Toxicology 333 (2015) 107–117

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Neuroprotective or neurotoxic effects of 4-aminopyridine mediated by KChIP1 regulation through adjustment of Kv 4.3 potassium channels expression and GABA-mediated transmission in primary hippocampal cells

Javier Del Pino [a,*,1], María Teresa Frejo [a,1], María José Anadon Baselga [b], Miguel Andrés Capo [a], Paula Moyano [b], José Manuel García [b], María Jesús Díaz [a]

[a] Department of Toxicology and Pharmacology, Veterinary School, Complutense University of Madrid, 28040 Madrid, Spain
[b] Department of Toxicology and Legal Medicine, Medicine School, Complutense University of Madrid, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 25 January 2015
Received in revised form 5 April 2015
Accepted 22 April 2015
Available online 24 April 2015

Keywords:
4-Aminopyridine
Cell death
KChIP1
Kv 4.3
GABA
Rats

ABSTRACT

4-Aminopyridine (4-AP) is a potassium channel blocker used for the treatment of neuromuscular disorders. Otherwise, it has been described to produce a large number of adverse effects among them cell death mediated mainly by blockage of K^+ channels. However, a protective effect against cell death has also been described. On the other hand, Kv channel interacting protein 1 (KChIP1) is a neuronal calcium sensor protein that is predominantly expressed at GABAergic synapses and it has been related with modulation of K^+ channels, GABAergic transmission and cell death. According to this KChIP1 could play a key role in the protective or toxic effects induced by 4-AP. We evaluated, in wild type and KChIP1 silenced primary hippocampal neurons, the effect of 4-AP (0.25 μM to 2 mM) with or without semicarbazide (0.3 M) co-treatment after 24 h and after 14 days 4-AP alone exposure on cell viability, the effect of 4-AP (0.25 μM to 2 mM) on KChIP1 and Kv 4.3 potassium channels gene expression and GABAergic transmission after 24 h treatment or after 14 days exposure to 4-AP (0.25 μM to1 μM). 4-AP induced cell death after 24 h (from 1 mM) and after 14 days treatment. We observed that 4-AP modulates KChIP1 which regulate Kv 4.3 channels expression and GABAergic transmission. Our study suggests that KChIP1 is a key gene that has a protective effect up to certain concentration after short-term treatment with 4-AP against induced cell injury; but this protection is erased after long term exposure, due to KChIP1 down-regulation predisposing cell to 4-AP induced damages. These data might help to explain protective and toxic effects observed after overdose and long term exposure.

1. Introduction

4-Aminopyridine (4-AP) is a K^+ channel blocker indicated for the treatment of neuromuscular disorders such as multiple sclerosis, botulism, spinal cord injury, Alzheimer's disease, myasthenia gravis and Eaton–Lambert syndrome, as it improves interneuronal and neuromuscular synaptic transmission (Murray and Newsom-Davis, 1981). The currently approved dosing regimen is 10 mg administered orally twice a day, but it presents a narrow safety index observing adverse effects with doses higher than 25 mg, especially at neurological (confusion, seizures, hyperexcitability, tremors, involuntary movements and choreoathetoid) and cardiovascular level (hypertension and heart arrhythmias as a result of a possible prolongation of the QT interval) (King et al., 2012).

4-AP has been reported to stimulate the release of both excitatory (glutamate) and inhibitory (GABA) neurotransmitters (Versteeg et al., 1995) and it produces intense epileptiform activity (Bijak and Misgeld, 1996; Faivre et al., 1999; Morales-Villagran et al., 1996). Recent studies have shown that 4-AP suppresses cell proliferation and induces apoptosis in several cell lines (Fieber et al., 2003; Kim et al., 2000; Rybalchenko et al., 2001). Moreover, these effects have been supported by *in vivo* studies. Thus, it has been described that intraperitoneal administration of 4-AP (5 mg/kg) to rats induced neuronal damage in hippocampus,

