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

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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 result from inhibiting the Na⁺,K⁺-ATPase (Na⁺,K⁺-pump) and therefore, after 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 millimolar ranges. At similar concentrations, 4-AP induced a neuronal death sensitive to attenuation by the caspase inhibitor Z-VAD-FMK (Z-Val-Ala-Asp[OMe]- fluoromethyl ketone) or Ca²⁺ chelator BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxyethyl ester). 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; Yamaguchi and Rogawski, 1992). Recent studies have shown 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 Ca²⁺ ([Ca²⁺]) was suggested for the pro-apoptotic effect of 4-AP (Kim et al., 2000). The role for Ca²⁺ in the induction of apoptosis, however, is controversial and complex. Increasing [Ca²⁺], may either induce or antagonize apoptosis (Dowd, 1995; Yu et al., 2001); furthermore, apoptosis may occur without alterations in [Ca²⁺], (Iseki et al., 1993; Beaver and Waring, 1994; Treves et al., 1994; Reynolds and Eastman, 1996; Ubol et al., 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, 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

4-AP, on the other hand, is well known as an experimental convulsant for seizure induction (Spyker et al., 1980; Murray and Newsom-Davis, 1981; Yamaguchi and Rogawski, 1992; Pickett and Enns, 1996). Recent studies have shown 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 Ca²⁺ ([Ca²⁺]) was suggested for the pro-apoptotic effect of 4-AP (Kim et al., 2000). The role for Ca²⁺ in the induction of apoptosis, however, is controversial and complex. Increasing [Ca²⁺], may either induce or antagonize apoptosis (Dowd, 1995; Yu et al., 2001); furthermore, apoptosis may occur without alterations in [Ca²⁺], (Iseki et al., 1993; Beaver and Waring, 1994; Treves et al., 1994; Reynolds and Eastman, 1996; Ubol et al., 1996).

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ABBREVIATIONS: 4-AP, 4-aminopyridine; AM, acetoxyethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; HS, horse serum; LDH, lactate dehydrogenase; MEM, minimal essential medium; MK-801, (−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMN, N-methyl-o-aspartate; TEA, tetraethylammonium; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)- fluoromethyl ketone.
depletion of intracellular K⁺ and simultaneous accumulations of Ca²⁺ and Na⁺, respectively (Xiao et al., 2002). Supporting the contribution of over-activated K⁺ channels to apoptosis, K⁺ channel blockers such as TMA 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- to 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- or 96-well plates or 35-mm dishes (Falcon Primaria; BD Biosciences, Franklin Lakes, NJ) 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, and 5% horse serum (HIS). Medium was changed after 1 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 days 1 to 3 mice. Glial cells were plated at a density of 0.06 hemispheres/ml in Eagle's MEM containing 20 mM glucose, 10% fetal bovine serum, 10% HS, and 10 ng/ml epidermal growth factor; a confluent glial bed was formed in 1 to 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, Darmstadt, Germany). Recording electrodes of 8 to 10 MΩ (fire-polished) were pulled from Corning Kovar Sealing 7052 glass pipettes (PG52151-4, WPI Instruments, Waltham, MA) by a Flaming-Brown micropipette puller (P-80/PC, Sutter Instrument Co., Novato, CA). Current and voltage signals were displayed on a computer monitor and collected by a data acquisition/analysis program PULSE (HEKE, Lambrecht, 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 125 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM sodium-HEPES, 10 mM glucose, and 0.01 or 0.1 μM tetrodotoxin. The electrode solution contained 60 mM cesium-acetate, 20 mM NaCl, 100 mM N-methyl-D-glucamine, 5 mM Mg-ATP, 1 mM BaPta, 10 mM TEA, and 10 mM HEPES. Tonic Na⁺,K⁺-pump activities were blocked by local applied strophanthidin (500 μM) to the surface of cell body by the DAD-12 drug delivery device (Adams and 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. Ba²⁺ (4 mM) was included in the external solution to block voltage-gated K⁺ channels. Gadolinium (1 μM) was added into the external solution to prevent opening of voltage-gated Ca²⁺ channels. Recordings were performed at room temperature (21 ± 1°C); all solutions had pH values of 7.3 to 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 NaHCO₃) using a multiple-plate reader (Molecular Devices Corp., Sunnyvale, CA). Validation of the LDH method for measuring apoptotic death has been performed before (Gottron et al., 1997). Neuronal death 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-h 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°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 h, dehydrated through a graded ethanol series, embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA), and polymerized in a 60°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 (Electron Microscopy Sciences, Fort Washington, PA). Sections were photographed using a transmission electronic microscope (Zeiss 902; LEO Electronic, Thornwood, NY).

