Effect of Chronic Exposure to Aluminium on Isoform Expression and Activity of Rat (Na⁺/K⁺)ATPase

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The ability of aluminum to inhibit the (Na⁺/K⁺)ATPase activity has been observed by several investigators. The (Na⁺/K⁺)ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both catalytic (α) and regulatory (β) subunits. For instance, three main α (α1, α2 and α3) and three β (β1, β2 and β3) subunit isoforms exist in vertebrate nervous tissue, whereas only α1 and β1 have been identified in kidney. However, no studies have focused on determining the change in (Na⁺/K⁺)ATPase isoforms caused by chronic exposure to aluminum and its relation with aluminum toxicity. In this study, adult male Wistar rats were submitted to chronic dietary AlCl₃ exposure (0.03 g/day of AlCl₃ for 4 months), and the activity and protein expression of (Na⁺/K⁺)ATPase isozymes were studied in brain cortex synaptosomes and in kidney homogenates. The intracellular levels of adenine nucleotides, plasma membrane integrity, and aluminum accumulation were also studied in brain synaptosomes. Aluminum accumulation upon chronic dietary AlCl₃ administration significantly decreased the (Na⁺/K⁺)ATPase activity measured in the presence of nonlimiting Mg-ATP concentrations, without compromising protein expression of α-subunit isoforms in brain and kidney. Aluminum-induced synaptosomal (Na⁺/K⁺)ATPase inhibition was due to a reduction in the activity of isozymes containing α₂-α₂ and α₂-β₂-subunits. The onset of enzyme inhibition was accompanied by a decrease of the (Na⁺/K⁺)ATPase sensitivity to submicromolar concentrations of ouabain, and it preceded major damage in plasma membrane integrity and energy supply, as revealed by the analysis of lactate dehydrogenase leakage and endogenous adenine nucleotides. The data suggest that, during chronic dietary exposure to AlCl₃, brain (Na⁺/K⁺)ATPase activity drops, even if no significant alterations of catalytic subunit protein expression, cellular energy depletion, and changes in cell membrane integrity are observed. Implications regarding underlying mechanisms of aluminum neurotoxicity are discussed.

Key Words: Aluminum; (Na⁺/K⁺)ATPase; ouabain; brain; kidney; rat.

The geochemical processes controlling the cycling and availability of aluminum are significantly modified under the influence of anthropogenic activity. These changes are well documented in the literature (see Exley, 2003 for a recent review), whereas the impact of increased bioavailability of aluminum in the biosphere are not as well known. Human health problems resulting from aluminum exposure are based mainly on acute aluminum poisoning, amyotrophic lateral sclerosis/Parkinson–dementia complex of Guam, renal osteodystrophy, anemia (microcytic, hypochromic), and dialysis encephalopathy produced by uptake of aluminum in excess in people treated with hemodialysis for renal disease. Three organ systems are clearly implicated in the toxic effects of aluminum: bone, the hematopoietic system, and the nervous system. Within the nervous system, aluminum is associated with morphological and biochemical changes that tend to reduce nerve synapses and conduction, promoting neurotoxicity (Yokel, 2000; Yokel et al., 2001).

The effects of aluminum on brain gene transcription have been investigated (Lukiw et al., 1998). Aluminum appears to be localized in the chromatin region of nuclei, where relatively strong interaction between Al(III) and DNA occurs (Wu et al., 2005). Moreover, aluminum compounds are capable of inducing a significant increase in cytogenetic damage, and it is recognized that aluminum interrupts peptide synthesis (Lukiw et al., 1998). Nevertheless, there are few indications of a causal relationship between aluminum-induced genetic damage and the appearance of neurotoxicity.

One of the mechanisms of neuronal destruction in degenerative brain damage is apoptosis, a regulated process of cell death with characteristic morphological changes that include nuclear condensation and fragmentation, DNA damage, cell shrinkage, membrane blebbing, and the formation of membrane-bound apoptotic bodies. Aluminum compounds have been shown to induce neurodegeneration and apoptotic effects in several experimental models (Johnson et al., 2005; Savory et al., 2003). Several studies have indicated that oxidative stress, release of calcium from intracellular stores,
and perturbation of mitochondrial function may represent important steps in the mechanisms underlying neuronal cell death induced by aluminum (Johnson et al., 2005; Savory et al., 2003). Furthermore, the mechanisms by which aluminum interacts with apoptotic pathways are only partially understood.

