JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.045013

JPET/2002/45013

Block of Na^+, K^+-ATPase and Induction of Hybrid Death by 4-Aminopyridine in Cultured Cortical Neurons

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JPET/2002/45013

Running title: 4-AP induced Na^+, K^+-ATPase failure and neuronal death

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Number of text pages: 24

Number of figures: 4

Number of tables: 0

Number of references: 42

Number of words: Abstract 139; Introduction 559; Discussion 600

Abbreviation: 4-AP, 4-aminopyridine; FBS, fetal bovine serum; HS, horse serum; I_A, A-type K^+ channel; I_K, outward delayed rectifier; LDH, lactate dehydrogenase; MEM, Eagle's minimal essential medium; NMDA, N-methyl-D-aspartate; TEA, tetraethylammonium.

Abstract

K^+ channel blockers such as 4-aminopyridine (4-AP) can be toxic to neurons; the cellular mechanism underlying the toxicity, however, is obscure. In cultured mouse cortical neurons, we tested the hypothesis that the toxic effect of 4-AP might be a result from inhibiting the Na^+, K^+-ATPase (Na^+, K^+-pump) and thereafter induction of a hybrid death of concomitant apoptosis and necrosis. The Na^+, K^+-pump activity, monitored as whole-cell membrane currents, was markedly blocked by 4-AP in concentration- and voltage-dependent manners in low mM range. At similar concentrations, 4-AP induced a neuronal death sensitive to attenuations by the caspase inhibitor Z-VAD-FMK or Ca^{2+} chelator BAPTA-AM. Electron microscopy confirmed hybrid ultrastructural features of coexisting apoptotic and necrotic components in same cells. We suggest that 4-AP is a potent antagonist of the Na^+, K^+-ATPase and an inducer of the hybrid death of central neurons.

Aminopyridines particularly 4-aminopyridine (4-AP) have been investigated as a means of beneficial symptomatic treatment in a variety of neurological conditions of demyelination diseases including multiple sclerosis, myasthenia gravis, and spinal cord injury (Murray and Newsom-Davis 1981; Jones et al. 1983; Targ and Kocsis 1985; Waxman 1993; Segal and Brunnemann 1997; Potter et al. 1998; Halter et al. 2000). 4-AP is a typical blocker of the voltage-gated, fast-inactivating A-type K^+ channels or I_A channels (Mathie et al. 1998); the inhibition of I_A channels, specifically those located in the inter- and para-nodal regions of axonal membranes, prolongs the duration of action potential and facilitates the propagation of action potentials in demyelinated nerve fibers (Targ and Kocsis 1985; Bostock et al. 1981; Schauf 1987; Kaji and Sumner 1988; Blight 1989; Shi and Blight 1997). K^+ channel blockers including 4-AP also increase Ca^{2+} influx that can increase transmitter release in a wide range of neuronal types (Bowman and Savage 1981; Glover 1982; Hayes et al. 1994; Soni and Kam 1982), beneficial for improving neurological conditions in diseases such as myasthenia gravis.

4-AP, on the other hand, is well known as an experimental convulsant for seizure induction (Murray and Newsom-Davis 1981; Spyker et al. 1980; Yamaguchi and Rogawski 1992; Pickett and Enns 1996). Recent studies have shown that 4-AP, at commonly used concentrations, can cause apoptosis in hepatoblastoma cells (Kim et al. 2000) and malignant astrocytoma cell lines (Chin et al. 1997). Another classical K^+ channel blocker tetraethylammonium (TEA) at high concentrations also shows toxic effects on cortical neurons (Yu et al. 1997). The mechanism of 4-AP- or TEA-induced neurotoxicity is unclear. A link to increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was suggested for the pro-apoptotic effect of 4-AP (Kim et al. 2000). The role for Ca^{2+} in the

induction of apoptosis, however, is controversial and complex. Increasing $[Ca^{2+}]_i$ may either induce or antagonize apoptosis (Dowd 1995; Yu et al. 2001); furthermore, apoptosis may occur without alterations in $[Ca^{2+}]_i$ (Iseki et al. 1993; Ubol et al. 1996; Beaver and Waring 1994; Treves et al. 1994; Reynolds and Eastman 1996).