Fig. 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 Ca²⁺ 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 hath-applied 5 mM 4-AP, much smaller pump current was recorded in the same cell. B, elevated extracellular K⁺ triggered an outward membrane current resulting from an enhanced pump activity; this outward pump current was markedly inhibited by 30 μM strophanthidin in the presence of 5 mM 4-AP. C, dose-response relationships of 4-AP effect on the Na⁺,K⁺-pump. The dose-response curve was fitted by the two exponential equation, yielding an IC₅₀ of 1.17 mM on the pump current recorded at the holding potential of −70 mV (n = 10 for each concentration point).

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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 analysis of variance test followed by Tukey’s test for multiple pairwise tests. Changes were identified as significant if the P value was less than 0.05. Mean values were reported together with the standard error of mean (S.E.M.).

Results

Suppression of Na⁺,K⁺-Pump Currents by 4-AP and TEA. 4-AP is regarded as a classical A-type K⁺ channel blocker. To further investigate its mechanism of action, we measured Na⁺,K⁺-pump currents in the absence and presence of 4-AP. As shown in Fig. 2A, 4-AP induced a significant suppression of the pump current, n = 7–10. 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, whereas 4-AP (5 mM) in the internal solution caused little effect (n = 8 for each column; 5- to 10-min application). Control currents were recorded before the 4-AP application in the same cells. C, as a drug application control, 1 mM 4-AP included in the intracellular solution drastically suppressed Iᵦ current 5 to 10 min after establishing the whole-cell configuration. *, significantly different (P < 0.05) from controls.

Fig. 2. Characterization of 4-AP effects on Na⁺,K⁺-pump currents. 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, n = 7–10. 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, whereas 4-AP (5 mM) in the internal solution caused little effect (n = 8 for each column; 5- to 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 Iᵦ current 5 to 10 min after establishing the whole-cell configuration. *, significantly different (P < 0.05) from controls.

Fig. 3. Neuronal death induced by 4-AP and TEA. Cortical neuronal death was assessed by LDH release 24 to 48 h 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 EC₅₀ for the 4-AP effect calculated from an exponential curve fitting was 5.7 mM at 24 h and 4.1 mM at 48 h. B, the 4-AP-induced neuronal death was partly attenuated by the caspase inhibitor Z-VAD-FMK or by the membrane-permeable Ca²⁺ chelator BAPTA-AM (10 μM) co-applied with 4-AP. Combined application of Z-VAD-FMK plus BAPTA-AM resulted in additional neuroprotection. The residual 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 exposure to 10 and 20 mM TEA for 48 h. Z-VAD-FMK (100 μM) significantly reduced TEA-induced cell death. n ≥ 12 cultures for each group. *, significant difference from 4-AP or TEA alone (P < 0.05).
Blocker TEA also inhibited \( I_{\text{pump}} \) with an \( I_{50} \) 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 \( I_{\text{pump}} \) associated with activation of the Na\(^+\),K\(^+\)-pump was blocked by bath-applied 4-AP with an \( I_{50} \) of 4.2 mM. Similar to a previous report on TEA (Eckstein-Ludwig et al., 1998), the 4-AP effect was voltage-dependent; stronger \( I_{\text{pump}} \) 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 \( I_{\text{pump}} \) (Fig. 2).

**Hybrid Neuronal Death Induced by 4-AP and TEA.**

4-AP at low millimolar concentrations exhibited dose-dependent toxicity to cortical neurons; significant cell death occurred after a 24-h incubation in \( \geq 5 \) mM 4-AP and after a 48-h incubation in 0.1 to 10 mM 4-AP (Fig. 3). The \( EC_{50} \) of 4-AP toxic effect was 4.1 mM at 48 h \( (n = 8 \) cultures). TEA also showed time- and concentration-dependent toxic effect on cortical neurons at relatively higher concentrations \( (\geq 10 \) 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 \( \mu \)M) (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 \([Ca^{2+}]_{i}\), coapplied membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM (10 \( \mu \)M) 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 \( \mu \)M), however, could neither eliminate the 4-AP toxicity nor change the hybrid nature of the cell death (Fig. 4).

**Discussion**

This study provides new insight into the 4-AP pharmacology and neurotoxicity. We showed that, in addition to being 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 a role 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., 1999, 2000; Krick et al., 2001); on the other hand, 4-AP is toxic in malignant astrocytoma cell lines and HepG2 human hepatoblastoma cells (Chin et al., 1997; Kim et al., 2000). Our data suggest that blocking the Na\(^+\),K\(^+\)-ATPase, but not the 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, 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 results 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 oversight 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 Ca²⁺. In this regard, it is possible that some previously observed effects induced by 4-AP are in fact at least partly a result of dysfunction of the Na⁺,K⁺-pump. For example, 4-AP at 1 μM 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 but 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 defined whether 4-AP blocks the pump via a direct competitive mechanism like TEA.

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 needed for therapeutic uses. The more selective compounds may avoid or reduce the side effects associated with membrane depolarization and disruption of ion homeostasis therefore preclude induction of the hybrid cell injury.

References


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