The \((\text{Na}^+/\text{K}^+)\)ATPase (EC 3.6.3.9) is the largest protein complex in the family of P-type ATPases expressed in all living organisms. It is essential for the generation and maintenance of \(\text{Na}^+\) and \(\text{K}^+\) gradients between the intracellular and extracellular milieux, a prerequisite for basic cellular homeostasis and for functions of specialized tissues. The \((\text{Na}^+/\text{K}^+)\)ATPase consists of two obligatory subunits, the catalytic \(\alpha\) subunit and a regulatory glycoprotein \(\beta\) subunit, and a third non-obligatory proteolipid component belonging to the FXYD gene family of small ion-transport regulators. Individual genes of at least four \(\alpha\)-subunit isoforms and three \(\beta\)-subunit isoforms of \((\text{Na}^+/\text{K}^+)\)ATPase have been identified in mammals (Serluca et al., 2001). Appropriate enzyme expression and activity adapted to changing physiological demands are assured by a variety of regulatory mechanisms for post-translational modification and short-term regulation and by complex regulation of the expression of isozymes (Blanco and Mercer, 1998; Cornelius and Mahmoud, 2003; Geering, 2001; Jorgensen et al., 2003; Kaplan, 2002; Mobasher et al., 2000). For example, in neurons where three \(\alpha\)-subunit isoforms are present, during rest conditions the basal ionic gradients are maintained mainly by isozymes containing the \(\alpha_1\) and \(\alpha_2\) subunits, whereas upon depolarization and repeated firing of action potentials those isozymes labor at saturation and maximal activation of \(\alpha_3\), assuring the restoration of the resting membrane potential.

Failure of the \((\text{Na}^+/\text{K}^+)\)ATPase has been implicated in the pathophysiology of neurodegenerative diseases. Yu (2003) has summarized the critical role of \((\text{Na}^+/\text{K}^+)\)ATPase in signal transduction and cell death pathways (apoptosis, necrosis, and hybrid cell death). Furthermore, the precise sequences of events at the cellular and subcellular levels that follow failure of \((\text{Na}^+/\text{K}^+)\)ATPase remain to be identified in vivo. Recent studies have demonstrated that tissue-specific \((\text{Na}^+/\text{K}^+)\)ATPase inhibition plays a fundamental role in apoptosis, and they also suggested that inhibition of this enzyme activity may directly trigger cell death or markedly increase cell susceptibility to other apoptotic insults (Xie and Cai, 2003; Yu, 2003).

The inhibitory effect of in vivo and in vitro exposure to aluminum on \((\text{Na}^+/\text{K}^+)\)ATPase activity has been observed by several investigators (Caspers et al., 1994; King et al., 1983; Lai et al., 1980; Lal et al., 1993; Rao, 1992; Sarin et al., 1997; Silva and Gonçalves, 2003). However, no studies have analyzed the changes in the \((\text{Na}^+/\text{K}^+)\)ATPase isoforms that result from chronic aluminum exposure. Thus in the present study we determined whether in vivo exposure to aluminum was associated with specific changes in activity and protein expression of membrane-bound \((\text{Na}^+/\text{K}^+)\)ATPase isozymes.

**MATERIALS AND METHODS**

**Materials.** The primary polyclonal antibodies anti-NASE, anti-HERED, anti-TED were kindly offered by Dr. Thomas Pressley from Texas Tech University, Health Sciences Center. Secondary antibodies were obtained from Chemicon International and from Amersham Biosciences (Little Chalfont, UK). The protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin, and antipain), the Kit Enzyline LDH/HBDH Optimisé Unitaire and the chemiluminescence system were purchased from Sigma Chemical Co. (St. Louis, MO), bioMérieux (Carnaxide, Portugal), and Amersham Biosciences (Buckinghamshire, UK), respectively. Amersham Biosciences (Little Chalfont, UK) was the source of Polyvinylidene difluoride (PVDF) Hybrid-P membranes. All other reagents were of analytical grade.

