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Apoptotic insults impair Na^+, K^+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress

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Summary

The Na^+, K^+-ATPase (Na^+, K^+-pump) plays critical roles in maintaining ion homeostasis. Blocking the Na^+, K^+-pump may lead to apoptosis. By contrast, whether an apoptotic insult may affect the Na^+, K^+-pump activity is largely undefined. In cultured cortical neurons, the Na^+, K^+-pump activity measured as a membrane current I_{pump} was time-dependently suppressed by apoptotic insults including serum deprivation, staurosporine, and C_2-ceramide, concomitant with depletion of intracellular ATP and production of reactive oxygen species. Signifying a putative relationship among these events, I_{pump} was highly sensitive to changes in ATP and reactive oxygen species levels. Moreover, the apoptosis-associated Na^+, K^+-pump failure and serum deprivation-induced neuronal death were antagonized by pyruvate and succinate in ATP- and reactive-oxygen-species-dependent manners. We suggest that failure of the Na^+, K^+-pump as a result of a combination of energy deficiency and production of reactive oxygen species is a common event in the apoptotic cascade; preserving the pump activity provides a neuroprotective strategy in certain pathological conditions.

Key words: Na^+, K^+-ATPase, Apoptosis, Potassium homeostasis, Neuron

Introduction

The Na^+, K^+-ATPase (Na^+, K^+-pump) acts as an electrogenic ion transporter in the plasma membrane of all mammalian cells, each cycle of Na^+, K^+-pump activity extrudes three Na^+ ions from the cell, moves two K^+ ions into the cell and uses 1 ATP (Rakowski et al., 1989). The primary role of the Na^+, K^+-pump is therefore to maintain high intracellular K^+ and low intracellular Na^+. 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 (Archibald and White, 1974; Lijnen et al., 1986; DiPolo and Beauge, 1991; Xiao et al., 2002). A central role for the Na^+, K^+-pump in pathogenesis has been widely implicated, particularly in heart ischemia (Ziegelhoffer et al., 2000). Blocking the Na^+, K^+-pump can induce apoptosis (Olej et al., 1998; Chueh et al., 2001) or a 'hybrid death' containing both apoptotic and necrotic components in individual neurons (Xiao et al., 2002). Conversely, a recent study showed that apoptotic thymocytes had decreased protein levels of the Na^+, K^+-ATPase (Mann et al., 2001). Whether the apoptotic process may alter the Na^+, K^+-pump activity, however, is still an open question and has so far not been directly investigated.

Excessive K^+ efflux and intracellular K^+ depletion are thought to be critical steps in cell body shrinkage and apoptotic death (Yu et al., 1997; Dallaporta et al., 1998; Bortner and Cidlowski, 1999). A significant reduction in intracellular K^+ concentration may be a prerequisite for key apoptotic events including caspase-3 cleavage and endonuclease activation (Dallaporta et al., 1998; Bortner and Cidlowski, 1999). The pro-apoptotic K^+ efflux may be mediated by voltage-gated K^+ channels in neurons (Yu et al., 1997; Colom et al., 1998; Nadeau et al., 2000); and other cells (Nietsch et al., 2000; Wang et al., 1999; Diem et al., 2001; Krick et al., 2001). In addition, K^+ loss may occur through NMDA or AMPA/kainate receptor channels (Yu et al., 1999a; Xiao et al., 2001). Theoretically, K^+ homeostasis may not be altered if K^+ efflux can be balanced by sufficient K^+ uptake. Since the Na^+, K^+-pump is the only major mechanism for K^+ uptake, we hypothesized that cells undergoing apoptosis, in addition to an enhanced K^+ efflux, might additionally suffer from dysfunction of the Na^+, K^+-pump. To test this hypothesis, we identified the membrane currents associated with the Na^+, K^+-pump activity in cortical neurons, examined the effects of several apoptotic insults on the Na^+, K^+-pump current and modeled the putative role of the Na^+, K^+-pump in neuronal apoptosis. This work was partly presented in an abstract (Wang et al., 2001).

Materials and Methods

Neocortical cultures

Near pure-neuronal cultures and mixed cortical cultures (containing neurons and a confluent glia bed) were prepared as described previously (Rose et al., 1993). Briefly, mice of 15-17 days gestation were anesthetized with halothane. Dissociated neocortices obtained from fetal mice were plated onto a poly-D-lysine and laminin coated base (near-pure culture) or a previously established glial monolayer (mixed culture) at a density of 0.35 to 0.40 hemispheres/ml in 35-mm dishes or 24-well plates (Falcon, Primaria), in Eagle's minimal essential medium (MEM, Earle's salts) supplemented with 20 mM glucose, 5% fetal bovine serum (FBS) and 5% horse serum (HS). For near-pure neuronal cultures, cytosine arabinoside (10 μM) was added 3 days later to inhibit glial cell growth and cell division. These neuronal cultures contained less than 1% of astrocytes (Dugan et al., 1995). For mixed neuronal and glial cultures, medium was changed after one week to MEM containing 20 mM glucose and 10% HS, as well as cytosine arabinoside (10 μM) to inhibit cell division. Subsequently, cultures were fed once weekly with MEM supplemented with 20 mM glucose. Glial cultures used for the mixed cultures were prepared from dissociated neocortices of postnatal day 1-3 mice. Cultures were kept in a 37°C, humidified incubator in a 5% CO_2 atmosphere. Cortical cultures of 9-12 days in vitro were used for experiments or otherwise specified.

Electrophysiological recordings of Na^+, K^+-pump currents

The 35-mm culture dish containing cortical neurons was placed on the stage of an inverted microscope, and membrane currents were recorded by whole-cell configuration or perforated patch using an EPC-9 amplifier (List-Electronic, Germany). Recording electrodes of 8-10 MΩ (fire-polished) were pulled from Corning Kovar Sealing #7052 glass pipettes (PG52151-4, WPI, USA) by a Flaming-Brown micropipette puller (P-80/PC, Sutter Instrument Co., USA). For perforated patches, gramicidin D was dissolved in DMSO (10 mg/ml) and freshly diluted to a final concentration of 50 μg/ml with the internal solution. After formation of a gigaohm seal, brief voltage steps of –10 mV were applied to monitor the changes in input resistance and capacitance for 15-20 minutes before the formation of the perforated patch. Series resistance compensation was routinely applied during recordings. Current and voltage signals were displayed on a computer monitor and collected by a data acquisition/analysis program PULSE (HEKE, Lambrect, Germany). Currents were digitally sampled at 0.33 kHz and filtered at 3 Hz by a 3-pole Bessel filter.

An inward current representing the tonic Na^+, K^+-ATPase activity was generated by application of the selective blocker ouabain (1 mM) or strophanthidin (10-1000 μM). Experimental testing solutions were locally applied to the cell surface using the DAD-12 drug delivery system (Adams & List, New York, NY). As a reversible inhibitor, strophanthidin could be repeatedly applied with intervals of ≥2 minutes. The extracellular solution contained (in mM): NaCl 125, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, Na-HEPES 10, Glucose 10, and 0.01 μM TTX. The electrode solution contained (in mM): Cs-acetate 60, NaCl 20, *N*-methyl-D-glucamine 100, Mg-ATP 5, BAPTA 1, TEA 10, and HEPES 10. To record an outward current at –70 mV associated with activation of the Na^+, K^+-pump, cells were first exposed to a K^+-free solution (10 seconds) to minimize the pump activity and then to a solution containing 4 mM K^+ (5 seconds) to activate the pump. Gadolinium (1 μM) was applied into the external solution to prevent openings of voltage-gated Ca^{2+} channels and stretch-sensitive channels. In the experiment examining outward currents, TTX concentration was raised to 0.1 μM to ensure complete block of Na^+ channels.

For effects of acute treatments (~30 minutes) on the pump current, whole-cell or perforated patch recordings was performed in the same cells before and after a treatment; group studies in different cells were used for chronic treatments (hours). Recordings were performed at room temperature (21±1°C); all solutions had a pH of 7.3-7.4.

Assessments of cell death

Neuronal cell death was assessed in 24-well plates by measuring lactate dehydrogenase (LDH) released into the bathing medium (MEM + 20 mM glucose and 30 mM $NaHCO_3$) using a multiple plate reader (Molecular Devices, Sunnyvale, CA), and confirmed by staining DNA with propidium iodide (PI) followed by quantification using a fluorometric plate reader (PerSeptive Biosystems, Framingham, MA). Neuronal loss is expressed as either a percentage of LDH release or fluorescence measured in each experimental condition normalized to the negative control (sham wash) and positive control (complete neuronal death induced by 24-hour exposure to 300 μM NMDA or cell death induced by ouabain alone). There was no significant glial death detected by Trypan Blue exclusion in these injury paradigms. In serum deprivation experiments, the NMDA receptor antagonist MK-801 (1 μM) was included in the serum-free medium to block excitotoxicity (Yu et al., 1997).

