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Na^{+}, K^{+}-ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death

Shan Ping Yu*

Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425, USA

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Abstract

The Na^{+}, K^{+}-ATPase is a ubiquitous membrane transport protein in mammalian cells, responsible for establishing and maintaining high K^{+} and low Na^{+} in the cytoplasm required for normal resting membrane potentials and various cellular activities. The ionic homeostasis maintained by the Na^{+}, K^{+}-ATPase is also critical for cell growth, differentiation, and cell survival. Although the toxic effects of blocking the Na^{+}, K^{+}-ATPase by ouabain and other selective inhibitors have been known for years, the mechanism of action remained unclear. Recent progress in two areas has significantly advanced our understanding of the role and mechanism of Na^{+}, K^{+}-ATPase in cell death. Along with increased recognition of apoptosis in a wide range of disease states, Na^{+}, K^{+}-ATPase deficiency has been identified as a contributor to apoptosis and pathogenesis. More importantly, accumulating evidence now endorses a close relationship between ionic homeostasis and apoptosis, namely the regulation of apoptosis by K^{+} homeostasis. Since Na^{+}, K^{+}-ATPase is the primary system for K^{+} uptake, dysfunction of the transport enzyme and resultant disruption of ionic homeostasis have been re-evaluated for their critical roles in apoptosis and apoptosis-related diseases. In this review, instead of giving a detailed description of the structure and regulation of Na^{+}, K^{+}-ATPase, the author will focus on the most recent evidence indicating the unique role of Na^{+}, K^{+}-ATPase in cell death, including apoptosis and the newly recognized ''hybrid death'' of concurrent apoptosis and necrosis in the same cells. It is also hoped that discussion of some seemingly conflicting reports will inspire further debate and benefit future investigation in this important research field.
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1. Introduction

The Na^{+}, K^{+}-ATPase, sometimes called Na^{+} pump or Na^{+}-K^{+} pump, is a transmembrane enzyme acting as an electrogenic ion transporter in the plasma membrane of all mammalian cells. Each cycle of the Na^{+}, K^{+}-ATPase activity extrudes three Na^{+} from the cell, moves two K^{+} into the cell and utilizes one ATP [1]. The primary role of the Na^{+}, K^{+}-ATPase is therefore to maintain high intracellular K^{+} and low intracellular Na^{+} [2]. Failure of the Na^{+}, K^{+}-pump results in depletion of intracellular K^{+}, accumulation of intracellular Na^{+}, and, consequently, leads to membrane depolarization and increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) due to activation of voltage-gated Ca^{2+} channels and a reversed operation of the Na^{+}-Ca^{2+} exchanger [3–5]. The ion gradients formed by the Na^{+}, K^{+}-ATPase are necessary for Na^{+}-coupled transport of nutrients and amino acids into cells, osmotic balance, cell volume regulation, and for maintenance and restoration of the resting membrane potential in excitable cells [6,7].

The Na^{+}, K^{+}-ATPase is composed of two subunits. The catalytic α subunit is a large polypeptide of $\sim$1000 amino acid residues that spans the plasma membrane 10 times, catalyzes the ion-dependent ATPase activity, carries the binding sites for ATP and the specific inhibitor ouabain. The β subunit is a smaller polypeptide of about 300 residues with a single transmembrane spanning segment [8,9]. The β subunit regulates conformational stability and activity of the α subunit [10,11]. It may be involved in the modulation of the K^{+} and Na^{+} affinity of the enzyme [12,13]. A third small polypeptide, the γ subunit, is found in association with the $\alpha\beta$ dimmer in a tissue-specific manner; this subunit does not seem to be required for

* Tel.: +1-843-792-2992; fax: +1-843-792-1712.
E-mail address: yusp@musc.edu (S.P. Yu).

Abbreviations: ROS, reactive oxygen species; TEA, tetraethylammonium; Z-VAD, Z-Val-Ala-Asp-fluoromethylketone.

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functional Na^+, K^+-ATPase and may play a regulatory role [14]. At present, four genes (α1–α4) encoding the α subunit, three genes (β1–β3) encoding the β subunit, and one gene for the γ subunit have been described in mammals [9]. All α and β isoforms are expressed in the nervous system. Among them, α1 is almost ubiquitously distributed with highest expression levels found in kidney, α2 predominates in skeletal muscle, brain and heart, and α3 appears to be the most abundant form in brain, but it is also present in heart. The temporal and spatial expression in the brain demonstrates that various α and β isoforms may be expressed in the same cell type [15,16].