**Chronic exposure to aluminium chloride.** One-month-old male Wistar rats (weight ~250 g) were obtained from Harlan Interfama Ibérica, S. L., and housed individually in standard laboratory cages in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The environment was maintained at a temperature of 20±1°C and a humidity of 50±10%. Animals \((n = 10)\) were randomly assigned to be chronically exposure to aluminum. AlCl₃ (0.03 g) was diluted in a small volume of deionized water and added to cheese curled with lemon juice just before administration, to ensure the voluntary and rapid ingestion of the total dose. This regime results in exposure to 3.60 g of AlCl₃, orally administered as 120 doses (once daily) over 4 months. Control animals \((n = 10)\) received cheese curled with lemon juice for the duration of the treatment. Food and water were provided ad libitum to both animal groups until sacrifice. Food and beverages represent the most common route of exposure to aluminium for the general population. The amount of AlCl₃ to be administered was established according to previously reported values of oral uptake that promote neurotoxicity.

**Tissue preparation and cellular fractionation.** All steps were carried out at 4°C. The collected organs were rapidly removed, chopped, and homogenized in a tenfold volume of ice-cold 320 mM sucrose and 10 mM HEPES-Tris at pH 7.4. Synaptosomal fractions were prepared from brain cortex homogenates by differential centrifugation as described elsewhere (Silva and Gonçalves, 2003). The final pellet was resuspended in 0.32 M sucrose, 10 mM HEPES-Tris (pH 7.4) at a final concentration of ~8 mg protein/ml, as determined by the Biuret method. The samples were frozen in liquid nitrogen and maintained at –80°C until used.

**Aluminium quantification.** The samples were prepared by chemical precipitation of the organic matter by the addition of ice-cold 0.2 N HNO₃ and 8% trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm for 10 min. The resulting supernatants were used for the determination of aluminum concentrations at 309.3 nm in an atomic absorption spectrometer equipped with a graphite furnace (PerkinElmer 4100-VL) with auto-sampler AS-70. The aluminum concentrations in the samples are expressed as ng Al³⁺/mg protein.

**Western blot analysis.** Western blot assays were performed as described previously (Pressley, 1992). Protein samples were pre-treated with a lysis buffer (1 mM EDTA, 1 mM EGTA, 2 mM MgCl₂, 25 mM HEPES, pH 7.5) supplemented with 100 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM 1,4-dithiothreitol (DTT), and 1:1000 of a mixture of protease inhibitors, containing chymostatin, pepstatin A, leupeptin, and antipain (1 mg/ml). Then, the samples were mixed (1:1) with SDS-loading buffer (6% SDS, 20% glycerol, 5 mM EDTA, 0.25 M Tris-HCl, at pH 6.8, and 0.02% of bromophenol blue). Samples were heated at 60°C for 5 min, centrifuged (14,000 × g for 10 min, at room temperature), and electrophoresed on a 7.5% sodium dodecyl sulfate (SDS)—polyacrylamide gel. Samples were transferred to PVDF membranes at 0.75 A for 90 min, at 4°C. The membranes were washed and incubated with TBS-T (0.1% Tween, 150 mM NaCl, and 25 mM Tris–HCl at pH 7.6), supplemented with 5% (w/v) skim dry milk (M-TBS-T) for 2 h to block nonspecific antibody binding. The membranes were incubated with antibodies...
directed against the $\alpha_1$, $\alpha_2$, and $\alpha_3$-subunit isoforms of the (Na$^+$/K$^+$)-ATPase, respectively, using the rabbit polyclonal antibodies, anti-NASE (1:1,000), anti-HERED (1:500), and anti-TED (1:1,000) in 0.5% (w/v) M-TBS-T overnight at 4°C. The antibodies were raised against NASE (KNPNASEPKHLL), HERED (KHEREDSPQSHVL), and TED (KHETEDPNDNRYL) sequences of the rat (Na$^+$/K$^+$)-ATPase $\alpha_1$, $\alpha_2$, and $\alpha_3$-subunits, respectively (Pressley, 1992). Actin expression was used as a loading control by incubating membranes with a mouse anti-actin monoclonal antibody (1:500) for 2 h at room temperature in 0.5% (w/v) M-TBS-T. The membranes were incubated with anti-rabbit and anti-mouse IgG secondary antibodies (1:20,000), for 2 h at room temperature with gentle shaking, and developed using ECF fluorescence reagent. Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA). The fluorescence signal was analyzed with the QuantityOne software.