ATP and ADP assays

For ATP determinations, neurons in 24-well plates were washed with ice cold PBS, scraped off with 0.125 ml of 5% trichloroacetic acid per well and collected into tubes left on ice for 5 minutes and centrifuged for 5 minutes. A fraction (0.4 ml) of the supernatant was mixed with 1.5 ml of diethylether, and the ether phase containing trichloroacetic acid was discarded. This step was repeated three times to ensure complete elimination of trichloroacetic acid. The extracts were then diluted with 0.4 ml of a buffer (buffer A) containing 20 mM HEPES and 3 mM $MgCl_2$, adequate KOH was added to adjust pH to 7.75.

ATP and ADP were assayed by a modified luminometric method (Detimary et al., 1996). For measurements of the sum of ATP + ADP, ADP was first converted into ATP by mixing 100 μl of the diluted extract with 300 μl of buffer A supplemented with 1.5 mM phosphoenolpyruvate and 2.3 units/ml pyruvate kinase, and incubated at room temperature for 15 minutes. Samples with known amounts of ADP but without ATP were run in parallel to check that the transformation was complete. An ATP assay kit (Sigma, St Louis, MO) was used and the emitted light was measured in a luminometer. For the measurements of ATP, the same procedure was followed except that the first incubation step was performed in the absence of pyruvate kinase. ADP levels were calculated by subtracting the concentration of ATP in the parallel lysate from this ATP + ADP value. Blanks and ATP standards were run through the entire procedure, including the extraction steps. For each measurement, the sample was collected from six culture wells; at least three different culture batches were used for each task. Protein concentrations were determined in the pellet after solubilization with 0.1 M NaOH by a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Confocal imaging of fluorescence measurement of ROS

The fluorescent dye dihydroethidium (DHE) was used for detecting the production of superoxide anion (O_2^-) during apoptosis. DHE was prepared as a 10 μg/ml stock solution, packed under N_2, and stored in –80°C; working stocks consisted of 1 μg/ml dilutions made in DMSO. A fresh aliquot was used for each experiment. Other drugs and test solutions were made in regular saline; all solutions contained (in mM): NaCl 144, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, and D-glucose 10 (measured osmolarity=312 mOsm, pH 7.4 adjusted with NaOH).

Cells were loaded with 1 μg/ml DHE for 1 hour, culture dishes were then placed on the stage of the confocal fluorescence microscope (Olympus IX-70, Japan). Fields of cells were randomly selected, and fluorescence images were obtained using excitation λ=488 nm and emission λ>590 nm. Frame-averaged confocal images were digitized at 640×480 pixels using the Fluoview image acquisition software

(Olympus, Japan). Fluorescence intensity was calculated as fluorescence pixel intensity for each cell.

Mitochondrial membrane potential determination

We examined mitochondrial membrane potential by monitoring mitochondrial uptake and distribution of rhodamine 123 (R123), a lipophilic cationic indicator that, driven by mitochondrial membrane potential, accumulates in mitochondrial matrix where it undergoes quenching (Scaduto and Grotyohann, 1999). Mitochondrial membrane depolarization results in the R123 release into the cytosol, unquenching and rapid fluorescence increase, which reflects the amount of dye taken up by the mitochondria, and the mitochondrial membrane potential, before depolarization.

Cells were loaded with 1 μM rhodamine 123 (Molecular Probes, Eugene, OR) by 20-minute incubation in HBBSS. After washing the dye off with HBBSS, the fluorescence of the dye was recorded for 10 minutes before and after depolarizing mitochondria with 10 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP). The cells were visualized at 530 nm emission filter with a cooled CCD camera (Cooke, Auburn Hills, MI) on a Nikon Diaphot inverted microscope (40×, 1.3 NA oil lens, 75 W xenon arc lamp). The images were collected at 485 nm at 120-second intervals and digitized. After subtracting background, the fluorescence was divided by the average rhodamine fluorescence before FCCP application for each individual cell. The MetaFluor software system (Universal Imaging, West Chester, PA) was used for image acquisition and analysis. Experiments were performed in 11-78 neurons per group from at least two separate cultures.

Protein phosphorylation assay

Immunoprecipitation

Cortical cultures in 24-well dishes were washed in ice-cold PBS and lysed for 10 minutes in 0.5 ml immunoprecipitation buffer containing 20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 30 mM NaF, 30 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, 10 μg/ml leupeptin, 4 μg/ml aprotinin,and 1% Triton X-100 (pH=7.45). After centrifugation (12,000 ***g***) and determination of protein concentration in the supernatants by the bicinchoninic acid method (BCA assay), equal amounts of protein from supernatants were incubated overnight at 4°C with antibodies. Antibodies were subsequently bound to a saturating amount of Protein A-Sepharose beads at 4°C for 4 hours. Centrifugation (12,000 ***g***) was performed twice in 1 ml ice-cold immunoprecipitation buffer and then once in TBS. After adding sample buffer (2% SDS, 50 mM Tris-Cl, 100 mM DTT, 10% glycerol, 0.1% Bromphenol Blue), the samples were heated at 65°C for 15 minutes and loaded onto SDS-PAGE gels. Phosphorylated proteins were immunoprecipitated with rabbit poly-clonal anti-pan phosphorylated protein antibody (5 μg for 200 μg protein) (Zymed Laboratories, CA) or with rabbit polyclonal anti-Na^+,K^+-ATPase α3-subunit antibody (5 μg for 200 μg protein) (Upstate Biotechnology, Lake Placid, NY).

Western blotting

Proteins were separated by electrophoresis on 6% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 3% BSA in Tris-buffered saline (TBS) containing 150 mM NaCl, 50 mM Tris, and 0.2% Tween 20 (pH=7.5) for 1 hour at room temperature. After three washes in TBS-Tween, the membranes were incubated overnight at 4°C with rabbit poly-clonal Na^+,K^+-ATPase α3-subunit antibody (1 μg/ml) (Upstate Biotechnology, Lake placid, NY) in TBS-Tween containing 1% BSA. The membranes were then washed three times and incubated for 1 hour at room temperature in TBS-Tween containing 1% BSA and anti-rabbit alkaline phosphatase-conjunated second antibody (Promega, WI) at dilution of 1:5000 (v/v). Protein bands were quantified under conditions of linearity by integration of the density of the total area of each band using the MetaMorph software (Universal Imaging, West Chester, PA). Protein A band was used as intra-control. Results were expressed as percentage ± s.e.m. of the intra-control optical density.

Chemicals

The caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-FMK) was purchased from Enzyme Systems Products (Dublin, CA), MK-801 was purchased from RBI (Natick, MA), rhodamine 123 and dihydroethidium were from Molecular Probes (Eugene, OR). Other chemicals including ouabain, strophanthidin, pyruvic acid, succinate acid, C_2-ceramide, and oxaloacetate were purchased from Sigma.

Statistics analysis

Student's two-tailed *t*-test was used for comparison of two experimental groups; multiple comparisons were done using one-way ANOVA test followed by Tukey test for multiple pairwise tests. Changes were identified as significant if *P* value was less than 0.05. Mean values were reported together with the standard error of mean (s.e.m.). The statistical analysis was performed using SigmaPlot or the statistical software SigmaStat (SPSS, Chicago, IL).