Emerging evidence now supports an ionic mechanism underlying apoptosis, associating with excessive K^+ efflux and loss of intracellular K^+ (Yu et al. 1997; Dallaporta et al. 1998; Hughes and Cidlowski 1999). The pro-apoptotic K^+ depletion can be mediated by K^+ permeable ion channels (Yu et al. 1997; Yu et al. 1999) or by blocking the Na^+, K^+-ATPase (Xiao et al. 2002). In the latter case, a "hybrid death" of concomitant apoptosis and necrosis in same cells was associated with depletion of intracellular K^+ and simultaneous accumulations of Ca^{2+} and Na^+, respectively (Xiao et al. 2002). Supporting the contribution of over-activated K^+ channels to apoptosis, K^+ channel blockers such as TEA attenuate caspase activation and apoptotic death (Yu et al. 1997; Colom *et al.*, 1998; Krick *et al*., 2001; Xiao et al. 2002).

As 4-AP and TEA may have potential clinical values in certain pathological conditions, understanding the mechanism of their adverse effects becomes necessary and important. Previous works showed that TEA was capable of blocking the Na^+, K^+-ATPase (Eckstein-Ludwig et al. 1998), thus the toxic effect of K^+ channel blockers might be linked to a dysfunction of the Na^+, K^+-ATPase. In the present study, we tested the hypothesis that 4-AP and TEA may induce apoptosis or the hybrid death mediated by blocking the Na^+, K^+-ATPase.

Materials and Methods

Neocortical cultures

Mixed cortical cultures (containing neurons and a confluent glia bed) were prepared as described previously (Rose et al. 1993). Briefly, neocortices were obtained at 15 - 17 days gestation from fetal mice; they were dissociated and plated onto a poly-D-lysine and laminin coated base (near-pure neuronal culture) or a previously established glial monolayer (mixed culture), at a density of 0.35 to 0.40 hemispheres/ml in 24-well or 96-well plates or 35 mm dishes (Falcon, Primaria) depending on experimental requests. Cultures were maintained 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). 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. Glial cultures used for mixed cultures were prepared from dissociated neocortices of postnatal day 1 - 3 mice. Glial cells were plated at a density of 0.06 hemispheres/ml in Eagle's MEM containing 20 mM glucose, 10% FBS, 10% HS and 10 ng/ml epidermal growth factor; a confluent glial bed was formed in 1 - 2 weeks.

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

The 35-mm culture dish containing cortical neurons was placed on the stage of an inverted microscope, membrane currents were recorded by whole-cell configuration 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). 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.

To record the Na^+, K^+-pump current, 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 or 0.1 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, HEPES 10. Tonic Na^+, K^+-pump activities was blocked by local applied strophanthidin (500 μM) to the surface of cell body by the DAD-12 drug delivery device (Adams & List, New York, NY). To record outward current associated with activation of the Na^+, K^+-pump, the above extracellular solution was switched to a K^+-free solution to minimize the pump activity and then jumped to a solution containing 4 mM K^+ to activate the Na^+, K^+-pump. Ba^{2+} (4mM) was included in external solution to block voltage-gated K^+ channels. Gadolinium (1 μM) was added into the external solution to prevent opening of voltage-gated Ca^{2+} channels. Recordings were performed at room temperature (21 ± 1°C), all solutions had pH of 7.3 – 7.4.

Assessment 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). Validation of the LDH method for measuring apoptotic death has been performed before (Gottron et al., 1997). Neuronal loss is expressed as the percentage of LDH released in

each experimental condition normalized to negative (sham wash) and positive (complete neuronal death induced by 24-hr exposure to 300 μM NMDA) controls.

Electron microscopy

Cultures in 35-mm dishes were fixed in glutaraldehyde (1% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH = 7.4) for 30 min at 4^{o}C, washed with 0.1 M sodium cacodylate buffer, and postfixed in 1.25% osmium tetroxide for 30 min. Cells were then stained *en bloc* in 4% aqueous uranyl acetate for 1 hr, dehydrated through a graded ethanol series, embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA), and polymerized in a 60^{o}C oven overnight. Thin sections (62 nm) were cut on a Reichert Ultracut Ultramicrotome (Mager Scientific, Dexter, MI), mounted on 150-mesh copper grids, and poststained in uranyl acetate and Reynold's lead citrate. Sections were photographed using a transmission electronic microscope (Zeiss 902, LEO Electronic, New York).

Chemicals

4-Aminopyridine, tetraethylammonium chloride, gadolinium chloride, and strophanthidin were purchased from Sigma-Aldrich (St. Louis, MO). The caspase inhibitor Z-Val-Ala-Asp(OMe)-Fluoromethyl Ketone (Z-VAD-FMK) was obtained from Enzyme Systems Products (Dublin, CA).

Statistics

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 (SEM).

