Influence of Pyridine and Urea on the Rat Brain ATPase Activity

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Abstract. The neurotoxicity of pyridine and urea was investigated in respect to their ability to alter the activity of synaptosomal membrane Na^+/K^+-ATPase and Mg^{2+}-ATPase. *In vitro* treatment with pyridine and urea stimulated Na^+/K^+-ATPase activity in a dose-dependent manner up to 40% and 60%, respectively Mg^{2+}-ATPase activity increased up to 40% after pyridine treatment, while urea had no effect at all. The neuroactive potencies of pyridine and urea were evaluated by estimating parameters K_m and ΔV_{max} for enzyme stimulation, as well as Hill coefficient to estimate the levels of cooperativity for pyridine and urea binding. The results suggest that pyridine stimulates both enzymes, probably by interacting with some neuronal membrane components, and altering the lipid micro-environment of the ATPases. In contrast, urea stimulates the Na^+/K^+-ATPase only, assumingly by acting on it directly or via some other regulatory mechanism. Stimulation of Na^+/K^+-ATPase and Mg^{2+}-ATPase by the substances tested and subsequent alteration of neuronal cell functioning could contribute to the CNS dysfunction upon chronic exposure to pyridine and urea.

Key words: Adenosine triphosphatase — Brain — Synaptic plasma membranes — Pyridine — Urea

Introduction

Pyridine and urea are stable compounds widely used in agricultural, pharmaceuti-

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cal and other industrial processes. They are common environmental contaminants and water pollutants and they can be easily found in waste waters from which they migrate to the ground water. Both drugs are molecules having a free electron pair on their heteroatoms.

Na^+/K^+-ATPase and Mg^{2+}-ATPase are membrane-bound enzymes essential for neuronal cell functioning and cell homeostasis. Na^+/K^+-ATPase is involved in the restoration and maintenance of Na^+ and K^+ equilibrium through neuronal membranes, both at rest and after the passage of nerve impulse (see reviews by Stahl 1986; Albers et al. 1994). Since sodium gradient is important for the Ca^{2+} transport (Nikezić and Metlaš 1985) and consequently for re-uptake and release of neurotransmitters into and from nerve cells (Albers et al.1994), changes in Na^+/K^+-ATPase activity result in the modulation of neurotransmission. Therefore, the enzyme has a key role in the functioning of neuronal cells. On the other hand, the physiologic function of Mg^{2+}-ATPase in the brain remains unclear. According to some concepts, the enzyme may regulate hydrolysis of extracellular ATP, controlling ATP and adenosine levels in the synaptic cleft (James and Richardson 1993). These two enzymes have proven to be an excellent *in vitro* model for the testing of neurotoxicity of organic solvents (Tanii et al. 1994), monoketones (Huang et al. 1993; Tanii 1996) and polychlorinated biphenyls (Maier et al. 1994), since membrane proteins are considered to be the most important targets for the action of neurotoxic substances.

The present study was undertaken to examine the neurotoxicity of pyridine and urea, as measured by their ability and potency to alter the activity of the Na^+/K^+-ATPase and Mg^{2+}-ATPase, as models of nerve cell membrane function.

Materials and Methods

Experiments were performed on 3-month-old male Wistar albino rats from the local colony. Animals were kept under controlled illumination (lights on: 5:00 a.m. 5:00 p.m.) and temperature (23 ± 2°C), and had free access to food and water. After decapitation, brains were rapidly excised and pooled (6/pool) for immediate preparation of synaptic plasma membranes (SPM). The SPMs were isolated according to the method of Cohen et al. (1977), as modified by Towle and Sze (1983). The preparation procedure and the purity of SPM preparations were described previously (Horvat et al. 1995). The level of mitochondrial contamination, based on both morphological and biochemical markers, was less than 7%. Protein content was determined by the method of Lowry et al. (1951), as modified by Markwell et al. (1978).