Results

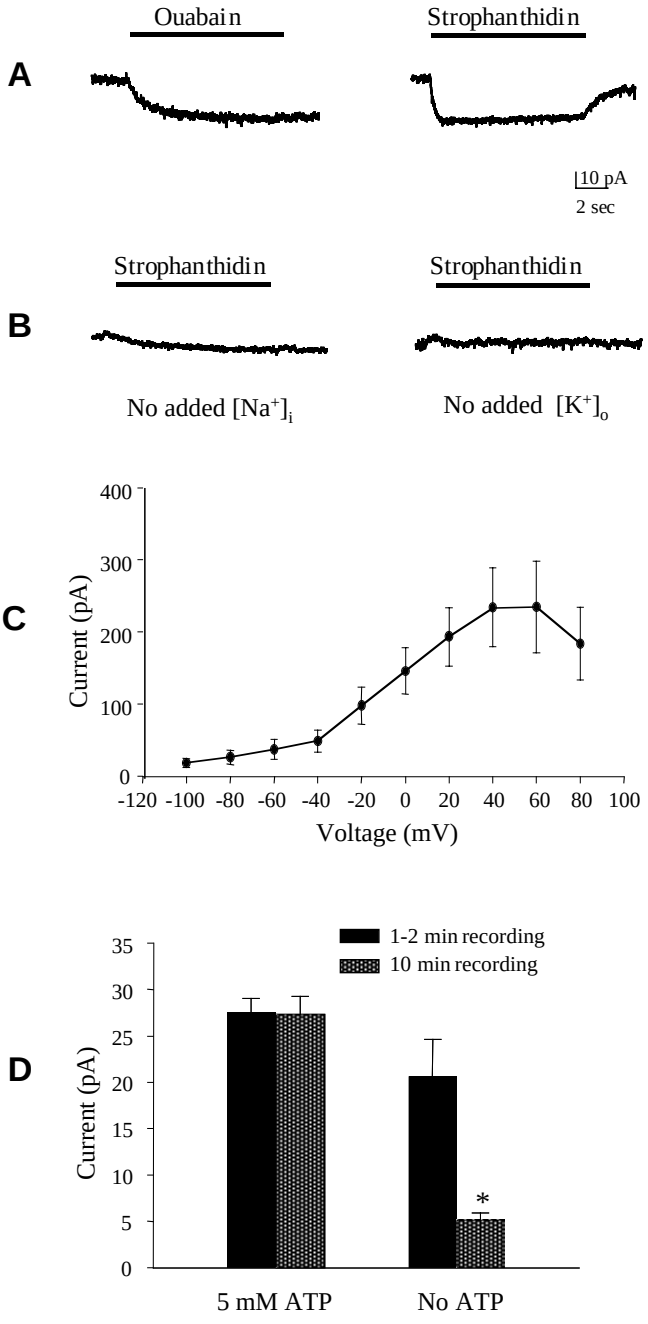
Inhibition of Na^+, K^+-pump currents by apoptotic insults

The Na^+, K^+-pump maintains its tonic activity in the presence of normal concentrations of intracellular Na^+ and extracellular K^+. This activity was revealed by an inward current I_{pump} upon application of the specific pump inhibitor ouabain (1.0 mM) or strophanthidin (500 μM) at the holding potential of –60 mV (Fig. 1A). The effect of strophanthidin was reversible and concentration-dependent with an IC_{50} of 76 μM. At 500 μM, strophanthidin triggered an I_{pump} of 29.37±1.70 pA (n=56 cells); the current density was 0.39±0.03 pA/pF, which is comparable to that reported in other neurons, cardiac myocytes, and epithelial cells (Gao et al., 1995; Senatorov et al., 1997; Gao et al., 2000; Hansen et al., 2000). The pump current had an estimated reversal potential of –133 mV, consistent with the voltage-dependent nature of the pump; the reversal potential was highly sensitive to changes in extracellular K^+, intracellular Na^+, and intracellular ATP levels (Fig. 1B-D).

In neocortical cultures, a typical apoptotic insult such as serum deprivation usually commits cells to die after about 10 hours; widespread cell death can be detected after 24-48 hours. To delineate activity changes of the Na^+, K^+-pump in cells undergoing the early stage of apoptosis, we examined I_{pump} in viable cells challenged by the classic apoptotic insult serum deprivation. I_{pump} progressively declined a few hours after serum withdrawal; the current density of I_{pump} was only 26±14% of controls after 9-hour serum deprivation (P<0.05, n=16) (Fig. 2A). Staurosporine is another widely used apoptotic insult and a pan inhibitor of protein kinases. When tested by acute application, I_{pump} was not altered by a 30-minute exposure to 0.1 μM staurosporine (Fig. 2B). Exposure to staurosporine (0.1 μM) for several hours, however, gradually downregulated I_{pump}. After 5 and 12 hours in staurosporine, the current density of I_{pump} was 42±5% and 24±3% of controls,

respectively (P<0.05 for both tests, n=8) (Fig. 2B,D). C_2-ceramide is a synthetic cell-permeable apoptotic signal mimicking the effect of endogenous ceramide; in the presence of C_2-ceramide (25 μM) I_{pump} was time-dependently inhibited too; the current density was 63±9% of controls after 20-hour exposure to C_2-ceramide (P<0.05, n=14) (Fig. 2C).

The above strophanthidin-sensitive inward current represents the background activity of the Na^+, K^+-pump; to delineate whether apoptosis might not only affect the tonic activity of the Na^+, K^+-pump but also impair its activation we measured the Na^+, K^+-pump outward current stimulated by raising extracellular K^+. Cortical neurons were first exposed to zero external K^+ followed by a sudden jump to 4 mM K^+; the raising in extracellular K^+ generated an outward current as a result of activating the Na^+, K^+-pump (Fig. 2E). This Na^+, K^+-pump current decreased in the course of apoptosis, corroborating that the ability of the Na^+, K^+-pump to transport Na^+ and K^+ across the membrane was reduced in cells undergoing apoptosis (Fig. 2E). These cells with depressed Na^+, K^+-pump activity were not noticeably deteriorated or dead. Although depolarized, they still had negative resting membrane potentials. For example, in 3 mM external K^+ the resting membrane potential was –62±1 mV (n=22) and –46±3 mV (n=13) before and 9-10 hours after staurosporine, respectively (P<0.05).



Preservation of the Na^+, K^+-ATPase activity by pyruvate and succinate in apoptotic cells

To understand whether a cellular energy failure might be responsible for the diminished I_{pump} during the apoptotic process, we studied the effect of intracellular ATP dialysis on I_{pump} in apoptotic cells. Supporting the ATP-dependence of the pump activity, intracellular dialysis of 10 mM ATP in the whole-cell configuration increased I_{pump} in apoptotic cells although the recovery was not complete (Fig. 2D). In the following experiments, pyruvate or succinate were tested during an apoptotic treatment. I_{pump} was recorded using the perforated patch configuration, so as not to interfere with the intracellular ATP level. Pyruvate is the product of glycolysis and is transported into mitochondria for the citric acid cycle. When 5 mM pyruvate was included in the serum-free medium or added together with 0.1 μM staurosporine, I_{pump} remained at normal levels even 12 hours after the onset of exposures (Fig.

Fig. 1. Na^+, K^+-pump currents in cortical neurons and its ATP dependence. Identification of the membrane current associated with activities of the Na^+, K^+-ATPase. (A) Whole-cell recordings of the Na^+, K^+-pump current, I_{pump}, in cultured cortical neurons. At the holding potential of –60 mV, an inward current was triggered by an acute application of the Na^+, K^+-pump inhibitor ouabain (1 mM) or strophanthidin (500 μM), corresponding to the block of a tonic activity of the Na^+, K^+-pump. The effect of strophanthidin was reversible, an outward current appeared owing to reactivation of the Na^+, K^+-ATPase. (B) The membrane current associated with the Na^+, K^+-pump was highly dependent on the presence of intracellular Na^+ ($[Na^+]_{i)}$ and extracellular K^+ ($[K^+]_o$). When Na^+ was removed from the pipette internal solution or K^+ was removed from the extracellular solution, little membrane current was observed upon application of 500 μM strophanthidin. (C) The I-V relationship of Na^+, K^+-pump current obtained by subtracting I-V curves constructed by membrane current responses to various voltage steps from the –100 mV holding potential in the presence and absence of 1 mM ouabain. The reversal potential of the pump current estimated from the I-V curve is about –133 mV. The relationship showed an outward rectification at depolarized potentials and a decrease of the current at very positive potentials, both are consistent with previous reports of the pump current (De Weer et al., 1988). Voltage pulses were applied every 4 seconds with increments of 20 mV starting at –100 mV. n=10. (D) The ATP-dependence of I_{pump} was revealed by comparison of the currents recorded with microelectrodes containing either 5 mM ATP or ATP-free internal solution. In the presence of ATP, there was no time-dependent decline of I_{pump}; without ATP in the internal solution, I_{pump} was greatly diminished 10 minutes after establishing the whole-cell configuration. n=10 and 8 for ATP and ATP-free groups, respectively. *P<0.05 compared with the current at 1-2 minutes.