Assessment of lactate dehydrogenase leakage. The integrity of the synaptosomal membrane was analyzed by monitoring the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the incubation medium. LDH activity was measured spectrophotometrically, by using the Enzylime LDH/HBDH Optimise™ Unitaire kit, which follows the rate of conversion of NADH to NAD$^+$ at 340 nm. The absorbance was recorded at different time intervals and enzymatic activity was calculated. One unit of enzymatic activity (U) was defined as the product of oxidation of 1 $\mu$mol of NADH/min at room temperature. Total lysis, corresponding to 100% of LDH activity on the sample, was determined after treatment with Triton X-100 (1%). Lactate dehydrogenase leakage was expressed as a percentage of the total LDH activity in the synaptosomes.

Assessment of adenine nucleotides. Aliquots (1 mg protein) of freshly prepared synaptosomal fractions were extracted with ice-cold 0.2 M perchloric acid and centrifuged at 15,800 $\times$ g for 5 min at 4°C. The resulting supernatants were neutralized with KOH/Tris (10/5 M), stored at −80°C and assayed for adenine nucleotides (ATP, ADP, and AMP) by separation via reverse-phase high performance liquid chromatography (HPLC), as described elsewhere (Stocchi et al., 1985). The results were expressed as nmol/mg protein, and the energy charge potential (ECP) was calculated following the equation:

$$ECP = \frac{ATP + 0.5 \times ADP}{ATP + ADP + AMP}$$ (1)

In vitro (Na$^+$/K$^+$)-ATPase activity assay. The (Na$^+$/K$^+$)-ATPase activity of freeze-thawed samples was determined by measuring the release of inorganic phosphate (P$_i$) associated with the hydrolysis of ATP (Silva and Gonçalves, 2005). Aliquots (100 $\mu$g protein) were incubated in 1 ml of reaction medium (128 mM NaCl, 5 mM KCl, 10 mM MgCl$_2$, 100 $\mu$M EGTA and 10 mM HEPES-Na, pH 7.4) and various concentrations of ouabain, a selective inhibitor of the (Na$^+$/K$^+$)-ATPase. The reactions were initiated by adding Mg-ATP and stopped by dilution with ice-cold TCA (5% final concentration). The precipitated protein was discarded by centrifugation, after which the supernatant was collected and the inorganic phosphate content of the samples was quantified by colorimetric reaction with the molybdate reagent (5% ferrous sulfate and 1% ammonium molybdate prepared in 1 N H$_2$SO$_4$). The reactions were carried out for 5 min at room temperature. Simultaneously, a standard curve of KH$_2$PO$_4$ (0–85 $\mu$M) was prepared. The (Na$^+$/K$^+$)-ATPase activity was taken as the difference between the amount of P$_i$ produced during 5 min in the absence or in the presence of 5 mM ouabain. The dose–response curves of (Na$^+$/K$^+$)-ATPase to ouabain were analyzed by nonlinear fitting with a sum of one to three terms of Equation 2, assuming the presence of one to three independent, non-interconvertible, saturable inhibitory processes, exhibiting different affinities for ouabain (Berrehbi-Bertrand et al., 1990):

$$(Na^+K^+)ATPase Activity(\% of maximal) = \frac{V_{\text{max}}}{10^{-(l-KIC\_50)}} - 1$$ (2)

where $V_{\text{max}}$ is the maximal velocity observed in the absence of ouabain, $l$ is the decimal logarithm of ouabain concentration, and $K_I$ is the decimal logarithm of the 50% inhibitory concentration ($IC_{50}$).

Data analysis. All the data obtained were treated statistically with the “Microcal Origin 6.0” computer program. The results are presented as means ± S.E. of the number of experiments indicated in Figures 1–4 and Tables 1–2. Statistical significance between the two groups was analyzed by the unpaired two-tailed Student’s $t$-test.

