porcelli et al., 2003; Dupere-Minier et al., 2004). In some cases, it has been suggested that Cl^- channel modulation during apoptosis occurs by second messengers including intracellular Ca^{2+}, ROS generation and kinases (Szabo et al., 1998; Nietsch et al., 2000; Kim, Kang & Lee, 2003; Shimizu, Numata & Okada, 2004). Moreover, Cl^--channel blockers have been widely reported to inhibit, with a distinct degree of potency and specificity, the progression of apoptosis (Fujita, Yanagisawa & Ishikawa, 1997; Szabo et al., 1998; Rasola et al., 1999; Maeno et al., 2000; Mizoguchi et al., 2002; Small, Tauskela & Xia, 2002; d'Anglemont de Tassigny et al., 2004; Myssina et al., 2004; Porcelli et al., 2004; Wei et al., 2004; Takahashi et al., 2005; Tanabe et al., 2005). Furthermore, media with reduced extracellular Cl^- have also been shown to prevent the progression of apoptosis in a few model systems (Lang et al., 2004c; Tsukimoto et al., 2005). Electrophysiological studies have suggested that Cl^--channels activated

during apoptosis have properties similar to the volume-regulated anion current (VRAC) that participates in volume recovery after cell swelling (Maeno et al., 2000; Souktani et al., 2000; d'Anglemont de Tassigny et al., 2004; Shimizu et al., 2004). However, the molecular identity of the Cl^--channels involved in the progression of apoptosis is still unclear. Recent reports have postulated the role of either the plasma membrane voltage-dependent anion channels (VDAC) (Elinder et al., 2005), the Ca^{2+}-activated Cl^--channels (Schumann, Gardner & Raffin, 1993; Kim et al., 2003) or the voltage-gated Cl^--channels (Wei et al., 2004) as possible mechanisms of Cl^- extrusion during apoptosis. Other reports have also suggested that Cl^- efflux occurs during apoptosis associated with K^+ loss and cell shrinkage or apoptotic volume decrease (AVD). According to the Cl^- concentration distribution across the plasma membrane (Fig. 1), the opening of a passive Cl^- flux pathway, like an anion channel, will drive an influx of Cl^- down its electrochemical gradient. However, during AVD and cell volume regulation after cell swelling (RVD), the intracellular concentration of Cl^- decreases (Arrebola et al., 2005; Zhou et al., 2005). Chloride loss in these cases has been postulated to be mediated by its extrusion coupled to the simultaneous loss of ionic K^+ due to the pronounced voltage-mediated coupling between both K^+ and Cl^- conductance pathways, which should not contribute to a change in PMP (Wehner et al., 2003; Okada et al., 2004). Alternatively, it has been reported that during RVD an initial activation of K^+ channels leads to a transient hyperpolarization, which then acts as the driving force for a sustained Cl^- efflux and PMP depolarization (Jakab et al., 2002). However, this phenomenon has not been reported for AVD.

Role of Ion Movements Associated with PMP Depolarization in the Progression of Apoptosis

As discussed earlier, the ionic mediators of plasma membrane depolarization must involve either a net cation influx or a net anion efflux across the plasma membrane. In general, the effects of PMP depolarization on biological systems are associated with the changes in the intracellular concentration of the ionic species that mediate it. This is also the case for apoptosis, where the effects of PMP depolarization might be ion-species specific. This observation is supported by the fact that PMP depolarization under high extracellular K^+ conditions is protective against apoptotic cell death (Chacon-Cruz et al., 1998; Lauritzen et al., 2003; Zhong et al., 2004; Johnson & D'Mello, 2005), although this effect has also been demonstrated to be related to the inhibition of K^+ loss during apoptosis (Bortner, Hughes & Cidlowski, 1997; Yu et al., 1997; Thompson et al., 2001).

In contrast, PMP depolarization by either Na^+ (Dargent et al., 1996; Jordan et al., 2000,2002, 2003; Koike et al., 2000; Gomez-Lazaro et al., 2005; Banasiak et al., 2004), or Ca^{2+} ionophores (Gwag et al., 1999; Gil-Parrado et al., 2002; Zhu et al., 2002; Lang et al., 2003d) has been consistently reported to induce apoptosis.