ATPase assay

The ATPase activities were determined by a slightly modified colorimetric inorganic

phosphate method of Pennial (1966) The standard assay medium contained (in mmol/l) 50 Tris-HCl, pH 7 4, 1 EDTA, 100 NaCl, 20 KCl, 5 $MgCl_2$, 2 ATP, 25 μg SPM proteins, and 20 μl pollutant (various final concentrations as indicated) in a final volume of 200 μl The activity obtained in the presence of Mg^{2+} alone was attributed to the Mg^{2+}-ATPase activity Na^+/K^+-ATPase activity was calculated by subtracting the Mg^{2+}-ATPase activity from the total ATPase activity measured in the presence of Na^+, K^+ and Mg^{2+} SPMs were pre-incubated 10 minutes at 37°C in the presence of various concentrations of pyridine or urea Control samples were incubated with the same volume of bidistilled water The ATPase reaction was initiated by the addition of ATP, and carried out for 10 minutes at 37°C The reaction was terminated with 22 μl of 3 mmol/l perchloracetic acid and immediate cooling in ice water Enzyme activities were measured as Pi released during ATP hydrolysis, and expressed as μmol Pi mg proteins^{-1} min^{-1}

Data analysis

All experiments were done in triplicate and the results were expressed as mean ± S E M of at least 5 separate determinations The results were analyzed using Student's t-test, and significance was determined at the $p < 0\ 05$ level

Results

The neuroactive potencies of pyridine and urea were estimated by determining their ability to affect the Na^+/K^+-ATPase and Mg^{2+}-ATPase activity Results obtained by the spectrophotometric determination of Pi liberated during the hydrolysis of ATP are presented as Michaelis-Menten plot (ATPase activation vs pollutant concentration) Kinetic parameters K_m (dissociation constant) and ΔV_{max} (maximum of enzyme activation) were estimated by analysis of the data using the Michaelis - Menten equation The same set of data from the Michaelis-Menten plots were linearized according to the Hill equation for ligands that display sigmoidal dose-response curves (Segel 1968) and the Hill coefficients (n) were estimated

Effect of pyridine and urea on Na^+/K^+-ATPase activity

Both pyridine and urea stimulated Na^+/K^+-ATPase activity in a dose-dependent manner Fig 1 represents a Michaelis-Menten plot of Na^+/K^+-ATPase activity (after subtracting the control value of 0 252 ± 0 003 μmol Pi mg protein^{-1} min^{-1}) in the presence of pyridine (0 04 – 1 47 mmol/l) The curve is sigmoidal, with a saturation plateau at a pyridine concentration of 1 17 mmol/l The maximum of the enzyme stimulation reached was $\Delta V_{max} = 0\ 115 \pm 0\ 012$ μmol Pi mg protein^{-1} min^{-1}, and represented 40% ($p < 0\ 05$) stimulation as compared to the control value (without pyridine) Half-maximum stimulation occurred at pyridine concentration estimated to be $K_m = 0\ 80 \pm 0\ 02$ mmol/l pyridine The value of the Hill coefficient