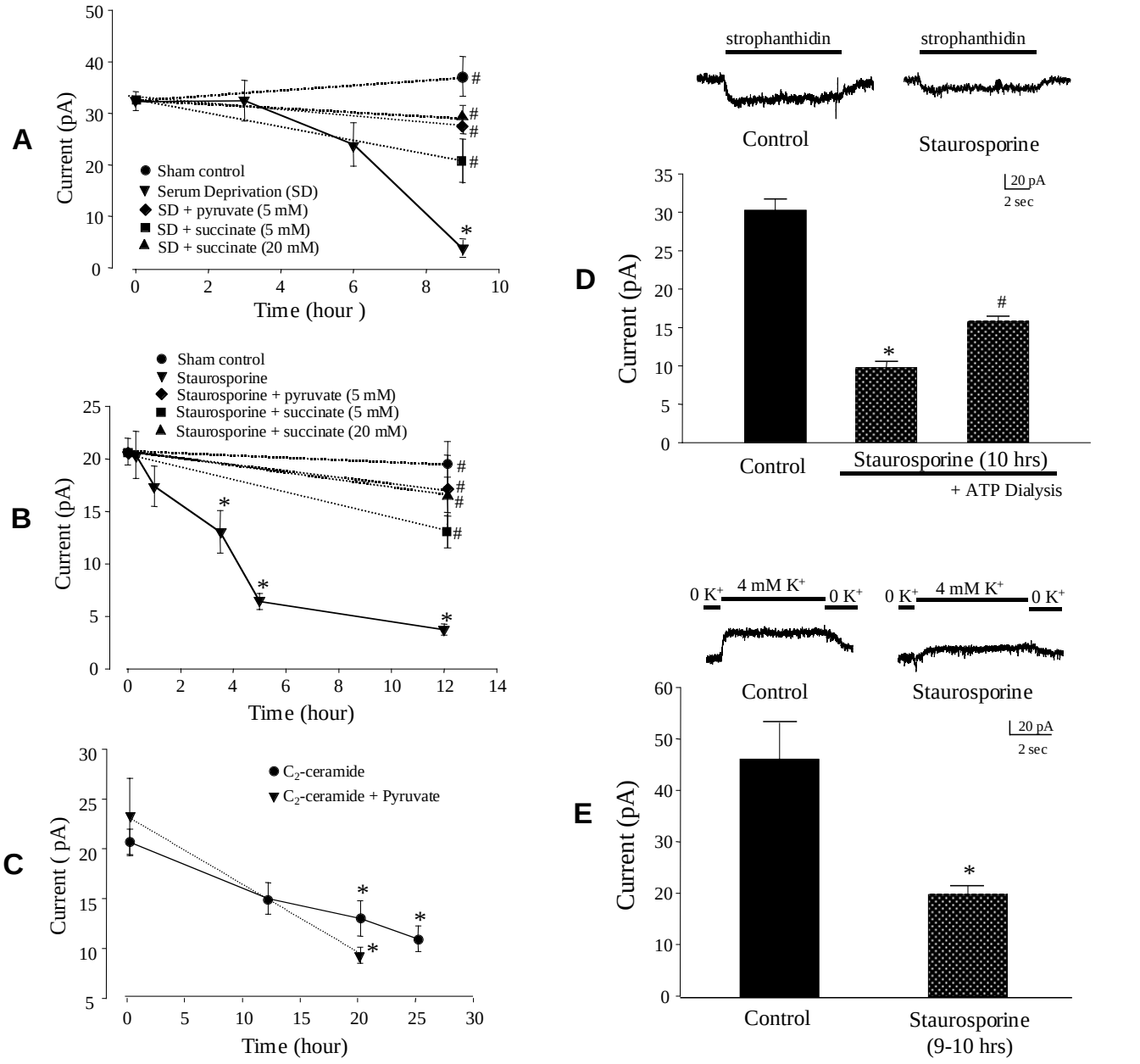


Fig. 2. Suppression of the Na^+, K^+-pump activity by apoptotic insults. I_{pump} was recorded at –60 mV in cortical neurons during exposure to the apoptotic insult serum deprivation or staurosporine, which induced neuronal apoptosis in 24-48 hours. (A) I_{pump} was gradually suppressed after 5 hours in a serum-free medium; a 9-hour serum deprivation profoundly blocked the Na^+, K^+-pump (n=16). Pyruvate (5 mM) and succinate (5 and 20 mM) preserved the pump activity during serum deprivation. Notably, succinate showed less effect than pyruvate at 5 mM concentration; 20 mM succinate protected the pump current similarly as 5 mM pyruvate did. n=6-21 cells. (B) Incubation with 0.1 μM staurosporine for 30 minutes did not show any effect on the current; prolonged incubation, however, resulted in progressive depression of I_{pump}. By 12 hours, there was little strophanthidin-sensitive current detected. Pyruvate (5 mM) or succinate (20 mM), co-applied with staurosporine, was able to retain the pump current at around control levels even after 12-hour exposure. Succinate at 5 mM also attenuated the suppression of I_{pump}. n=7-17. (C) C_2-ceramide (25 μM) gradually suppressed I_{pump}; co-applied pyruvate (5 mM), however, could not prevent the Na^+, K^+-pump failure (n=8). Note that the effect of C_2-ceramide on the pump activity was slower and milder than that of serum deprivation and staurosporine, in agreement with less cell death induced by C_2-ceramide (see Fig. 7D). (D) Top: Representative inward Na^+, K^+-pump current induced by 500 μM strophanthidin in control cells and cells exposed to staurosporine (0.1 μM, 10 hours). Bar graph: control Na^+, K^+-pump current and the current in cells undergoing apoptosis (0.1 μM staurosporine, 9-10 hours); the Na^+, K^+-pump current in staurosporine-treated cells was significantly higher after 10-minute dialysis with an internal solution containing 10 mM ATP. n=5 for control group and the staurosporine group without ATP dialysis; n=6 for the staurosporine group with ATP dialysis. (E) Top: Representative outward current generated by activation of the Na^+, K^+-pump in control cells and cells exposed to 0.1 μM staurosporine (10 hours). Whole-cell recordings were established in regular external solution of 3 mM K^+; cells were exposed to a K^+-free extracellular solution for 10 seconds followed by 5 seconds exposure to 4 mM K^+. The outward current triggered by external K^+ was fully blocked by strophanthidin (data not shown), consistent with a pump current. Bar graph: The apoptotic insult staurosporine (0.1 μM, 9-10 hours) markedly suppressed the pump current (n=8). *P<0.01 compare with controls at time zero; #P<0.01 compare with apoptotic insult alone at same time point.

2A,B). Succinate, the middle product of the citric acid cycle, also prevented the I_{pump} depression induced by serum-free medium or staurosporine (Fig. 2A,B). Pyruvate and succinate, nevertheless, could not preserve the Na^+, K^+-pump current depressed by C_2-ceramide (Fig. 2C), probably as a result of a ceramide-induced direct damage to mitochondrial respiratory chain (Gudz et al., 1997).

Apoptosis, Na^+, K^+-pump activity and ATP metabolism

The experiments reported above suggested that intracellular ATP level is relevant but not fully responsible for the pump failure. To further delineate the relationship between ATP production and activity of the Na^+, K^+-pump during apoptotic process, we measured ATP levels in control cells and cells treated with the serum-free medium or staurosporine for 9-10 hours. In agreement with the reduced Na^+, K^+-ATPase activity, diminished cellular ATP levels were found in both apoptotic settings (Fig. 3). By contrast, pyruvate (5 mM) and succinate (20 mM) increased ATP levels during serum deprivation; ATP levels were also markedly increased by succinate co-applied with staurosporine (Fig. 3). Pyruvate, however, failed to prevent staurosporine-induced ATP depletion (Fig. 3C). To understand why pyruvate improved I_{pump} without increasing the ATP level, we examined the effect of pyruvate on cellular ADP/ATP ratio, which is an indicator for ATP hydrolysis or ATP use. The ADP/ATP ratio decreased either by serum deprivation or staurosporine, consistent with a diminished ATP metabolism and dysfunction of the Na^+, K^+-ATPase (Figs 2, 3). The ADP/ATP ratio was raised by co-applied pyruvate or succinate (Fig. 3B,D), implying an enhanced ATP consumption that might be favorable for preservation of the Na^+, K^+-pump activity.

Phosphorylation state of the Na^+, K^+-pump in cells undergoing apoptosis

Phosphorylation is a primary regulatory mechanism for activities of the Na^+, K^+-pump (Borghini et al., 1994; Feraille et al., 1997; Feschenko et al., 1997). To understand whether the reduced ATP level might affect the phosphorylation state of the Na^+, K^+-pump, its phosphorylation status was assessed using the antibody (anti-pan) recognizing serine, threonine, and tyrosine phosphorylated proteins, and the antibody against the pump α3 subunit that is commonly and abundantly expressed in brain neurons (Juhaszova and Blaustein, 1997; Habiba et al., 2000). After a 9-hour incubation in serum-free medium or 0.1 μM staurosporine, the α3 subunit phosphorylation level was reduced (Fig. 4). Phosphorylation of the α1 subunit, regarded as a 'housekeeping' subunit in different cells (Juhaszova and Blaustein, 1997; Crambert et al., 2000), was not tested.