Results

Suppression of Na^+, K^+-pump currents by 4-AP and TEA

4-AP is regarded as a classical A-type K^+ channel blocker and a blocker for certain delayed rectifier channels (Mathie et al. 1998). In cortical neurons it selectively inhibited I_A current triggered by voltage pulses from –110 mV to -10 mV, with an IC_{50} of 0.7 mM (n = 5 – 9 cells; the holding potential between pulses was –70 mV). To measure the tonic Na^+, K^+-pump activity, an inward membrane current, I_{pump}, was triggered by brief application of the selective inhibitor strophanthidin (500 μM) in the presence of voltage-gated channel blockers (Fig. 1). Bath-applied 4-AP at low mM concentrations markedly suppressed I_{pump} recorded at –70 mV (IC_{50} = 1.2 mM) (Fig. 1). The pan K^+ channel blocker TEA also inhibited I_{pump} with an IC_{50} of 5.2 mM at –70 mV (n = 8). Elevating extracellular K^+ stimulates the Na^+, K^+-pump activity and generates an outward current. This outward I_{pump} associated with activation of the Na^+, K^+-pump was blocked by bath-applied 4-AP with an IC_{50} of 4.2 mM (Fig. 1). Similar to a previous report on TEA (Eckstein-Ludwig et al. 1998), the 4-AP effect was voltage-dependent; stronger I_{pump} inhibition was achieved at depolarized membrane potentials (Fig. 2). 4-AP blocked the Na^+, K^+-pump activity only at an extracellular site, intracellular application of 5 mM 4-AP exhibited no effect on I_{pump} (Fig. 2).

(Figure 1 and 2 near here)

Hybrid neuronal death induced by 4-AP and TEA

4-AP at low mM concentrations exhibited dose-dependent toxicity to cortical neurons; significant cell death occurred after 24 hr-incubation in ≥ 5 mM 4-AP and after 48 hr-incubation in 0.1 - 10 mM 4-AP (Fig. 3). The EC_{50} of 4-AP toxic effect was 4.1 mM at 48 hrs (n = 8 cultures). TEA also showed time- and concentration-dependent toxic effect on cortical neurons at relatively higher concentrations (≥ 10 mM) (Fig. 3).

Consistent with an apoptotic component, the neuronal death induced by 4-AP or TEA was attenuated by the irreversible pan caspase inhibitor Z-VAD-FMK (100 μM) (Fig. 3). However, a significant portion of the neuronal death was not prevented by blocking caspases, in agreement with the recent notion that blocking the Na^+, K^+-pump induces concurrent apoptosis and necrosis (Xiao et al. 2002). In accordance with the necrotic component that may be triggered by accumulation of $[Ca^{2+}]_i$, co-applied membrane permeable Ca^{2+} chelator BAPTA-AM (10 μM) attenuated the 4-AP toxicity (Fig. 3). Combined treatment with Z-VAD-FMK plus BAPTA-AM produced additional neuroprotection (Fig. 3). Finally, electron microscopy revealed the mixed futures of ultrastructural alterations in 4-AP- or TEA-treated neurons; cells showed coexistence of nuclear condensation of apoptotic changes sensitive to caspase inhibition and necrotic disruptions of the cytoplasm (Fig. 4). K^+ channel blockers may depolarize the membrane and enhance glutamate release; blocking the NMDA receptor with MK-801 (1 μM), however, could neither eliminate the 4-AP toxicity nor change the hybrid nature of the cell death (Fig. 4).

(Figure 3 and Figure 4 near here)

Discussion

This study provides new insight into the 4-AP pharmacology and neurotoxicity. We showed that, in addition to be a classical K^+ channel blocker, 4-AP is a potent antagonist of the Na^+, K^+-ATPase. The latter property is likely responsible for the induction of a hybrid death of cortical neurons, consistent with our previous demonstration that the hybrid death was induced by failure of the Na^+, K^+-ATPase (Xiao et al. 2002 and see below). The additional protection gained from co-applied Z-VAD-FMK and BAPTA-AM supports the mixed nature of 4-AP toxicity; it is not clear why the residual cell death was not sensitive to caspase inhibition and Ca^{2+} buffering. Caspase-independent apoptosis and/or BAPTA toxicity (Fig. 3) may play roles in this observation.

Inhibiting K^+ channels by TEA or 4-AP are protective against apoptosis in several cell types (Yu et al. 1997; Colom et al. 1998; Dallaporta et al. 1999; Wang et al. 2000; Krick et al. 2001; Wang et al. 1999); on the other hand, 4-AP is toxic in malignant astrocytoma cell lines and HepG2 human hepatoblastoma cells (Kim et al. 2000; Chin et al. 1997). Our data suggest that blocking the Na^+, K^+-ATPase but not K^+ channels is likely the primary mechanism underlying the toxic effect of 4-AP. Supporting this notion, both the 4-AP block of Na^+, K^+-ATPase and 4-AP toxicity are similarly concentration-dependent; more importantly, the Na^+, K^+-ATPase blocker ouabain-induced hybrid death was attenuated but not exaggerated by blocking K^+ channels or reducing K^+ efflux (Xiao et al. 2002). Additional evidence can be found in the study where 4-AP inhibits outward K^+ currents and cell proliferation with similar efficacy in malignant astrocytoma U87 and A172 cells, however, 4-AP induces apoptosis only in U87 cells but not in A172 cells (Chin et al. 1997). It will be interesting and important to

know whether this discrepancy is a result from different effects of 4-AP on the Na^+, K^+-ATPase in these cells.