Multiple mechanisms can regulate the activity of Na^+, K^+-ATPase, which satisfies the functional roles of the enzyme in different conditions and makes the pump protein vulnerable to pathogenic insults and a potential target for therapeutic treatments [14,17]. In addition to its ATP dependence, the Na^+, K^+-ATPase activity is regulated by phosphorylation state [14], endogenous ouabain-like substances [18–20], neurotransmitters such as dopamine (inhibitory) and norepinephrine (stimulatory) [21], hormones such as insulin [22,23], oxidant stress such as reactive oxygen species (ROS) [24], and, more directly, by ionic distributions across the membrane [14,25,26]. Excellent reviews about the structure, distribution, and regulation of Na^+, K^+-ATPase are available as listed above for additional information.

2. Alterations of Na^+, K^+-ATPase in pathological conditions

A central role for the Na^+, K^+-ATPase in pathogenesis has been widely implicated, particularly in cardiovascular, neurological, renal, and metabolic diseases [27–29]. In general, a down regulation of Na^+, K^+-ATPase is found under these conditions. The Na^+, K^+-ATPase activity was 34% lower in ischemic cortex and 40% lower in ischemic basal ganglia after 30-min ischemia; after 60-min ischemia, both the Na^+, K^+-ATPase activity and K^+ concentration were decreased in the ischemic hemisphere [30,31]. This is consistent with the observations that ischemia or hypoxia induce energy crisis [32], increase the production of ROS [33–36], and release endogenous inhibitors of Na^+, K^+-ATPase [37]. The functional subunits, α2 and α3, were the ones that were mostly affected by a focal cerebral ischemia [38]. An inhibition of Na^+, K^+-ATPase secondary to cellular energy depletion might contribute to delayed membrane depolarization of cortical neurons after traumatic brain injury [39]. The Na^+, K^+-ATPase activity was reduced or insufficient to maintain ionic balances during and immediately after episodes of ischemia, hypoglycemia, epilepsy, and after administration of glutamate agonists. It was proposed that a reduction and/or inhibition of Na^+, K^+-ATPase contributed to the central neuropathy found in those disorders [40]. Dysfunction or deficiency of Na^+, K^+-ATPase has been identified in chronic neurodegenerative diseases; for example, the α3 subunit mRNA was ~30–45% lower in Alzheimer's brain relative to controls [41]. The possibility that a deficiency in the Na^+, K^+-ATPase activity might be a common pathogenesis of central nervous system disorders was tested in patients of CNS glioma, multiple sclerosis, systemic lupus erythematosis, subacute sclerosing panencephalitis, primary generalized epilepsy, Parkinson's disease, Down syndrome, syndrome X with multiple lacunar state, and several other neurodegenerative disorders [42]. The study revealed that the Na^+, K^+-ATPase activity on the red blood cell membrane were generally lower, along with increased serum level of endogenous digoxin and decreased level of serum Mg^{2+}; although changes in K^+, Na^+, and Ca^{2+} were not tested. Collectively, it appears factual that reduced Na^+, K^+-ATPase activity is a common event in a number of neural degenerative and metabolic diseases.

3. Na^+, K^+-ATPase, cellular K^+ depletion, and apoptosis

Recent evidence suggest that impaired Na^+, K^+-ATPase function can be linked to apoptosis. Apoptosis is a programmed cell death identified in development as well as under pathological conditions [43,44]. Apoptotic cells undergo cell body shrinkage, nuclear condensation, chromatin margination, DNA fragmentation (laddering), and formation of apoptotic bodies [45,46]. Apoptosis is mediated by a cascade of events including activation of caspases, release of cytochrome *c* from mitochondria, formation of the apoptosome, and activation of endonucleases [47–49]. Apoptosis is closely regulated by anti- and pro-apoptotic genes such as *bcl-2* and related genes [50–52]. Recent works, including ours, reveal that apoptosis is also regulated by ion homeostasis mainly involving the K^+ homeostasis [53–56]. Apoptotic cells commonly lose huge amounts of intracellular K^+, the cellular K^+ concentration can be reduced from well over 100 mM to as little as 30–50 mM in apoptotic cells [5,57–59]. This reduced K^+ concentration is likely a prerequisite for executing a number of apoptotic processes, including cell volume decrease, caspase-3 cleavage, cytochrome *c* release, and endonuclease activation [53,58,60–63]. Changes in K^+ homeostasis may also affect the mitochondrial function and its membrane potential as part of apoptotic process [59,124]. Depleting intracellular K^+ alone is often sufficient to induce apoptosis; for example, K^+ ionophores such as valinomycin or overexpression of K^+ channels can cause apoptosis in central neurons and peripheral cells [64–68]. In some cases, the cellular K^+ depletion acts as a necessary cofactor to promote apoptosis [58,67]. Several reviews have provided more detailed information about the intrinsic link between K^+ and apoptosis [54,56,59,69].