Reactive oxygen species (ROS) and Na^+, K^+-pump activity

Since above results implied a more complex mechanism underlying the apoptotic pump failure, we hypothesized that, in addition to ATP level and ADP/ATP ratio, additional factors especially the ROS production might contribute to the pump failure. In neurons treated by serum deprivation or

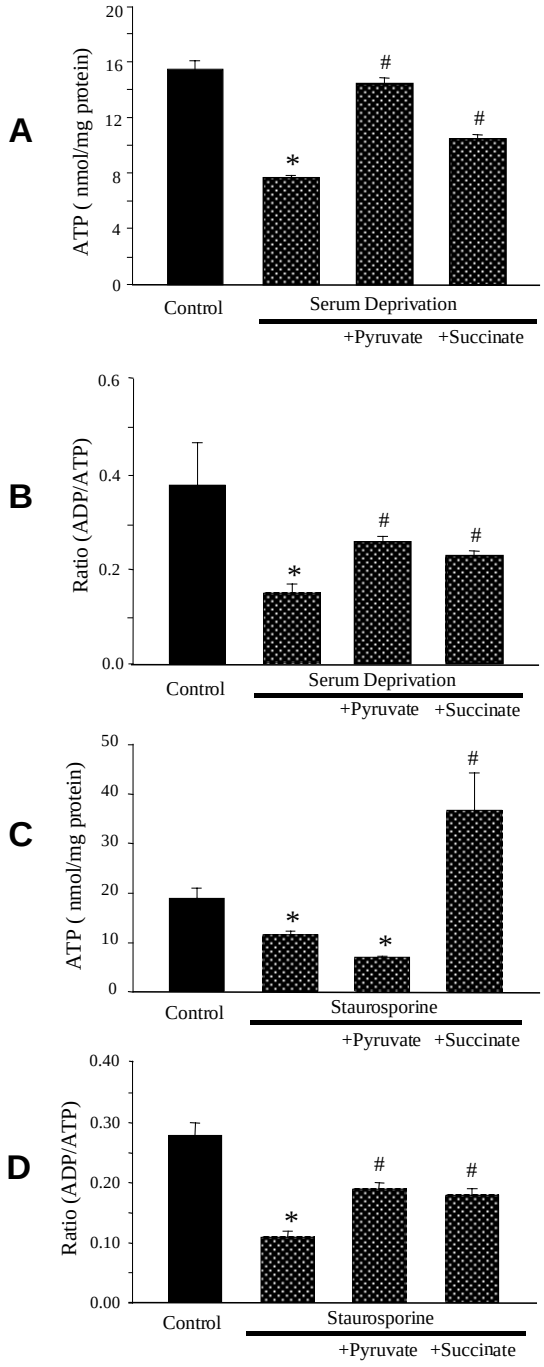


Fig. 3. ATP synthesis and ADP/ATP ratio during apoptotic process. ATP production and ADP/ATP ratio were measured in sham control and treated cortical cultures after 9–10-hour exposures. (A) Cellular ATP level was markedly reduced by serum deprivation; pyruvate (5 mM) and succinate (20 mM) each enhanced the ATP production. n=6 measurements. (B) ADP/ATP ratio was decreased during serum deprivation, and was improved by co-applied pyruvate or succinate (n=6). (C) ATP level was reduced by 0.1 μM staurosporine; in contrast to the effect in A, pyruvate (5 mM) did not increase ATP levels: the ATP level was even lower in the presence of pyruvate. Nevertheless, succinate (20 mM) markedly enhanced the ATP level. n=8. (D) Staurosporine decreased the ADP/ATP ratio, which was improved by both pyruvate and succinate. n=8. *P<0.05 compared with controls; #P<0.05 compared with serum deprivation or staurosporine alone.

staurosporine-enhanced production of superoxide radical anion (O_2^-) was indeed detected by the fluorescent dye dihydroethidium (DHE) 9-10 hours after the onset of exposure (Fig. 5). Superoxide production was much higher upon exposure to staurosporine than to serum deprivation, correlating with a more severe block of I_{pump} by staurosporine at this time point (Fig. 2). Pyruvate (5 mM) completely prevented the superoxide production induced either by staurosporine or by serum deprivation (Fig. 5B,C). Succinate (20 mM) also attenuated the superoxide production induced by apoptotic insults (Fig. 5B,C).

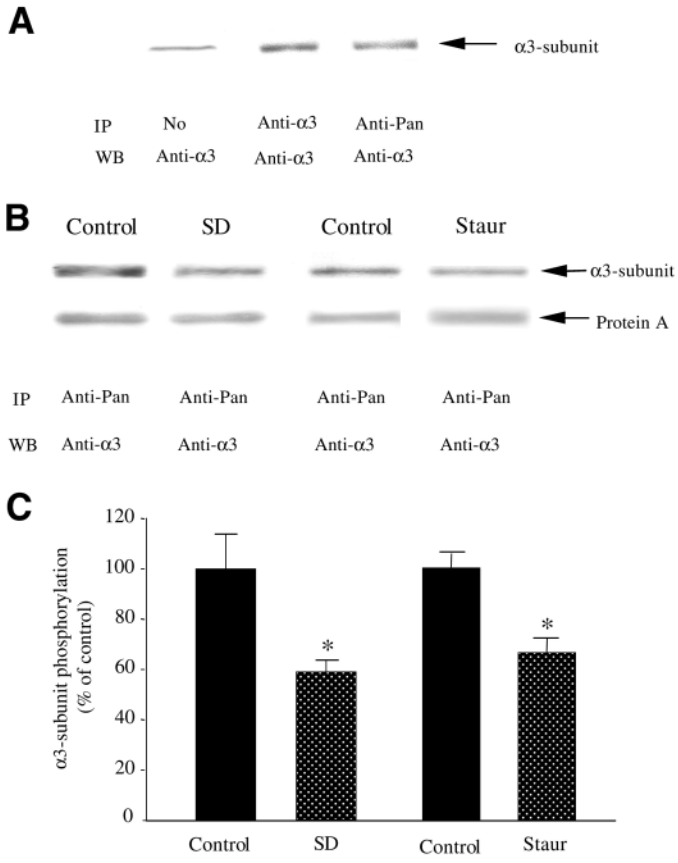


Fig. 4. Phosphorylation state of the Na^+, K^+-pump during apoptotic process. The protein phosphorylation level of the Na^+, K^+-pump was assessed using the anti-phosphorylated protein antibody. (A) In the left column, cell lysates were directly subjected to SDS-PAGE; anti-Na^+, K^+-ATPase α3 subunit antibody (anti-α3) was used as primary antibody. It demonstrated the specific binding of the anti-α3 antibody to the protein. In the last two columns, an anti-α3 or anti-phosphorylated protein antibody (anti-pan) was used as antibody in immunoprecipitation. Western blotting showed a clear α3 subunit protein band. (B) Cortical neurons were treated for 9 hours in control medium, serum-free medium (SD) or in 0.1 μM staurosporine (Staur). Anti-pan antibody was used to precipitate phosphorylated α3 subunit. Antibodies were linked to saturated amount of protein A-sepharose beads. Using anti-α3 as primary antibody, western-blotting showed reduced phosphorylation level of the α3 subunit after serum deprivation or exposure to staurosporine. (C) Protein phosphorylation levels of the α3 subunit was measured by band relative gray intensity and corrected by corresponding protein A band intensity. Both serum deprivation (9 hours) and staurosporine exposure (9 hours) reduced the phosphorylation. As a control, more matured neuronal cultures of more than 15 days in vitro were subjected to serum deprivation (9 hours) and showed no decreased phosphorylation state (data not shown), consistent with diminished apoptosis in these cells. n=8 independent experiments for serum deprivation and n=3 for staurosporine. *P<0.05 compared with controls.