In spite of a long research history on 4-AP, the inhibitory effect of 4-AP on the Na^+, K^+-pump activity has never been recognized before. This overlook is probably due to the fact that blocking K^+ channels and blocking the Na^+, K^+-pump result in similar consequences including membrane depolarization and increases in intracellular Ca^{2+}. In this regard, it is possible that some previously observed effects induced by 4-AP are in fact at least partly a result from dysfunction of the Na^+, K^+-pump. For example, 4-AP at 1 mM suppressed axonal conductance accompanied by marked membrane depolarization (Shi and Blight 1997), which can be partly explained by an inhibitory effect on the Na^+, K^+-pump. In addition, an enhanced membrane depolarization and disruption of ion homeostasis are likely important contributors to the convulsant side effects of 4-AP.

The K^+ channel blocker TEA can directly block the Na^+, K^+-ATPase in a voltage-dependent manner (Eckstein-Ludwig et al. 1998). 4-AP is structurally unrelated to TEA yet shows even stronger inhibition on the Na^+, K^+-ATPase. Similar to TEA, we confirmed that the 4-AP effect is voltage-dependent, with high inhibition at more depolarized membrane potentials. Like TEA, 4-AP blocks the Na^+, K^+-pump only at an extracellular site. It remains to be delineated whether 4-AP blocks the pump via a direct competitive mechanism as TEA does.

The block of Na^+, K^+-pump and induction of hybrid neuronal death by 4-AP and TEA suggest that more selective K^+ channel blockers without the adverse action on the Na^+, K^+-pump will be necessarily desirable for therapeutic uses. The more selective compounds may avoid or reduce the side effects associated with membrane

depolarization and disruption of ion homeostasis, and preclude induction of the hybrid cell injury.

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Footnotes

This work was supported by grants from National Science Foundation (9817151 to S.P.Y.), American Heart Association and Burgher Foundation (0170063N to W.L, and 0170064N to S.P.Y), and National Institute of Health (NS42236 to S.P.Y. and NS37337 to W.L.).

Figure Legends

Figure 1. Block of Na^+, K^+-pump currents by 4-AP

Na^+, K^+-pump currents were recorded in cultured cortical neurons using whole-cell recordings in the presence of K^+, Na^+, and Ca^{2+} channel blockers. **A**. The inward current associated with the tonic pump activity was induced by local application of the Na^+, K^+-ATPase inhibitor strophanthidin (500 μM). In the presence of bath-applied 5 mM 4-AP, much smaller pump current was recorded in the same cell. **B**. Elevated extracellular K^+ triggered an outward membrane current resulted from an enhanced pump activity; this outward pump current was markedly inhibited by 500 μM strophanthidin or 5 mM 4-AP. **C**. Dose-response relationship of 4-AP effect on the Na^+, K^+-pump. The dose-response curve was fitted by the two exponential equation, yielding an IC_{50} of 1.17 mM on the pump current recorded at the holding potential of –70 mV (n = 10 for each concentration point).

Figure 2. Characterization of 4-AP effects on Na^+, K^+-pump currents

4-AP effects on the Na^+, K^+-pump outward current were characterized. **A**. The inhibitory effect of 4-AP on the Na^+, K^+-pump activity was voltage-dependent; at depolarized membrane potentials 5 mM 4-AP induced greater suppression of the pump current. **B**. 4-AP affected the Na^+, K^+-pump activity only from an extracellular site. Bath application of 4-AP (5 mM) blocked about 50% of the outward pump current while 4-AP (5 mM) in the internal solution caused little effect (n = 8 for each column; 5-10 min application). Control currents were recorded before the 4-AP application in same cells. **C**. As a drug application control, 1 mM 4-AP included in the intracellular solution

drastically suppressed I_A current 5-10 minutes after establishing the whole-cell configuration. *. Significant difference ($P < 0.05$) from controls.