The pro-apoptotic K^+ efflux can be carried out by voltage-gated K^+ channels and ionotropic glutamate receptors [70–79]. Blocking the Na^+, K^+-ATPase while allowing normal K^+ efflux likewise causes substantial intracellular K^+ depletion, 50–80% K^+ loss was observed in ouabain-treated cells [5,80]. Theoretically speaking, cellular K^+ depletion may not occur as long as K^+ efflux can be compensated by a sufficient K^+ uptake. In this regard, Na^+, K^+-ATPase plays a critical role in K^+ homeostasis and apoptosis.

The cytotoxicity induced by the Na^+, K^+-ATPase inhibitors such as ouabain and digitalis has been known for many years, although the mechanism of action and the form of cell death were not well defined [63,66,81]. The first evidence linking the Na^+, K^+-ATPase to apoptosis was from a study a few years ago on the mechanism by which bcl-2 protected against apoptosis in PW and HL60 cells [82,83]. Elevated Na^+, K^+-ATPase activity and hyperpolarized membrane potentials found in these cells of overexpressing bcl-2 were thought to be a protective mechanism. Cells overexpressing bcl-2 were less susceptible to ouabain, suggesting that bcl-2 might act at the level of the Na^+, K^+-ATPase [84]. Consistently, the Na^+, K^+-ATPase activity remained less affected following irradiation in bcl-2 transfectants [83]. Furthermore, a lower concentration of ouabain (1 μM) eliminated the protective effect of bcl-2 expression on irradiation-induced apoptosis [83]. Another early indication was from the study showing that bufalin, an active principle of Chinese medicine, chan'su, and a Na^+, K^+-ATPase inhibitor, induced typical apoptosis in human leukemia U937 cells [84]. Ouabain-induced apoptosis was shown later by Olej *et al*. in lymphocytes [85]. The pro-apoptotic effects of blocking Na^+, K^+-ATPase have been demonstrated in lymphocytes [84,86,87], human prostatic smooth muscle cells [86], human prostate cancer cell lines [88], and neurons [5,89,90]. In the bufalin-induced apoptosis, nPKCδ may play a mediating role [87]. The inhibitory effect of radiation on the Na^+, K^+-ATPase activity was mediated by ROS generation [83]. A recent study showed that apoptotic thymocytes had decreased protein levels of Na^+, K^+-ATPase [91]. Consistently, acute ethanol intoxication decreased Na^+, K^+-ATPase activity by 55% in the lungs of rats mediated by oxidative stress [92]. Ouabain injection *in vivo* induced apoptotic injury in the animal brain [93]. Recognizing the critical role of K^+ efflux and cellular K^+ depletion in apoptosis, it was specifically proposed that inhibition of the Na^+, K^+-ATPase is a key player in the K^+ regulation of apoptosis [5,80,90].