Na^+ Overload

In contrast to reports for Ca^{2+}, evidence for a direct modulation of Na^+ on enzyme activity or signaling protein function is scarce. However, there are data that suggest a direct role of Na^+ ions on the regulation of apoptosis. For example, early phosphatidylserine exposure during apoptosis has been reported to be dependent on Na^+, but not Ca^{2+} influx (Courageot et al., 2004). Sodium overload has also been demonstrated to modulate cytoskeleton organization (Chifflet et al., 2003, 2004) and activate the Rho-ROCK signaling pathways (Szaszi et al., 2005), which are necessary for apoptotic body formation (Croft et al., 2005).

An indirect effect of Na^+ overload causing further ionic gradient imbalances during apoptosis might also modulate the activation of the apoptotic machinery. For example, sodium overload has been suggested to drive H^+ entry via the Na^+/H^+ exchanger (Koike et al., 2000), which can further regulate the progression of apoptosis via acidification of the intracellular milieu. Additionally, intracellular Na^+ increase has been shown to precede the activation of K^+ efflux associated with cell shrinkage or apoptotic volume decrease (Bortner et al., 2001). A rise in the intracellular Na^+ concentration has also been reported to directly activate G-protein–gated inwardly rectifying K^+ channels but the role of these channels in apoptosis is unclear (Migheli et al., 1999). Sodium overload-induced plasma membrane depolarization has been reported to activate VGCC that mediates intracellular Ca^{2+} rise. Moreover, sodium overload can induce further intracellular Ca^{2+} increases by means of the activation of the Na^+/Ca^{2+} exchanger which has been widely associated with apoptosis (Howes et al., 2003; Annunziato, Pignataro & Di Renzo, 2004; Elgel, Gursahani & Hadley, 2004). Finally, sodium overload has been recently reported to be necessary for cell shrinkage or AVD, but the mechanisms involved and implications are still elusive (Bortner & Cidlowski, 2003).

Ca^{2+} Overload

Ionic Ca^{2+} is a highly versatile intracellular signal that regulates numerous cellular processes. To date there are many examples describing how Ca^{2+} can directly regulate protein function by modulating either enzymatic activity or conformational changes

(Berridge, Bootman & Roderick, 2003), and changes in intracellular Ca^{2+} clearly have a role in apoptotic cell death. Calcium overload has also been suggested to be the final common pathway of all types of cell death (Rizzuto et al., 2003). Intracellular Ca^{2+} increases have been suggested to act not only as apoptotic inducers, but also as regulators of the amplification loop of the death signal. In contrast, there is also evidence suggesting that apoptosis induced by different stimuli including death receptor activation, radiation and DNA damage is either Ca^{2+}-independent (Jornot, Petersen & Junod, 1998; Rozental et al., 2004), or that it participates in only certain components of the cell death program (Scoltock et al., 2000).

Ionic Ca^{2+} has been reported to interact and modulate the apoptotic signaling machinery at different stages, and several studies have shown that cytosolic Ca^{2+} is elevated during both early and late stages of apoptosis (Tombal, Denmeade & Isaacs, 1999; Rizzuto et al., 2003). Cytosolic Ca^{2+} overload enhances mitochondrial Ca^{2+} uniport uptake that results in matrix swelling, mitochondrial depolarization and release of apoptogenic proteins. These effects have been suggested to be mediated by the direct opening of the permeability transition pore (PTP), the generation of ROS, cardiolipin peroxidation and activation of Ca^{2+}-activated K^+ channels (mitoK_{Ca}) (Hajnoczky, Davies & Madesh, 2003). Calcium overload may also activate apoptogenic effectors that control the cell death process independent from the mitochondria, by modulating the activity of kinases and phosphatases involved in apoptosis, such as calmodulin and calmodulin-dependent kinase II (Nutt et al., 2005; Wu et al., 2005). Furthermore, calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase has also been demonstrated to mediate the translocation of Bad (a Bcl-2 family member of the pro-apoptotic proteins) to the mitochondria. Another important mediator of Ca^{2+}-dependent apoptosis is the family of Ca^{2+}-dependent proteases, calpains. Calpains are cysteine proteases that act in a similar way as caspases and are activated from an inactive proenzyme by Ca^{2+}-dependent autocatalytic cleavage. Calpains have been reported to mediate apoptosis by the further cleavage of various cellular apoptogenic proteins including caspases, calcineurin, Bcl-2 family members and X-linked inhibitors of apoptosis. Additional effectors of apoptosis mediated by cytosolic Ca^{2+}-overload include DNases, nitric oxide synthases, phospholipases and transglutaminases (Orrenius, Zhivotovsky & Nicotera, 2003).