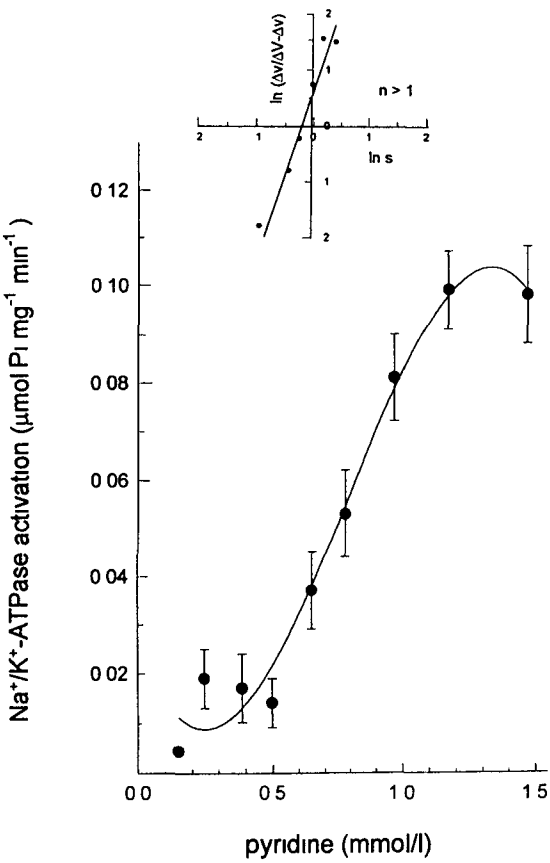

Figure 1. Michaelis-Menten plot of synaptosomal plasma membrane Na^+/K^+-ATPase activation in the presence of pyridine within 0 04–1 47 mmol/l The enzyme activity was measured in the incubation mixture as described in Materials and Methods, in the pres ence of increasing pyridine concentrations (0 04–1 47 mmol/l) The results are mean stimulation (μmol Pi mg^{-1} min^{-1}) ± S E M , from five separate experiments, each assayed in triplicate Control enzyme activity was measured in the absence of pyridine Mean control activity was 0 252 ± 0 003 μmol Pi mg $protein^{-1}$ min^{-1}
Inset to Figure 1 Hill plot ($\ln \Delta v/\Delta V - \Delta v$ vs $\ln S$) Δv – pyridine induced enzyme activation, ΔV – maximum enzyme stimulation, S – pyridine concentration (mmol/l)

$n > 1$ (inset to Fig 1) implies the presence of positive cooperativity in the binding of pyridine

Similar pattern of Na^+/K^+-ATPase stimulation (after subtraction of the control value) was observed in the presence of increasing concentrations of urea, in

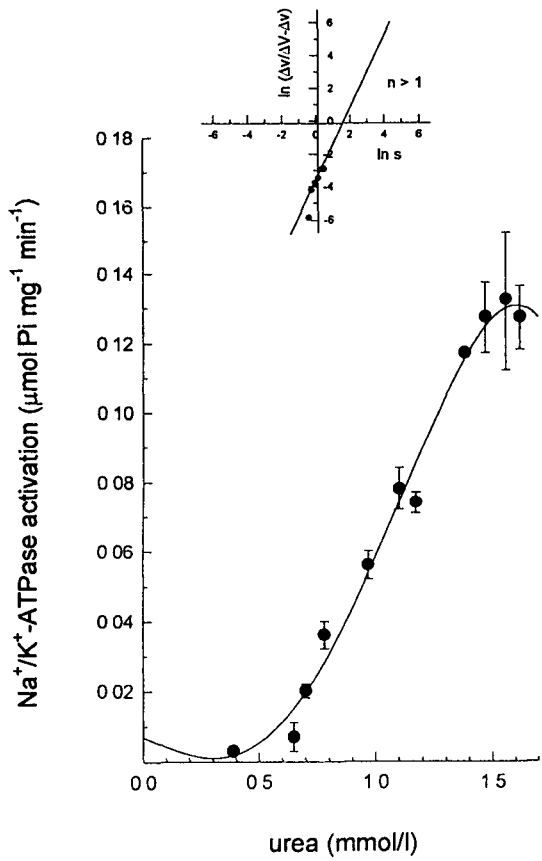

Figure 2. Michaelis-Menten plot of synaptosomal plasma membrane Na^+/K^+-ATPase activition in the presence of urea The enzyme activity was measured in the presence of increasing urea concentrations (0 04–1 63 mmol/l) The results are mean enzyme stimulation (μmol Pi mg protein^{-1} min^{-1}) after subtraction of the control value ± S E M , as determined from six separate experiments, each assayed in triplicate Control enzyme activity was measured in the absence of urea
Inset to Figure 2 Hill plot (ln $\Delta v/\Delta V - \Delta v$ vs ln S) Δv - urea - induced enzyme activation, ΔV - maximum enzyme stimulation, S - urea concentration (mmol/l)