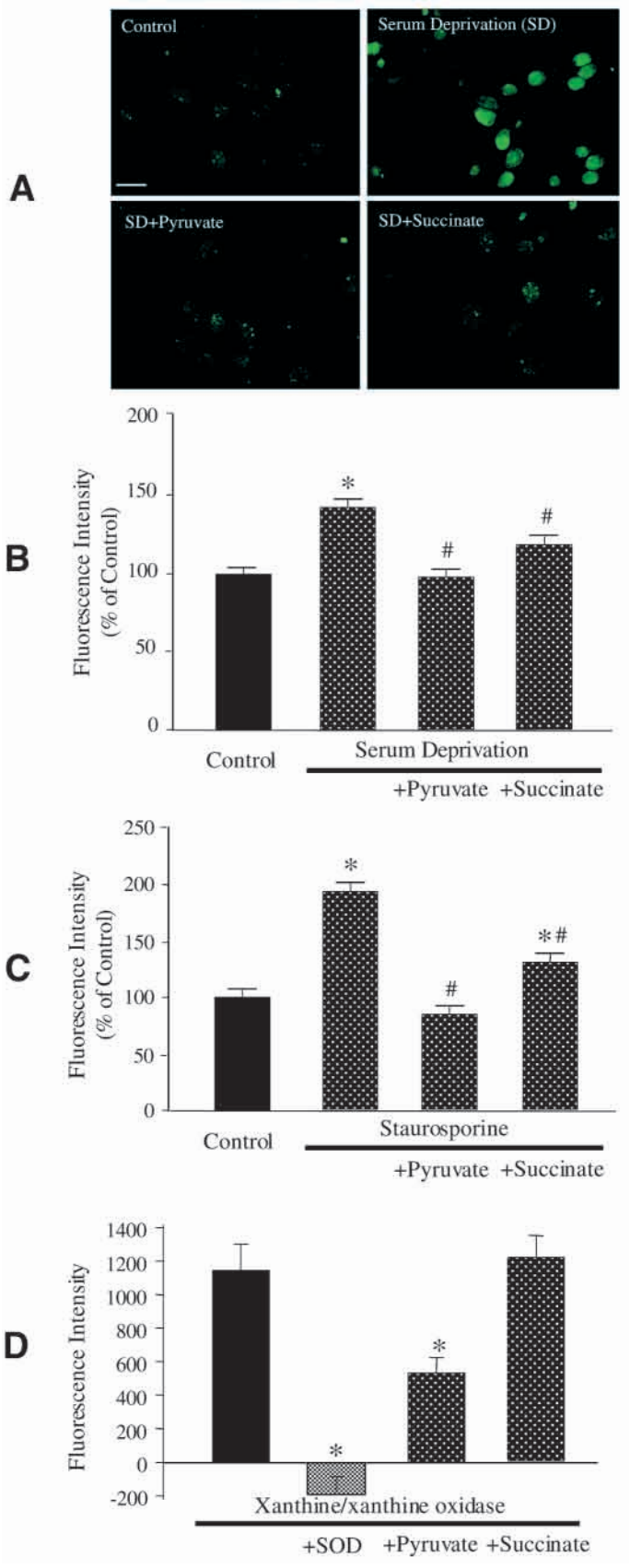
To understand whether the ROS production was mitochondria associated, we used the fluorescent indicator, R123, to determine whether conditions affecting the pump activity involved loss of mitochondrial membrane potential. During staurosporine exposure, the mitochondrial indicator uptake was initially (at 5 hours) over 30% larger (n=66 cells, P<0.05) but later (9 hour) 30% smaller (n=84, P<0.05) than that in untreated controls (n=60), indicating an early mitochondrial membrane hyperpolarization followed by depolarization after prolonged incubation with staurosporine. Serum deprivation in pure neuronal cultures reduced the ability of mitochondria to accumulate R123 by 30% at 5 hours (n=41, P<0.05) but not at 9 hours (n=34), suggesting an initial drop and subsequent recovery of mitochondrial membrane potential. At each time point, the cells maintained some mitochondrial membrane potential as they accumulated R123, as demonstrated by indicator release upon full mitochondrial membrane depolarization with 10 μM FCCP (data not shown).

To verify the ROS effect on the Na^+, K^+-pump activity and further delineate the antagonizing mechanism of pyruvate and succinate, oxidative stress was generated by exogenous hydrogen peroxide (H_2O_2). H_2O_2 (0.25 mM, 10 minutes) blocked more than 50% of I_{pump}; the marked acute effect of H_2O_2 was attenuated by the ROS scavenger catalase (250 unit/ml) and by pyruvate (5 mM), applied 5 minutes before and during H_2O_2 exposure (Fig. 6A). The acute H_2O_2 suppression of I_{pump}, however, was not prevented by succinate even at the high concentration of 20 mM (Fig. 6A). We then examined the effect of endogenous O_2^- induced by menadione or 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) on I_{pump}. Menadione (20 μM, 10 minutes) blocked about 50% of the pump current; the menadione effect was prevented by co-applied SOD (25 unit/ml) (Fig. 6B). Similarly to the experiment with H_2O_2, the effect of menadione on I_{pump} was fully prevented by 5 mM pyruvate but not by 20 mM succinate (Fig. 6B). In chronic experiments, exposure to 8 μM menadione for 9-10 hours suppressed I_{pump}, the inhibitory effect was prevented by pyruvate (Fig. 6C). Interestingly, the menadione-induced chronic suppression of I_{pump} was also attenuated by succinate (Fig. 6C), suggesting that succinate might diminish an oxidant stress via slow or indirect means. As shown in Fig. 5, succinate indeed reduced ROS production during the apoptotic process of several hours. DMNQ is an intracellular superoxide- and H_2O_2-forming compound metabolized in the mitochondria; a 20-minute exposure to 5 or 20 μM DMNQ caused dose-dependent block of I_{pump} (Fig. 6D). Pyruvate (5 mM) and succinate (20 mM) largely prevented the acute DMNQ effect on the pump activity (Fig. 6D). In chronic experiments of 15-20-hour incubations, the DMNQ inhibitory effect on I_{pump} was again attenuated by co-applied pyruvate or succinate (Fig. 6D). These results confirmed an inhibitory effect of endogenous ROS on the Na^+, K^+-pump and its antagonism by pyruvate and succinate.

Protective effects of pyruvate and succinate against neuronal apoptosis

We next examined the hypothesis that a failure of the Na^+,

K^+-pump was not only a consequence of apoptotic pathophysiology, but also affected the fate of neurons subjected to an apoptotic offense. Consistent with their effects of retaining I_{pump} in cells undergoing apoptosis, pyruvate or succinate added into serum-free medium attenuated apoptotic cell death 24-30 hours after the onset of the exposure (Fig. 7). The neuroprotective effect was concentration dependent; the effective concentrations were consistent with their effects on preserving the Na^+, K^+-pump activity. The protective effect persisted even when application of pyruvate was delayed for up to 4 hours after serum withdrawal (Fig. 7B), consistent with the observation that I_{pump} was not noticeably suppressed during the first 4 hours of serum deprivation (Fig. 2A). α-Cyano-4-hydroxycinnamate (4-CIN), an inhibitor of the monocarboxylic acid transporter and mitochondrial pyruvate carrier, blocked the neuroprotective ability of pyruvate, suggesting the involvement of mitochondria in the protection (Fig. 7B). In support of the notion that succinate may antagonize apoptotic damage via its downstream pathway, its metabolite oxaloacetate (5 mM) exhibited similar anti-apoptotic effect (Fig. 7C). As pyruvate failed to prevent the Na^+, K^+-pump failure induced by C_2-ceramide, it could not prevent C_2-ceramide-induced apoptosis (Fig. 7D).



Discussion

The identification of I_{pump} in cortical neurons provides the opportunity of directly monitoring the Na^+, K^+-pump activity in these cells. We demonstrate for the first time that in neurons undergoing various models of apoptosis the I_{pump} is progressively and markedly suppressed. This time-dependent dysfunction of the Na^+, K^+-pump is not just a result from ATP shortage alone or ROS production alone, but probably caused by concurrent ATP depletion and oxidant stress developed during apoptotic process. Furthermore, we show that the failure of the Na^+, K^+-pump may not merely be a consequence of apoptotic insults; instead it may play a critical role in cell survival following apoptotic insults such as those related to trophic factor deficiency. We demonstrate a powerful inhibitory effect of oxidant stress on the Na^+, K^+-pump activity, and show that pyruvate and succinate may act as potent antioxidant reagents via different mechanisms. The protective effects of

Fig. 5. Production and regulation of ROS during apoptosis. Superoxide (O^-_2) production was measured by the fluorescent dye DHE. The association of increased fluorescence intensity to ROS production was confirmed with the positive control xanthine/xanthine oxidase (see D). (A) Large increases in fluorescence intensity were detected after 9–10-hour treatment in the serum-free medium. This chronic increase in ROS was effectively prevented in the presence of 5 mM pyruvate or 20 mM succinate. Bar, 20 μm. (B) Superoxide production was increased after 9–10-hour serum deprivation. Pyruvate (5 mM) prevented the O_2^- production; succinate (20 mM) showed a less but significant inhibitory effect on superoxide stress. (C) Staurosporine (0.1 μM, 9-10 hours) induced a robust increase in O_2^- production; the increase was prevented or attenuated by co-applied 5 mM pyruvate and 20 mM succinate, respectively. (D) Measured in cell-free culture solution, the antioxidant scavenger property of pyruvate was confirmed by a marked reduction in DHE fluorescence intensity induced by xanthine/xanthine oxidase. Succinate was not effective. n=211-330 cells for serum deprivation experiments; n=92-148 cells for staurosporine experiments; n=128 wells in D. *P<0.05 compared with controls; #P<0.05 compared with serum deprivation or staurosporine alone.

pyruvate observed in this study are consistent with previous observations that pyruvate normalized ATP and NAD^+ levels in zinc-exposed neurons and protected them from zinc toxicity (Sheline et al., 2000) and that pyruvate induced a robust neuroprotection against transient forebrain ischemia in rats (Lee et al., 2001).