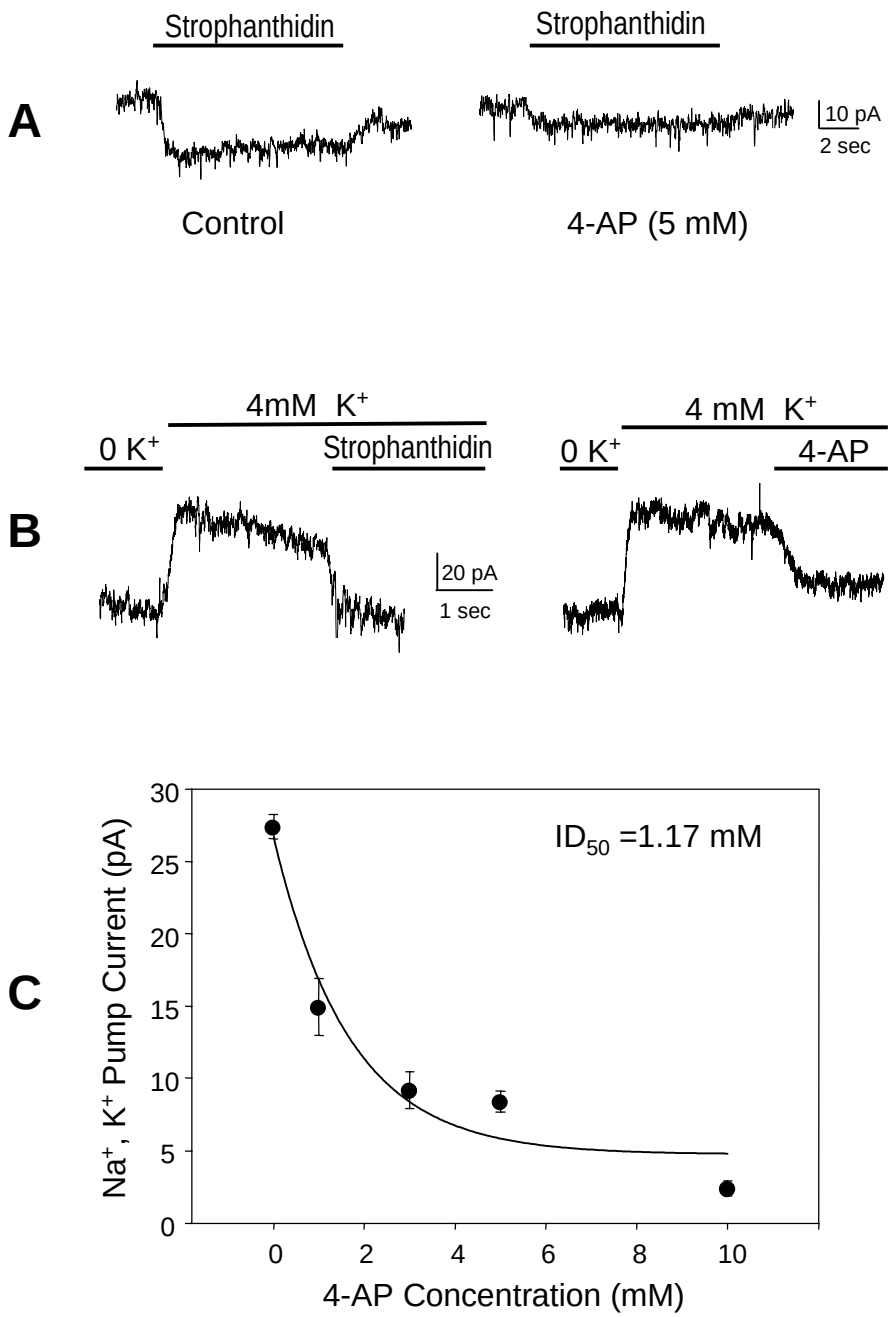
Figure 3. Neuronal death induced by 4-AP and TEA

Cortical neuronal death was assessed by LDH release 24 – 48 hrs after adding 4-AP, and expressed as the percentage of full damage induced by excessive activation of NMDA receptors (300 μM plus 10 μM glycine). **A**. Dose-response relationship for 4-AP toxicity; the EC_{50} for the 4-AP effect calculated from an exponential curve fitting was 5.7 mM at 24 hrs and 4.1 mM at 48 hrs. **B**. The 4-AP-induced neuronal death was partly attenuated by the caspase inhibitor Z-VAD-FMK or by the membrane permeable Ca^{2+} chelator BAPTA-AM (10 μM) co-applied with 4-AP. Combined application of Z-VAD-FMK plus BAPTA-AM resulted in additional neuroprotection. The residue death may be partly due to the BAPTA toxicity and undefined mechanism. **C**. The classical K^+ channel blocker TEA showed neurotoxicity at higher concentrations and after longer exposure; significant cell death was observed after exposed to 10 and 20 mM TEA for 48 hrs. Z-VAD-FMK (100 μM) significantly reduced TEA-induced cell death. $N \geq 12$ cultures for each group. *. Significant difference from 4-AP or TEA alone ($P < 0.05$).