In addition to directly triggering apoptosis, ouabain may sensitize cells to other apoptotic insults such as TNF in rodent tumor cell lines [94], irradiation in several human cell lines [95], CD-95-induced apoptotic shrinkage in Jurkat cells [80], and anti-Fas-induced apoptosis in Jurkat cells [96]. Based on the rationale that K^+ uptake depended on the Na^+, K^+-ATPase, we hypothesized in our investigation that although a slight impairment of the pump activity might not be disruptive, the reduced pumping ability might amplify the deteriorating effect of an apoptotic distress on K^+ homeostasis and cell survival. This is based on the observations that apoptotic insults including serum withdrawal, staurosporine, ceramide, and β-amyloid peptides can increasing K^+ efflux by increasing the delayed rectifier K^+ channels [70,71,73]. In cortical neuronal cultures, 0.1 μM ouabain alone showed minimum effect on the Na^+ pump activity measured as the membrane current; neither intracellular K^+ content nor cell viability was changed by this concentration of ouabain. On the other hand, the apoptotic insult C_2-ceramide at a low concentration of 5 μM also did not affect intracellular K^+ content and cell viability. Combination of 0.1 μM ouabain and 5 μM C_2-ceramide, however, caused about 50% intracellular K^+ depletion, significant caspase-3 activation, DNA laddering, and substantial neuronal death [90]. Similar synergistic toxicity was observed when 0.1 μM ouabain was co-applied with the sub-lethal concentration of 5 μM β-amyloid 1–42. The synergy-induced cell death was essentially blocked by the caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone (Z-VAD) [90]. These data indicate a vital role of the Na^+, K^+-ATPase in pathogenesis of some apoptosis-related diseases and suggest that even a slight deficiency of the pump activity may markedly increase the susceptibility of central neurons to some apoptosis-related diseases. Such risky situations may be particularly true when endogenous ouabain-like substances are released or ROS is produced in pathological conditions.

4. Blocking the Na^+, K^+-ATPase: a direct antagonism against apoptosis or an insult of pre-conditioning effect?

Orlov *et al*. reported [97] that blocking the Na^+, K^+-ATPase attenuated apoptosis triggered by serum withdrawal, staurosporine, or okadaic acid in vascular smooth muscle. They showed that 1–2 hr pre-treatment with ouabain prevented caspase-3 activation and DNA degradation [97]. To demonstrate that the effects of blocking Na^+, K^+-ATPase were mediated by an inversion of the intracellular Na^+/K^+ ratio, these investigators confirmed increases in intracellular Na^+ and decreases in intracellular K^+; moreover, they showed that neither the intracellular content of Na^+ and K^+ in vascular smooth muscle nor chromatin fragments (as a sign of apoptosis) was affected by ouabain in a K^+-enriched, Na^+-depleted medium [97]. A few details of the reported observations and conclusions remain open to debate. First, a key point in this study was the experimental protocol of pre-treatment with ouabain, which was often overlooked in later studies to the mechanism of protection. The protective effect induced by pre-treatment with ouabain appears to be a result from activation of a pre-conditioning or tolerance mechanism as

reported by others [98,99]. This was supported by subsequent studies of the same group showing that ouabain pretreatment induced concentration- and time-dependent RNA/DNA synthesis and overexpression of many stress-induced signals including mortalin, a member of the heat shock protein 70, and c-Fos, c-Jun proteins [97,100,101]. In a different study, ouabain treatment activated PI3 kinase and phosphorylation of Akt [102]. In addition, the ouabain effect could be mimicked by a free-radical generating system [99]. It is therefore feasible that ouabain acts as an insult but not as an antagonist of apoptosis. With respect to the mechanism of action, it is necessary to distinguish an antagonist from an insult that can generate a pre-conditioning effect. Ignorance of or failure to distinguish the differences has caused confusion in terms of what could be the pro- and anti-apoptotic mechanism. The second point that needs to be addressed is that in the study of Orlov *et al.* [97], concentrations of both Na^+ and K^+ were manipulated simultaneously; the possibility that changes of only one ion would be sufficient for the observed effect was never specifically tested. Therefore, the conclusion that the ouabain effect was mediated by an inversion of the Na^+/K^+ ratio in cells was speculative. It can be rationally argued that the ouabain pre-conditioning effect was due to the depletion of intracellular K^+, regardless of changes in Na^+, or due to the changes in intracellular Na^+ alone. As a matter of fact, the assumption is consistent with the data reported by the same group [97] that intracellular K^+ was unchanged in the free-Na^+/high-K^+ medium and thereafter ouabain showed no protection against serum deprivation-induced apoptosis. In a later study, the ouabain toxicity unrelated to the Na^+/K^+ ratio was observed in epithelial cells; the cell death was likely a hybrid death dominated by necrosis characterized by cell swell, transient caspase activation, and insensitivity to the pan caspase inhibitor Z-VAD [103].