It is well established that blocking the Na^+, K^+-pump diminishes intracellular K^+ concentration (Archibald and White, 1974; Lijnen et al., 1986; Xiao et al., 2002). This event has been linked to apoptotic cell shrinkage in Jurkat cells (Nobel et al., 2000) and cortical neurons (Xiao et al., 2002). In our previous studies, we showed that serum deprivation, staurosporine, and C_2-ceramide augmented the outward delayed rectifier K^+ current after 3–6-hour exposures (Yu et al., 1997; Yu et al., 1999b). Based on previous and present data, we now propose that an excessive K^+ efflux mediated by upregulated K^+ channels and a belated Na^+, K^+-pump failure are the joined forces contributing to intracellular K^+ depletion. Considering the key contribution of the Na^+, K^+-pump in K^+ uptake against the large K^+ gradient across the plasma membrane, the Na^+, K^+-pump failure may play an even more decisive role in the disruption of ion homeostasis and cell death.

The Na^+, K^+-ATPase can be stimulated by external K^+ in a voltage-dependent manner (Vasilets et al., 1991; Omay and Schwarz, 1992). It is expected that following an excessive K^+ efflux and accumulation of extracellular K^+, the Na^+, K^+-pump would be stimulated at least during some early period of apoptosis. In the present study, however, no such augmented pump activity (i.e. enhanced I_{pump}) was observed. This is perhaps mainly due to the huge dilution of escaped K^+ by extracellular solutions in the recording dish. Accumulations of extracellular K^+ and intracellular Na^+ do occur in the brain under pathological conditions such as cerebral ischemia (Siesjo, 1992) and hypoxia (Haddad, 1997); it is thus predicted that an enhanced Na^+, K^+-pump activity could take place under such conditions in vivo. Although a high activity of the Na^+, K^+-pump with sufficient ATP supply can be beneficial, in the event of mitochondrial damage and lack of continuous ATP production, such a transient increase of pump activity may magnify the energy crisis and exacerbate the cell injury.

The intracellular ATP level may be a factor influencing the fate of cells in the direction of either apoptosis or necrosis. Depletion of intracellular ATP prevents Fas/Apo-1-stimulated apoptosis and induces necrotic death in Jurkat cells; replenishment of ATP restores the ability of these cells to undergo apoptosis (Eguchi et al., 1997; Leist et al., 1997). This is consistent with the finding that cytochrome c release and perhaps caspase activation are ATP-dependent (Li et al., 1997; Volbracht et al., 1999). Therefore, during the early phase of apoptosis depletion of ATP can preclude caspase activation and consequently switch execution of cells towards necrosis

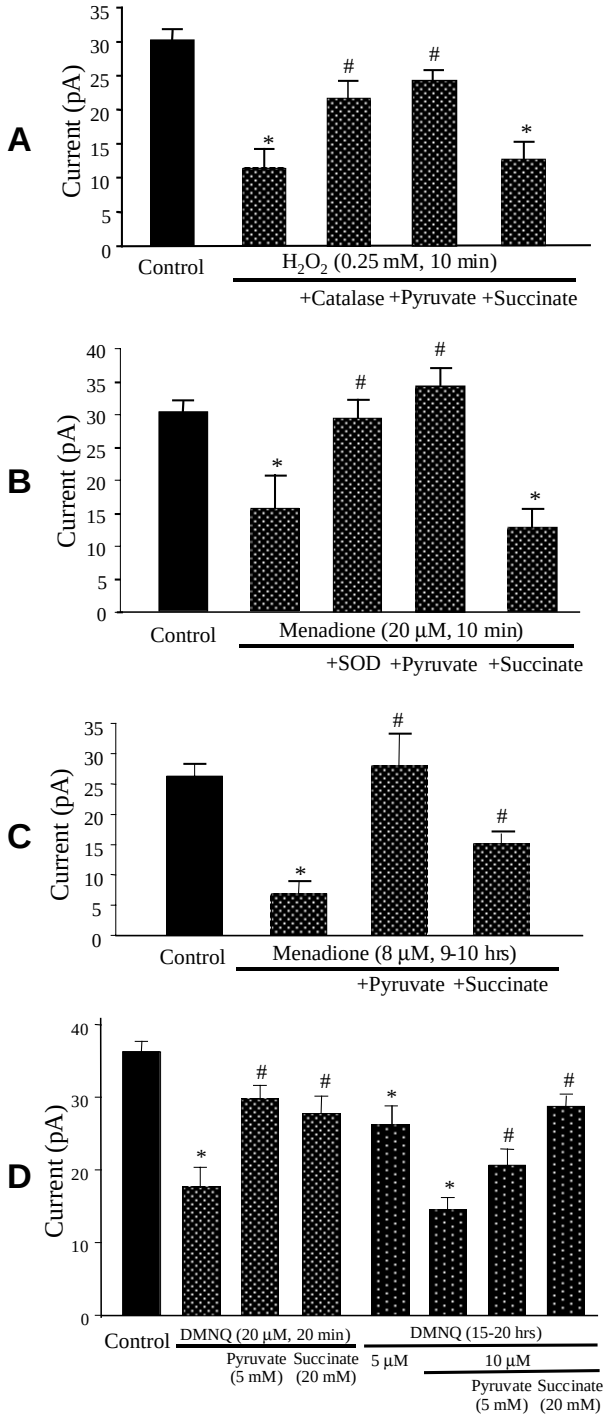


Fig. 6. Inhibitory effects of ROS on I_{pump}. I_{pump} was recorded in cortical neurons at –60 mV before and after an oxidant insult. (A) Hydrogen peroxide (0.25 mM) showed a marked inhibitory effect on I_{pump} after a few minutes application. This suppression of the Na^+, K^+-pump activity was markedly prevented in the presence of catalase (250 U/ml) or pyruvate (5 mM) added 5 minutes before and during the exposure. Succinate, on the other hand, showed no such effect even at high concentration of 20 mM (n=5-6 for each test). (B) Acute application (10 minutes) of 20 μM menadione, a stimulator of endogenous production of O_2^-, suppressed I_{pump}; this inhibition was prevented by 25 U/ml SOD or 5 mM pyruvate but not by 20 mM succinate ($n \geq 6$ in each test). (C) Chronic exposure to 8 μM menadione for 9-10 hours also diminished I_{pump}; both pyruvate (5 mM) and succinate (20 mM) protected the Na^+, K^+-pump activity from damage by the endogenous ROS (n=5-7). (D) Acute (20 minutes) and chronic (15-20 hours) applications of DMNQ (5-20 μM) inhibited I_{pump}, pyruvate (5 mM) or succinate (20 mM) antagonized the inhibition in both conditions (n=5-8).*P<0.05 compared with controls; #P<0.05 compared with hydrogen peroxide, menadione, or DMNQ alone.

(Nicotera and Lipton, 1999). By contrast, mitochondrial damage and energy insufficiency are shown in apoptotic cells (Fiskum, 2000). A reduction in ATP production by mitochondria (caused by hypoxia or mutations in genes encoding mitochondrial proteins of the electron transport chain) can induce apoptosis in neurons or increase their sensitivity to apoptosis (Gorman et al., 2000). It is specifically hypothesized that the cellular ATP level is an important determinant for apoptosis; a cell stays alive as long as a certain ATP level is maintained (Richter et al., 1996). When ATP falls below this level, apoptosis ensues provided adequate ATP is still available for energy-requiring apoptotic processes. However, the exact machinery that is responsible for the ATP-dependent control of apoptosis was ambiguous. Recent studies suggest that binding of ATP to the ATP-binding domain in an ATPase can protect it from ROS injury (Wei and Richardson, 2001). The present investigation provides novel evidence that dysfunction of the Na^+, K^+-ATPase is probably one of the main mechanisms mediating the ATP deficiency-induced apoptosis or exacerbating other insult-induced apoptotic injury. Thus reversing the consequences of ATP depletion in neurons prevents apoptosis. This idea is further supported by results demonstrating that retaining I_{pump} during serum-deprivation by stimulating endogenous ATP synthesis attenuated apoptosis and that pyruvate could not prevent Na^+, K^+-pump failure nor apoptosis induced by C_2-ceramide.