Figure 4. Ultrastructural alterations of hybrid injury induced by 4-AP and TEA

Apoptotic and necrotic morphological alterations were examined by electron microscopy. **A**. Normal control cells showed large nuclei, intact and distinguishable cellular organelles, and the intact membranes. **B**. After 15-hr treatment in the medium containing 5 mM 4-AP, many cells showed shrunken nuclei of apoptotic change, but

meanwhile the vacuolization of swollen cytoplasm and disruption of the organelles were signs of necrotic damage. **C**. About 30 hrs after onset of 4-AP treatment, numerous cells showed even more condensed nuclei and chromatin clamps (arrow), typical of apoptosis; on the other hand, the chaotic disruption of the cytoplasm was consistent with necrosis. **D**. The apoptotic nuclei condensation was largely prevented by the caspase blocker Z-VAD-FMK (100 μM) while cytoplasm and the plasma membrane still underwent necrotic changes (30-hr 4-AP plus Z-VAD-FMK). **E**. The 4-AP-induced hybrid nature was not affected by co-applied MK-801 (1 μM). **F**. A representative cell after 30-hr incubation in 20 mM TEA; the changes in nucleus and cytoplasm suggested apoptosis and necrosis, respectively, in the same cell. Magnification = X 4,000.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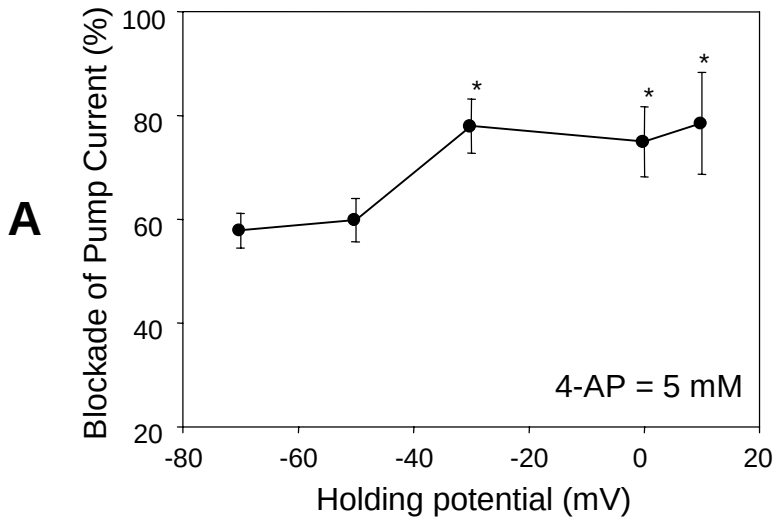
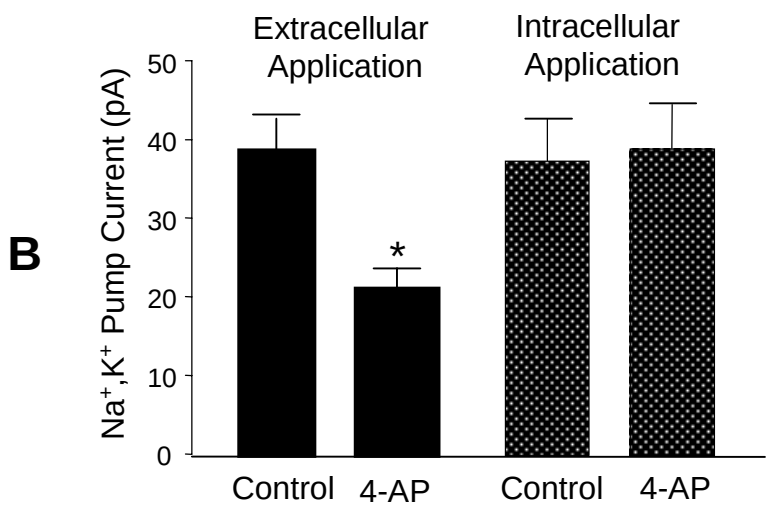
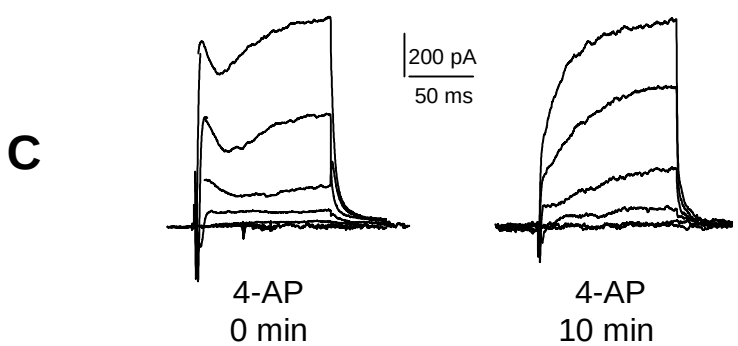
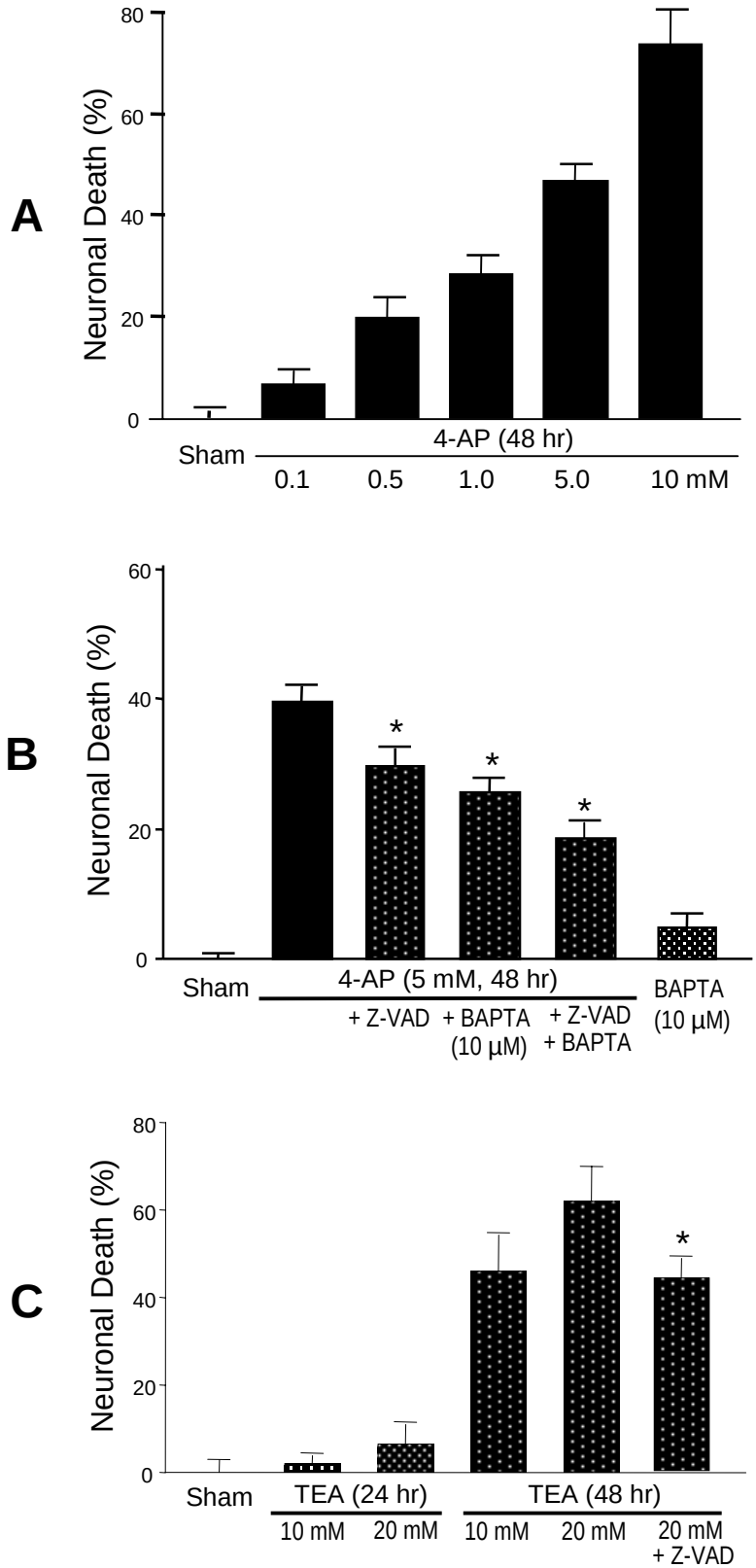
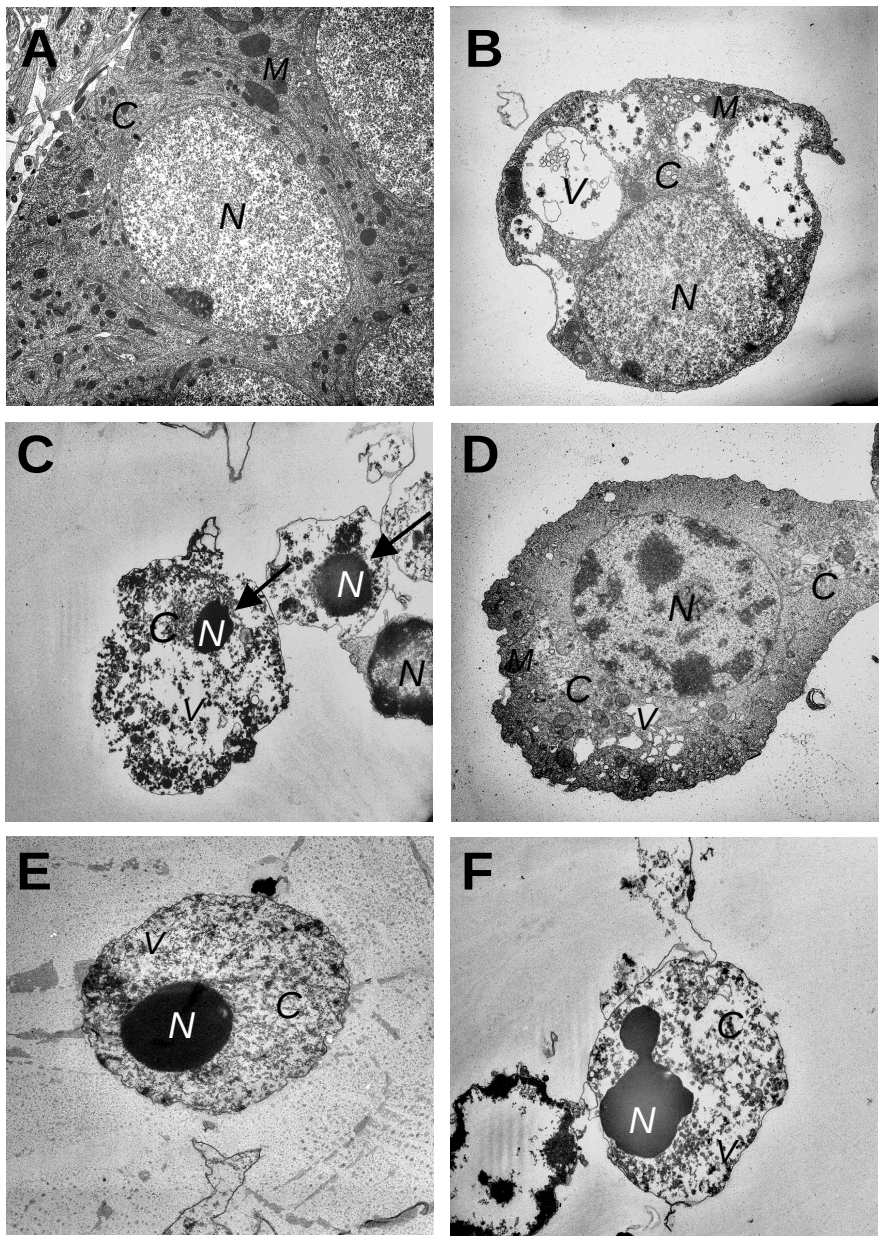
4mM K+
0 K+
Strophanthidin