* Corresponding author at: Department of Toxicology and Pharmacology, Veterinary Medicine School, Complutense University of Madrid, Avda. Puerta de Hierro s/n 28040 Madrid, Spain. Tel.: +34 913550920; fax: +34 916716224.
E-mail address: jdelpino@pdi.ucm.es (J. Del Pino).
[1] These authors have contributed equally to this work.

http://dx.doi.org/10.1016/j.tox.2015.04.013
0300-483X/

cortex and amygdala (Fabene et al., 2006). Besides, hippocampal infusion of this drug (35 mM) has also been described to induce cell death in rat hippocampal neurons *in vivo,* which was incremented by potentiating GABAergic transmission (Pena and Tapia, 2000; Salazar and Tapia, 2012). Furthermore, we reported previously that 4-AP induces cell degeneration in rat liver and kidney with features of both apoptosis and necrosis after subchronic exposure to therapeutic doses (Frejo et al., 2014). However, there have been discrepancies in recent reports such that 4-AP appears also to protect against cell apoptosis and necrosis trough K^+ channel blocking (Hu et al., 2006; Smith et al., 2009). These discrepancies have been hypostatized to be likely due to 4-AP actions on different ion channels in different cell models (Hu et al., 2006).

The mechanisms by which cell death occurs are not clearly determined. Several authors suggest that 4-AP produces this effect by causing an increase in intracellular calcium levels (Wang et al., 2011). Moreover, the inhibition of the ATPase Na^+, K^+ dependent has been reported to produce "hybrid death", after a 24 h incubation in mice cortical neurons of $\geq$5 mM 4-AP concentrations, originating necrosis and apoptosis in the same cells (Xiao et al., 2002; Yu, 2003). Furthermore, it has been shown that inhibition of potassium voltage-gated channel, Shal-related subfamily, member 3 (Kv 4.3) by 4-AP (2 mM) or Kv 4.3 small interfering RNA induces in human embryonic kidney-293 (HEK) cells, apoptosis and necrosis, which are completely rescued by the specific calcium/calmodulin-dependent proteinkinase II (CaMKII) inhibitor KN-93, suggesting that Kv 4.3 channels contribute to cell apoptosis and necrosis through CaMKII activation (Li et al., 2012). Immunohistochemical studies have also revealed that GABAergic interneurons in the hippocampus (Menegola et al., 2008), striatum, and cortex (Rhodes et al., 2004) selectively express the Kv 4.3 channels. Also, co-immunoprecipitation studies showed that Kv channel interacting protein 1 (KChIP1) is associated with all three Kv 4 channels (Kv 4.1, Kv 4.2, and Kv 4.3), but Kv 4.3 showed an almost complete colocalization with KChIP1 in the soma and dendrites of a distinct subpopulation of basolateral amygdala neurons (Dabrowska and Rainnie, 2010). In this way, it has been described that KChIP1 modulates Kv 4.3 channels activity in hippocampal interneurons (Bourdeau et al., 2007), and has been also related with necroptotic cell death (Frejo et al., 2014; Hitomi et al., 2008). However, KChIP1 knockout mice were apparently normal and did not show any abnormality of cell death (Xiong et al., 2009), which suggests that KChIP1 gene expression alteration could induce a higher vulnerability of cells to death.

Furthermore, KChIP1 gene expression has been described to be increased in rat hippocampus following lithium–pilocarpine induced status epilepticus (SE) whereas it was down-regulated during the chronic-epilepsy phase (Su et al., 2008). It has also been reported that when SE is induced an increase of KChIP1, Kv 4.2 and Kv 4.3 is produced, suggesting a neuroprotective role of KChIP1 against epilepsy through potassium channels regulation (Chang et al., 2006). On the other hand, KChIP1 has been hypothesized to modulate GABAergic transmission, and KChIP1 deletion has been shown to increase susceptibility to seizures induced by anti-GABAergic convulsive drug pentylenetetrazole, indicating that KChIP1 might play a pivotal role in the regulation of GABAergic inhibitory system (Xia et al., 2010; Xiong et al., 2009) which could contribute to this neuroprotective role.