the range of 0.04–1.63 mmol/l (Fig. 2). A plateau of the enzyme stimulation was observed in the presence of 1.5 mmol/l urea and was estimated to be ΔV_{max} = 0 156 ± 0 08 μmol Pi.mg proteins^{-1}.min^{-1}, which corresponded to 60% ($p < 0.05$) stimulation as compared to the control activity (without urea). Half-maximum stimulation of the enzyme was achieved in the presence of K_m= 1.12±0.03 mmol/l

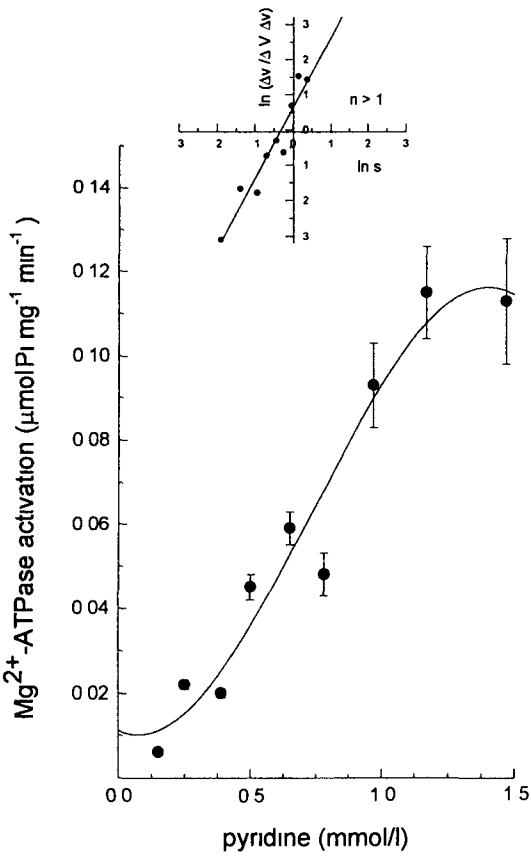

Figure 3. Michaelis-Menten plot of activation of synaptosomal plasma membrane Mg^{2+}-ATPase in the presence of pyridine The enzyme activity was measured (as described in Materials and Methods) in the presence of pyridine at the concentration range 0 04 1 47 mmol/l The results are expressed as mean enzyme stimulation (μmol Pi mg protein^{-1} min^{-1}) after subtraction of the control value ± S E M , as determined from five separate experiments each assayed in triplicate Mean control activity (obtained in the absence of urea) was 0 321 ± 0 011 μmol Pi mg protein^{-1} min^{-1}
Inset to Figure 3 Hill plot (ln $\Delta v/\Delta V - \Delta v$ vs ln S) Δv - pyridine induced enzyme activation ΔV maximum enzyme stimulation, S - pyridine concentration (mmol/l)

urea The value of the Hill coefficient $n > 1$ (inset to Fig 2) indicates the positive cooperativity of the enzyme for urea

Effect of pyridine and urea on Mg^{2+}-ATPase activity

Pyridine, at all concentrations tested within the range of 0 1–1 47 mmol/l, in-

creased the Mg^{2+}-ATPase activity Fig 3 presents the Michaelis-Menten plot of the enzyme activity (after subtraction of the control value of 0 321 ± 0 011 μmol Pi mg protein^{-1} min^{-1}) The curve is sigmoidal and the maximum of the enzyme stimulation, corresponding to 40% ($p < 0$ 05) stimulation compared to the control value, obtained in the presence of 1 17 mmol/l pyridine, was estimated ΔV_{max}= 0 142 ± 0 024 Half-maximum stimulation of the enzyme activity was detected at the pyridine concentration K_m= 0 78 ± 0 07 mmol/l The Hill coefficient $n > 1$ implies positive cooperativity for pyridine