4 mM K+
0 K+
4-AP
20 pA
1 sec
C
ID50 =1.17 mM
Na+, K+ Pump Current (pA)
4-AP Concentration (mM)
0
5
10
15
20
25
30
0
2
4
6
8
10


A
Blockade of Pump Current (%)
100
80
60
40
20
-80
-60
-40
-20
0
20
Holding potential (mV)
4-AP = 5 mM
*
*
*


B
Extracellular Application
Intracellular Application
Na+,K+ Pump Current (pA)
50
40
30
20
10
0
*
Control
4-AP
Control
4-AP


C
200 pA
50 ms
4-AP
0 min
4-AP
10 min


A
Neuronal Death (%)
80
60
40
20
0
Sham
4-AP (48 hr)
0.1
0.5
1.0
5.0
10 mM
B
Neuronal Death (%)
60
40
20
0
*
*
*
Sham
4-AP (5 mM, 48 hr)
+ Z-VAD
+ BAPTA (10 μM)
+ Z-VAD + BAPTA
BAPTA (10 μM)
C
Neuronal Death (%)
80
60
40
20
0
*
Sham
TEA (24 hr)
10 mM
20 mM
TEA (48 hr)
10 mM
20 mM
20 mM + Z-VAD


A
M
C
N
B
M
V
C
N
C
C
N
N
N
V
D
N
C
M
C
V
C
E
V
N
C
F
C
N
V