RESULTS

Dietary administration of 3.60 g of AlCl$_3$ for a period of 120 days (0.03 g/day) was sufficient to produce a significant enrichment in aluminum of synaptosomes isolated from rat brain cortex, because the total aluminum content increased from 10.9 ± 1.2 in control rats to 17.5 ± 0.7 ng Al$_{3+}$/mg protein ($p < 0.01$) in AlCl$_3$-exposed rats during the experimental period. Measurement of the leakage of components from the cytoplasm into the surrounding extracellular medium has been widely accepted as a valid method to estimate cell viability based on the integrity of the cell membrane. Determination of leakage of the cytosolic lactate dehydrogenase (LDH) activity is one of the indicators of cell damage. Table 1 shows that LDH released from synaptosomes isolated from rat brain after dietary administration of 3.60 g of AlCl$_3$ for 120 days (0.03

FIG. 1. Inhibition of synaptosomal (Na$^+$/K$^+$)-ATPase activity by chronic exposure to aluminum. Freeze-thawed synaptosomes (0.1 mg protein/ml) were incubated during 5 min, at 35°C, in a medium containing 128 mM NaCl, 5 mM KCl, 10 mM MgCl$_2$, 100 $\mu$M EGTA and 10 mM HEPES-Na (pH 7.4), in the absence and in the presence of 5 mM ouabain. The reactions were started by adding Mg-ATP to obtain final concentrations of 1 mM. After 5 min, the reactions were stopped and the (Na$^+$/K$^+$)-ATPase activity was determined as described in the text. □, Control conditions (synaptosomes isolated from control rats); and ■, in vivo exposure to AlCl$_3$ (synaptosomes isolated from rats that received 0.03 g/day of AlCl$_3$ during 4 months). Data are the mean ± S.E. of six independent determinations. Statistical significance ****$p < 0.001$, compared to the control.
Day) was comparable to the LDH released from synaptosomes isolated from control rats, indicating that chronic exposure to aluminum failed to produce significant damage of synaptosomal membranes. It should be noted that the activity of the cytosolic marker measured in lysed synaptosomes was 0.018 U/mg protein, in synaptosomes isolated from brain cortex of either control rats or AlCl3-treated rats. Synaptosomal susceptibility was also evaluated on both synaptosomal preparations by the analysis of intrasynaptosomal adenine nucleotides. Adenine nucleotides provide ready-to-use substrates for carrying out normal cellular activities, and their levels are maintained by a balance between their production and degradation by specific enzymatic reactions. The levels of ATP/ADP and energy charge potential (ECP), which reflects the molar fraction of high-energy bond (Hardie and Hawley, 2001), were thereby analyzed to provide information on the metabolic status of nerve terminals after exposure to dietary aluminum. Upon exposure to aluminum, this balance was not significantly perturbed, and both ATP/ADP and ECP remained unchanged (Table 2). In fact, we did not observe significant changes in the amount of ATP (from 4.91 ± 0.15 to 5.03 ± 0.48 nmol/mg protein), ADP (from 1.49 ± 0.27 to 2.07 ± 0.43 nmol/mg protein) or AMP (from 4.56 ± 0.03 to 4.03 ± 0.50 nmol/mg protein). ATP represented 44.8% and 45.2% of the total adenine nucleotide content of synaptosomes isolated from control and aluminum-exposed rats, respectively.

As shown in Figure 1, in the presence of 1 mM Mg-ATP, which corresponds to a substrate-saturating concentration, the (Na+/K+)ATPase [ATP phosphohydrolase (Na+/K+-exchanging) (EC 3.6.3.9)] activity of freeze-thawed synaptosomes isolated from brain cortex of AlCl3-treated rats was significantly reduced (30%) when compared to the activity exhibited by freeze-thawed synaptosomes isolated from brain cortex of control rats. Since previous results demonstrated that AlCl3 concentrations up to 100 µM in the reaction medium does not change the ouabain-binding affinity of the (Na+/K+)ATPase
Effect of AlCl₃ Exposure on Synaptosomal Energy Adenine Nucleotides Balance

<table>
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<th>ATP/ADP</th>
<th>ECP</th>
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<tr>
<td>Control</td>
<td>3.42 ± 0.73</td>
<td>0.52 ± 0.01</td>
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<tr>
<td>In vivo exposure to AlCl₃</td>
<td>2.82 ± 0.40</td>
<td>0.55 ± 0.01</td>
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Note. Quantification of adenine nucleotides was performed by HPLC in perchloric acid extracts of synaptosomes isolated from control rats (control condition) and from rats that received 0.03 g/day of AlCl₃ during 4 months (in vivo exposure to AlCl₃). Data are the mean ± S.E. of 2–6 independent determinations.