5. Na^+, K^+-ATPase and "hybrid" cell death of concurrent apoptosis and necrosis in the same cells

Both Ca^{2+} influx and K^+ efflux have been proposed to mediate the ouabain cytotoxicity [5,104]. We specifically tested the roles of Ca^{2+} and K^+ in ouabain-induced cell death in cultured cortical neurons. Ouabain blocks the Na^+, K^+-ATPase in neural cell with IC_{50} of low micromolar concentrations (~1–10 μM) depending on the low and high affinity binding sites and α subunits [105–107]. At high concentrations of 80–100 μM ouabain severely blocked the Na^+, K^+-ATPase and caused cell swelling in minutes, as seen in previous reports [108,109]. However, long-term inspections revealed that the cell swelling was followed by gradual cell shrinkage in the following 24 hr accompanied with approximately 80% of intracellular K^+ depletion [5]. This chronic shrinkage over several hours cannot be accounted for by the mechanism of regulatory volume decrease or RVD that typically lasts for several minutes [110]. The accompanied cellular K^+ depletion suggested that the persistent shrinkage was an apoptotic event. Supporting this idea, the K^+ channel blocker tetraethylammonium (TEA) effectively prevented the K^+ depletion, cell shrinkage, cytochrome *c* release, caspase-3 cleavage, and DNA laddering [5]. Interestingly, ouabain-induced cell death was only partially blocked by TEA or the caspase inhibitor Z-VAD. The partial protection suggested to us that (1) ouabain cytotoxicity contained an apoptotic component mediated by caspase activation; (2) the caspase-independent component raised a possibility of a necrotic element in ouabain toxicity.

Blocking Na^+, K^+-ATPase not only reduces intracellular K^+ but also increases intracellular Ca^{2+}; the latter event is generally believed to be a trigger for excitotoxicity of necrotic cell death [111]. We hypothesized that the caspase-independent cell death might be associated with the ouabain-induced $[Ca^{2+}]_i$ increase. Supporting this proposition, the Ca^{2+} channel antagonist nifedipine (1 μM) attenuated ouabain-induced cell death, but it was ineffective in cytochrome *c* release, caspase activation, and DNA fragmentation [5]. Furthermore, co-applied Z-VAD and nifedipine conferred virtually complete neuroprotection against ouabain-induced neuronal death. Electron microscopy confirmed that both necrotic and apoptotic features coexisted in ouabain-injured neurons (Fig. 1). Individual cells showed morphological changes of shrunken nuclei and chromatin condensation; meanwhile, necrotic changes are apparent in the same cells, including deteriorated membranes and chaotic disruption of the cytoplasm and cellular organelles. The mixed nature of ultrastructural changes was detected throughout the time course of the ouabain treatment (Fig. 2). This mixed cell death was identified as a distinct form of 'hybrid cell death' to distinguish it from 'pure' necrosis and 'pure' apoptosis. The high concentration ouabain-induced hybrid death may not be limited to neurons, ouabain caused simultaneous apoptosis and necrosis and profound caspase-3 activity in human smooth muscle cells [86].

In fact, cell death bearing both apoptotic and necrotic features can be found in previous investigations in which the cell death was induced by glutamate, zinc, or oxygen-glucose deprivation in mouse cortical neurons [112–114], by hypoxia in rat PC12 and hepatoma 7316A cells [115], by nitric oxide in PC12 and HeLa cells (a caspase-independent but Bcl-2 dependent cell death) [116], by oxalate in renal epithelial cells [117], by IL-3 deprivation in murine bone marrow-derived mast cells [118], by serum and K^+ withdrawal in cerebellar granule cells [119], and by complement C9, which forms large transmembrane channels, in Ehrlich ascites tumor cells [120]. Even in experiments in which cells appear to die by necrosis, some aspects of apoptosis, such as DNA strand breaks, are observed under conditions of delayed neurotoxicity induced by glutamate [121]. A similar example was reported in a