According to all exposed above, we hypothesized that 4-AP after short-term exposure could induce a protective effect against cell death up to certain concentrations through induction of KChIP1, Kv 4.3 and GABAergic transmission, but after long-term exposure it could contribute to induction of cell death through down-regulation of them. To prove this hypothesis, a study on primary hippocampal neuronal culture after both 24 h and long term exposure (14 days) to several 4-AP concentrations (0.25 μM to 2 mM) was undertaken to study 4-AP effects on KChIP1, Kv4.3 and GABAergic transmission. In order to determine the 4-AP neuroprotective effects mediated by GABAergic potentiation after short term exposure, hippocampal cells were co-treated with 4-AP and semicarbazide, an inhibitor of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) (Santos et al., 2008), to see if the cell viability was reduce due to the decrease of GABA levels. Moreover, we evaluated Kv 4.3 knockdown effects on cell viability to prove that its down-regulation induces cell death, and thus its induction could be a neuroprotective mechanism. After that, we studied on KChIP1 silenced cells with or without 4-AP treatment, the effect on cell viability, Kv 4.3 expression and GABAergic transmission to prove that KChIP1 mediates the neuroprotective effects of 4-AP. Besides, we studied the effect of semicarbazide on cell viability, KChIP1 and Kv 4.3 expression and GABAergic transmission to confirm that the effect of this compound is only due to the reduction of GABA levels.

2. Methods

2.1. Chemicals

The compounds, 4-aminopyridine (98%), semicarbazide hydrochloride, gamma-aminobutyric acid (GABA), poly-L-lysine, dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (Madrid, Spain). Anti-microtubule associated protein-2 (MAP2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody were obtained from Millipore (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Primary hippocampal neuron culture

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals. Pregnant Wistar rats on embryonic days 17 and 18 (Charles River, Barcelona, Spain) were anaesthetized with sodium pentobarbital, the embryos were removed and their brains dissected under a stereomicroscope (Olympus SZ51, Barcelona, Spain). Hippocampus was collected in ice-cold Hibernate E (Life Technologies, Madrid, Spain) and dissociated by incubation for 10 min at 37 °C in 1 mL of trypsin-EDTA solution (0.25%; Invitrogen, Madrid, Spain) and 0.5 mL DNase solution (Sigma, D4263, Madrid, Spain). Following trypsinization, trypsin/DNase solution was aspirated and added 0.5 mL of 4% bovine serum albumin (BSA, Sigma, Madrid, Spain), 1 mL Dulbecco's modified Eagle's medium (DMEM/F-12 with Glutamax-1, Life Technologies, Madrid, Spain) supplemented with 20% fetal bovine serum (FBS, Sigma, Madrid, Spain) antibiotics (penicillin/streptomycin 1 mL/L, Sigma, Madrid, Spain) and 2 mL DNase solution. Cells were subsequently dissociated by gentle trituration using a fire-polished Pasteur pipette. The suspension was centrifuged at 250 × *g* (Eppendorf 5804R, Madrid, Spain) for 5 min and the pellet was resuspended in growth medium (see above) and forced to pass through a 40 mm nylon mesh filter. This suspension was re-centrifuged (250 × *g*, 5 min) and resuspended in growth medium (see above). Cells suspension was plated at a density of 1×10^5 cell/mL onto poly-L-lysine 0.10 mg/mL coated glass coverslips (22 mm diameter) placed in plastic Petri dishes (35 mm diameter) coated with poly-L-lysine. After incubation for 12 h at 37 °C in a humidified 95% air/5% CO2 atmosphere, the medium was changed with a serum-free nutritive medium composed of DMEM, insulin (400 μL, Sigma, Madrid, Spain), transferrine (50 μL, Sigma, Madrid, Spain), progesterone (100 μL, Sigma, Madrid, Spain), putrescine (16 mg/L, Sigma, Madrid, Spain),