In contrast, urea, at the same concentrations that stimulated Na^+/K^+-ATPase activity (0 1–1 63 mmol/l), produced no substantial change in Mg^{2+}-ATPase activity

Discussion

The first-hand effect of neurotoxic substances on the central nervous system is their impact on membrane-bound proteins, based on changes in the membrane lipid bilayer, or on their direct binding to proteins The structure of neurotoxic substances, in addition to their lipid solubility (Vaalavirta and Tahti 1995), seems to be important when considering the neuroactive potency of different organic compounds

In this work, we tested the neurotoxicity of pyridine and urea by their ability to alter the activities of synaptosomal membrane ATPases, previously shown to be good biomarkers for testing neurotoxicity of different organic compounds

In our study, both pyridine and urea at concentrations higher than 0 1 mmol/l stimulated the Na^+/K^+-ATPase activity The maximum stimulation obtained was 40% and 60% with pyridine and urea, respectively, compared to the control activity Half-maximum enzyme stimulation was observed in the presence of 0 80 mmol/l pyridine and 1 12 mmol/l urea, suggesting that Na^+/K^+-ATPase is more sensitive to pyridine than urea On the other hand, it seems that brain Mg^{2+}-ATPase is insensitive to urea, while pyridine exhibited a stimulating effect, with K_m being 0 78 mmol/l pyridine The Mg^{2+}-ATPase was stimulated by pyridine up to 40% compared to the control value, at the same pyridine concentration that evoked maximum stimulation of Na^+/K^+-ATPase

Comparing the effects of pyridine on Na^+/K^+-ATPase and Mg^{2+}-ATPase activity it could be seen that pyridine stimulated both enzymes with a uniform potency and to the same extent In addition, the Hill coefficients $n > 1$ of the two enzymes for pyridine indicated that pyridine has similar mode of action with respect to both enzymes Extensive studies have demonstrated that the neurotoxic potency of some organic compounds is due to their lipophilic nature, they disrupt the lipid micro-environment of the membranes, thereby changing the activity of membrane-bound enzymes such as ATPases Because of its lipophilic nature,

it can be assumed that pyridine interacts with some neuronal membrane component(s), inducing some disordering and changing the conformational state of membrane-bound proteins, consequently altering the activity of Na^+/K^+-ATPase and Mg^{2+}-ATPase and probably the neuronal receptor function, since they are all highly dependent on the lipid micro-environment (Ciofalo 1981)

Dissimilar to the uniform effect of pyridine on both ATPases, urea displayed selective action, stimulating the Na^+/K^+-ATPase solely. One previous study (Ratnakumari et al 1995) reported a significant increase in brain Na^+/K^+-ATPase activity in rats with congenital and acquired disorders of urea cycle enzymes. The mechanism by which urea activates Na^+/K^+-ATPase is ambiguous. Possible explanations involve direct action on the ATPase protein or indirect action via some ATPase regulatory mechanism, as was suggested for ammonia-induced stimulation of brain Na^+/K^+-ATPase (Kosenko et al. 1994). In conclusion, results presented herein show that both pyridine and urea possess neurotoxic potential, as judged by their ability to stimulate the activity of Na^+/K^+-ATPase and/or Mg^{2+}-ATPase *In vitro* exposure of the brain tissue to pyridine and urea is associated with the stimulation of membrane ATPases, followed by hyperpolarisation of the neuronal membrane and depression of neuronal firing. The stimulation of Na^+/K^+-ATPase in such conditions could indirectly alter the transport of amino-acids, sugars, phosphates and Ca^{2+}, consequently interfering with the overall mechanism of neurotransmission and neural cell functioning. Such changes could play a significant role in the pathophysiology of chronic CNS exposure to pyridine and urea. Further studies will be performed to test *in vivo* effects of those drugs

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