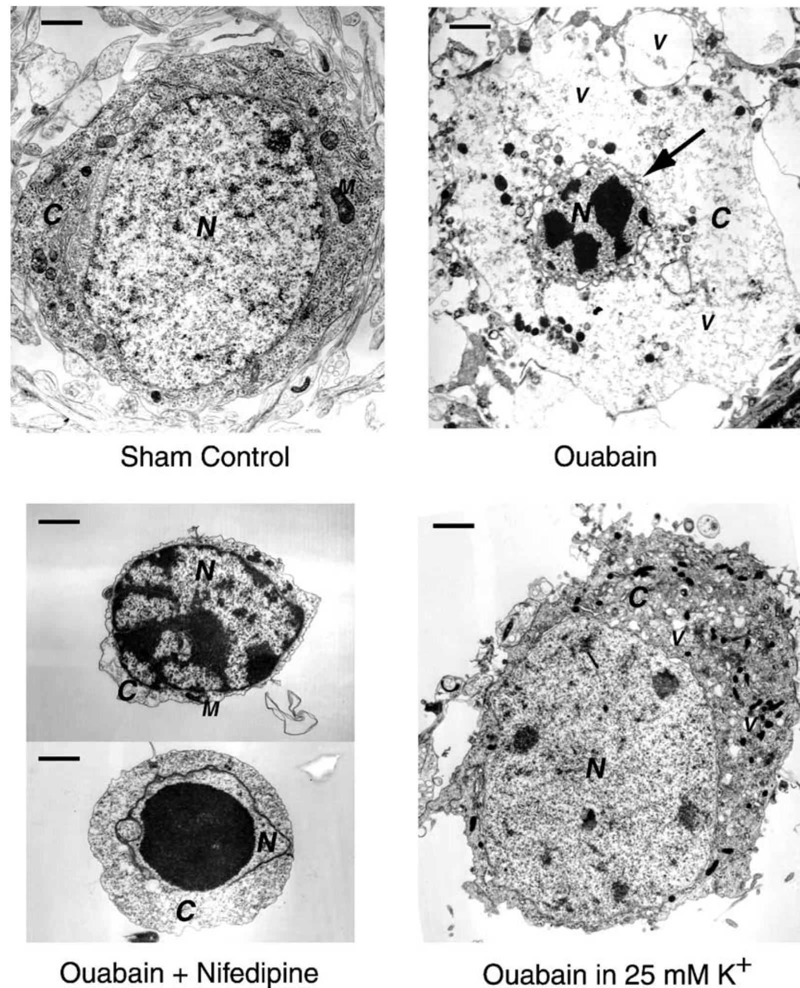

Fig. 1. Ouabain-induced ultrastructural alterations of hybrid death in cortical neurons. Electron micrographs show a control neuron and reveal striking morphological distinctions after different treatments. The normal cortical neuron has a relatively small cytoplasm and a large nucleus; the cell and cellular organelles are surrounded by intact membranes. About 15 hr after incubation in 100 μM ouabain and 1 μM MK-801, injured cells show apoptotic features such as highly condensed nuclei and dark chromatin clumps (arrow), accompanied by necrotic changes including cytoplasmic edema manifested by vacuolization and decreased cytoplasmic density, loss of cellular organelles, and breakdown of the plasma membrane. In another experiments, the co-applied Ca^{2+} channel antagonist nifedipine (1 μM) primarily eliminated necrotic alterations. Two representative injured cells show typical apoptotic morphology, including highly condensed nuclei and cytoplasm, dark chromatin masses (pyknosis) with or without fragmentation, intact cellular organelles and intact plasma membrane. Reducing K^+ efflux, on the other hand, by raising extracellular K^+ to 25 mM resulted in the morphological pattern of necrotic injury in most cells. A representative cell shows that ouabain in the high K^+ medium induced chaotic alterations in the swollen cytoplasm. No single intact cellular organelle can be detected in the cell; instead, lucent vacuoles appear in the cytoplasm. The cell membrane is deteriorating, but there is little or no nuclear/cellular shrinkage, and no chromatin condensation or fragmentation. Bar = 2.0 μm in all five electron micrographs. N: nuclear; C: cytoplasm; M: mitochondria; V: vacuole. From Fig. 9 in [5].