Cl^-/Anion Efflux

Chloride/anion flux pathways have also been reported to modulate the progression of apoptosis. This has been studied primarily through the inhibitory effect of chloride channel blockers and reduced extracellular Cl^- media on the apoptotic signaling cascade. However, the exact role of reduced intracellular Cl^- concentration on the cell death program is far from being understood. Chloride/anion efflux is necessary for cell shrinkage or AVD during apoptosis (Szabo et al., 1998; Okada & Maeno, 2001; d'Anglemont de Tassigny et al., 2004; Okada et al., 2004; Porcelli et al.., 2004). However, the idea of AVD as a necessary signal for the activation of apoptosis (Orlov et al., 1996; Maeno et al., 2000; Friis et al., 2005) has been challenged by studies showing either the occurrence of apoptosis in the absence of cell shrinkage (Bortner & Cidlowski, 2003; Vereninov et al., 2004), or the inhibition of apoptosis by cell shrinkage (Gulbins et al., 1997; Uhlemann et al., 2000).

Possible role of Voltage Changes

The role of PMP depolarization in the cell death program appears to depend on the ionic species implicated, however, one cannot also discard the existence of voltage-sensitive steps acting on the signaling cascade. Changes in membrane potential affect ion flux pathways with an intrinsic voltage sensor (ion channels), which alters intracellular chemical conditions and modulates a variety of biological processes. Evidence concerning the direct effect of the changes in PMP potential on enzyme activity or other signaling pathways is limited. A recent study has demonstrated the presence of an intrinsic voltage sensor in non-channel signaling proteins, which suggests that other enzymes, including apoptogenic signaling proteins, might also possess a similar mechanism (Murata et al., 2005). Apoptosis induced by different stimuli leads to the activation of voltage-activated ion channels, including VGCC and voltage-gated K^+ channels that have been reported to be involved in the progression of apoptosis (Yu et al., 1997; Storey et al., 2003; Yu, 2003b; Remillard & Yuan, 2004; Wang, 2004). Electrical activity has also been demonstrated to regulate programmed cell death in neurons by activation of Na^+ channels (Svoboda, Linares & Ribera, 2001). Other kinds of electric-mediated signaling have been described as well for ion channels functionally linked to membrane receptors involved in apoptosis (Arcangeli et al., 1993; Olivotto et al., 1996; Brassard et al., 1999; Lewis, Truong & Schwartz, 2002). Thus, it is plausible to hypothesize that other death receptors or membrane domains might employ similar mechanisms for signal transduction during apoptosis.

Concluding Remarks and Perspectives

Apoptosis is an evolutionarily conserved process involved in both physiological and pathophysiological phenomena. Despite a large number of studies dedi-

cated to the elucidation of the signaling machinery involved in apoptosis, there are still many aspects of this process to be resolved. Changes in the intracellular milieu of the cells have been reported to be a determinant for the activation, modulation and progression of apoptotic cell death, and maintaining a normal ionic homeostasis may be an important inhibitory mechanism for apoptosis in cells. Ionic homeostasis regulation is a transcendental phenomenon for the normal physiology of all cell types, and accordingly, ionic homeostasis deregulation is a common hallmark of apoptosis. Particularly, PMP depolarization has been observed to be an initial feature of apoptosis associated with either cation overload or anion efflux; however, the interrelationship of these phenomena is still far from being understood. In this review, we have summarized the current knowledge and evidence about the role of electrogenic ion transport (including channels, transporters and ATPases) and PMP depolarization in apoptosis. Evidence suggests that direct PMP depolarization is able to trigger the cell death program in some cell types, as shown by the observation that direct activation of Na^+ and Ca^{2+} ionophores induces apoptosis. These effects seem to be ion species- specific. Notwithstanding, and less studied is the possibility of intrinsic voltage-sensors in non-channel signaling proteins, as suggested by recent studies (Murata et al., 2005). In addition, the level of complexity is raised by the fact that there is evidence suggesting that not only changes in the concentration of ionic Ca^{2+}, but also of Na^+ and Cl^-, might have direct effects on the signaling machinery of apoptosis. As summarized here, both cation overload and/or anion efflux associated with PMP depolarization can modulate or activate the apoptotic signaling machinery at different steps in the cell death program. However, current evidence is insufficient to make a clear synthesis of the pathways involved, and more studies are necessary to clarify the role of ionic imbalance and PMP depolarization in apoptosis.

We appreciate the input of Dr. James W. Jr. Putney.

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