Mitochondrial dysfunction has been strongly implicated in mediating both apoptosis and necrosis (Kroemer et al., 1998). We observed the mitochondrial potential changes in apoptotic cells although the change pattern was different following different insults. Interestingly, mitochondria were hyperpolarized after 5-hour staurosporine exposure when the Na^+, K^+-pump current was already substantially blocked (Fig. 2B); and, despite the pump failure, only transient

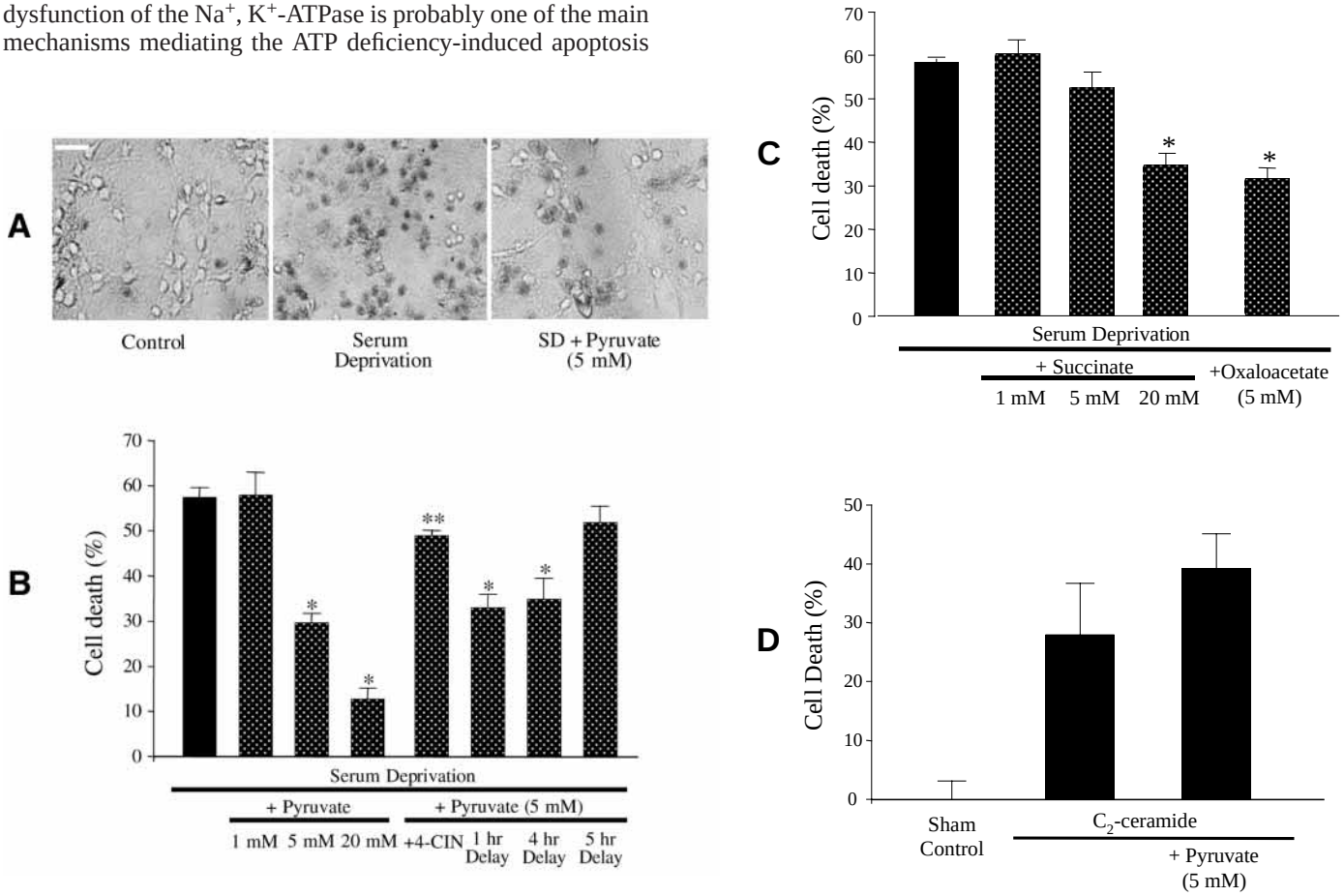


Fig. 7. Neuroprotection of pyruvate and succinate against apoptosis. Serum withdrawal from the culture medium resulted in 50-60% cell death in 30 hours, measured by LDH release and Trypan Blue extrusion. (A) Bright phase photos of Trypan-Blue-treated pure-neuronal cultures before and after serum deprivation. Normal cells do not show positive staining with Trypan Blue (dark color); after a 30-hour incubation in the serum-free medium many Trypan-Blue-positive cells represent the injured or dead cells lack of ability to extrude the dye from the intracellular space. Addition of 5 mM pyruvate in the serum-free medium attenuated Trypan Blue staining and cell death. Bar, 50 μm. (B) Pyruvate attenuated serum deprivation-induced cell death in a concentration-dependent manner; the neuroprotection was mostly reversed by co-applied 4-CIN (2 mM). Neuroprotective effects were achieved even when pyruvate was given up to 4 hours after the onset of serum deprivation. (C) Succinate at 5 mM showed little neuroprotective effect, increasing its concentration to 20 mM reduced serum deprivation-induced apoptosis, consistent with its effect on I_{pump} and ROS production at this concentration. The succinate downstream metabolite oxaloacetate (5 mM) also showed comparable neuroprotection against serum deprivation-induced apoptosis. (D) Co-applied pyruvate (5 mM) showed no attenuating effect on the neuronal death induced by C_2-ceramide (25 μM, 48-hour exposure). In fact, pyruvate appeared to increase the C_2-ceramide toxicity, which is consistent with a lower pump current in the presence of pyruvate and C_2-ceramide (see Fig. 2C). n=8-31 cultures. *P<0.05 compared with serum deprivation alone; #P<0.05 compared with serum deprivation plus pyruvate.

mitochondrial depolarization took place during 9-hour serum deprivation. Comparing these data, it appears that the observed pump failure does not necessarily require loss of mitochondrial membrane polarization and that the ROS production related to the pump failure may be mediated by mechanisms mostly independent of the depolarization of the mitochondrial membrane. These observations are generally in agreement with recent reports that staurosporine induced mitochondria hyperpolarization at an earlier stage of apoptosis (Poppe et al., 2001) and that mitochondrial depolarization may not be required for neuronal apoptosis (Krohn et al., 1999).

In addition to its role in ATP synthesis, H_2O_2 was shown to uncouple the Na^+, K^+-ATPase from ATP hydrolysis (Garner et al., 1983). The failure of ATP consumption and the inability to provide adequate ADP (unbalanced ATP and ADP ratio) for the adenine nucleotide transporter during oxidative stress may promote cytochrome c release and initiate apoptosis (Kantrow et al., 2000). It is conceivable that both ATP and ADP levels have to be considered so the effects of pyruvate and succinate on energy metabolism (e.g. ATP synthesis, available ATP and ADP, ATP consumption, and ADP/ATP ratio) will be evaluated. In the present investigation, both pyruvate and succinate increased the ADP/ATP ratio concurrently with improvements in Na^+, K^+-pump currents, supporting the hypothesis that an increased ATP hydrolysis along with sufficient ATP production is essential for a more balanced ADP/ATP ratio and may act as a potential anti-apoptotic mechanism.

The Na^+, K^+-pump activity is highly sensitive to oxidant stress and production of free radicals (Kourie, 1998; Shattock and Matsuura, 1993). Previously observed anti-apoptotic effects of ROS scavengers (Lieberthal et al., 1998; Pong et al., 2001) are consistent with the preserved Na^+, K^+-pump function observed in this study. Pyruvate is well-known for its direct antioxidant effect of reacting with H_2O_2 to form water and carbon dioxide (Crestanello et al., 1998; Varma et al., 1998); it may also possess a hydroxyl radical scavenging property (Dobsak et al., 1999) or stimulate NADPH-dependent peroxide scavenging systems (Cavallini et al., 1990). In our study both exogenous H_2O_2 and endogenous ROS induced by menadione, DMNQ, or apoptotic insults inhibited I_{pump}. Succinate showed little effect against the acute effect of menadione on I_{pump}, however, it did antagonize acute and chronic inhibitory effects of DMNQ. Its metabolite oxaloacetate showed a neuroprotective effect, presumably due to the oxidation of the carbonyl carbon and carboxyl groups in the structure of oxaloacetate, which reduces the production of hydrogen peroxide (Ramsay, 1949).

Information from this and previous investigations suggests that failure of the Na^+, K^+-pump can either be causative or contributory in neuronal injury, depending on whether the Na^+, K^+-pump is the original target of the insult. For example, increased endogenous ouabain in some pathological conditions can be the causal factor for disruptions of the ionic homeostasis and hybrid cell death (Budzikowski et al., 1998; Ferrandi and Manunta, 2000; Xiao et al., 2002); while a secondary destruction of the Na^+, K^+-pump may occur following energy deficiency and ROS production induced by apoptotic and other insults. Based on available knowledge and our data, it also appears conceivable that pyruvate and succinate preserve the pump activity through multiple actions as the primary mechanism of promoting cell survival. We propose that the Na^+, K^+-ATPase can be a therapeutic target for neuroprotection in apoptosis-related diseases.

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