human monocytic cell line and monocytes where ouabain induced a caspase-dependent cell death with features of necrosis and the cell death was mediated by K^+ depletion [122]. Mice deficient in the adhesion molecule on glia (AMOG, the β 2 subunit of the Na^+, K^+-ATPase) showed cell swelling and subsequent degeneration of astrocytes endfeet in the brainstem and cell death of photoreceptor cells in the retina. The terminal deoxynucleotidyl transferase nick-end-labeling and electron microscopy examinations revealed apoptotic alterations [123]. In studies of *in vivo* animal models, concurrent apoptotic and necrotic features in the same cells were observed after coronary artery occlusion and reperfusion in myocardial cells [124]. In the adult or newborn rat brain, injection of kainic acid induced a continuum of apoptotic, necrotic and overlapping morphologies [125]. After hypoxic ischemia in the newborn rat, ''hybrid'' neuronal cells showed intermediate ultrastructural characteristics similar to the mixed death induced by ouabain [126]. These data dictate reassessment of 'mixed cell death' as a heterogeneous entity combining both active and passive cell death [5,72,127,128] and in line with an *in vivo* ''apoptosis-necrosis continuum'' in excitotoxically lesioned rat brain [129].

It is expected that further investigations on the unique role of Na^+, K^+-ATPase in apoptosis and hybrid cell death

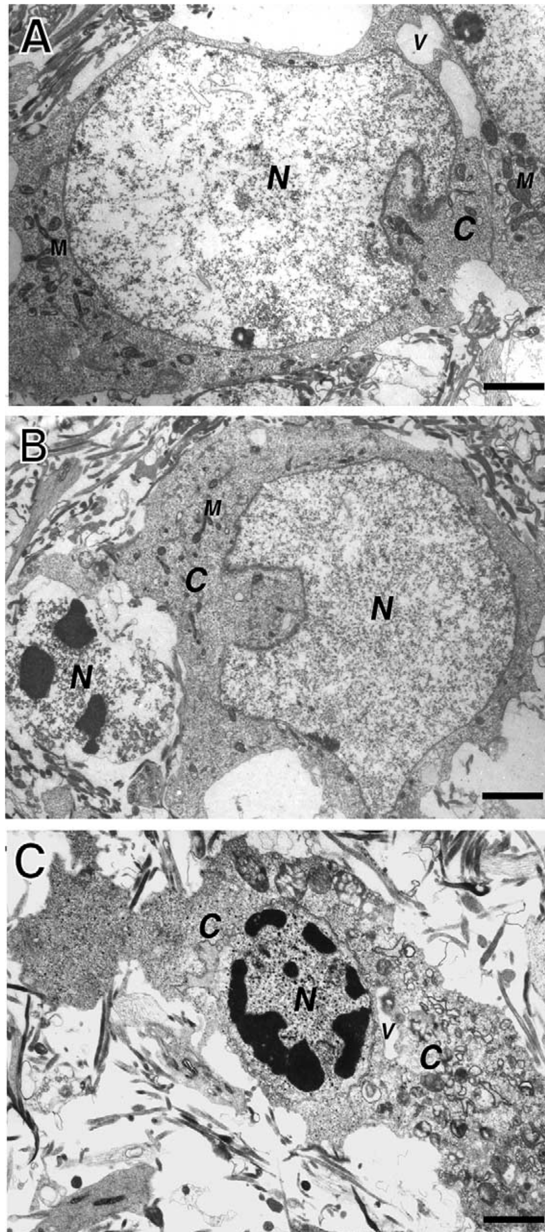

Fig. 2. Morphological changes of hybrid cell death at early time points of ouabain exposure. Ultrastructural morphology of a normal neuron can be seen in Fig. 1. (A) Two hours after adding 100 μM ouabain plus 1 μM MK-801, some cells started to show signs of nuclear changes; the electron micrograph shows an irregular shape of the nuclear, implying a volume decrease. Meanwhile, mitochondria swelling were observed in many cells. (B) Apoptotic features such as nuclear shrinkage and condensation of the nuclear chromatin were advanced after 5 hr in ouabain. Necrotic changes such as cytoplasm swelling, formation of vacuoles, and disruptions of cellular organelles and the plasma membrane also appeared at earlier hours. The two cells shown in this micrograph represent different stages of morphological changes observed at this time. (C) Ten hours after onset of ouabain exposure, injured cells with highly condensed nuclei, chaotic cytoplasm, and disrupted plasma membrane were easily detected. Bar = 3.0 μm. N: nuclear; C: cytoplasm; M: mitochondria; V: vacuole. From Fig. 8 in [5].

will provide a more comprehensive understanding of cell death mechanism and may provide novel approaches to cell protection in pathological conditions of altered regulatory systems of ionic homeostasis.

Acknowledgments

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