(Na⁺/K⁺)ATPase activity by aluminum (Fig. 1) appears to precede impairment of energy balance because ATP level was maintained at near control levels for the total period of aluminum exposure.

The (Na⁺/K⁺)ATPase is composed of stoichiometric amounts of two obligatory major polypeptides, the α-subunit (~112 kDa) and the β-subunit (~45 kDa). The binding sites for ATP, cations and ouabain are localized in the α-subunit, which is responsible for the catalytic activity of the enzyme. Three distinct isoforms of the α-subunit have been identified in the central nervous system of vertebrates (Mobasheri et al., 2000; Sweadner, 1992). To confirm the expression of α₁, α₂, and α₃ subunits of (Na⁺/K⁺)ATPase in rat brain synaptosomal fraction (Foley and Linnoila, 1993), three antibodies against the catalytic subunit were used in the present study, namely anti-NASE, anti-HERED, and anti-TED. Western blot analysis revealed the presence of all the three α-subunit isoforms in the synaptosomes, because major immunoreactive bands of the three specific antibodies with relative molecular weights of 93 kDa (α₁-subunit), 92 kDa (α₂-subunit), and 98 kDa (α₃-subunit) were detected (Fig. 2A), although α₂-subunit was shown to be highly expressed (Fig. 2A and B). The (Na⁺/K⁺)ATPase α₁-, α₂-, and α₃-subunits are known to have different sensitivities to ouabain (Blanco and Mercer, 1998). Taking that into account, the synaptosomal isoform pattern was also checked by measuring the ATP phosphohydrolase dependence on the concentration of ouabain presented in the reaction medium (Fig. 2C). The dose–response curves for ouabain inhibition of membrane-bound (Na⁺/K⁺)ATPase activity exhibited the typical profile, currently interpreted as reflecting the presence of three (Na⁺/K⁺)ATPase α-subunit isoforms with distinct sensitivities to ouabain in brain tissue. To discriminate the ouabain sensitivities of the isozymes presented in the synaptosomal preparation, the experimental data were fitted to a curve described by an equation consisting of the sum of three functions assuming the presence of a very high, a high, and a low affinity, independent, non-interconvertible, saturable inhibitory processes exhibiting different affinities for ouabain (Fig. 2D). Since the experimental data were close to those obtained by nonlinear fitting of the titration curves, this approach was used to
calculate the 50% inhibitory concentration ($IC_{50}$) for ouabain of the hydrolytic reaction carried out by synaptosomal ($Na^+/K^+$)ATPase. We verified that the enzyme activity could be depicted in a very-high-sensitive ($IC_{50} = 5.4 \times 10^{-10}$ M), a high-sensitive ($IC_{50} = 3.7 \times 10^{-7}$ M), and a low-sensitive ($IC_{50} = 5.2 \times 10^{-5}$ M) component. These values were consistent with previously reported ouabain sensitivity of rat brain ($Na^+/K^+$)ATPase. The expression of the three subunit isoforms from control and AlCl$_3$-treated rats ($\alpha_1$-subunit, $\alpha_2$-subunit, and $\alpha_3$-subunit) remained almost unchanged upon exposure to AlCl$_3$ (Fig. 2C), whereas the $\alpha_1$-isoform of the total enzyme activity results from concurrent ATP depletion as a result of mitochondrial impairment and/or formation of non-hydrolysable ATP complexes (Kohila et al., 2004; Panchalingam et al., 1991). Aluminum appears to act as a noncompetitive inhibitor of the ATP hydrolysis by the ($Na^+/K^+$)ATPase (Caspers et al., 1994; Silva and Gonçalves, 2003), and total inhibition has never been reported in the range of micromolar concentrations. The functional activity of ($Na^+/K^+$)ATPase in brain homogenates (Fig. 4A). In accordance with data obtained in brain synaptosomes, a decrease in ($Na^+/K^+$)ATPase activity was observed in kidney homogenates after chronic exposure to aluminum (Fig. 3). Nevertheless, no significant changes in ($Na^+/K^+$)ATPase $\alpha_1$-subunit expression were observed in the AlCl$_3$-treated group, compared to the control (Fig. 4B). It is interesting to note that the isoform pattern in AlCl$_3$-exposed rat heart and brain total homogenates, in which the $\alpha_1$, $\alpha_2$, and $\alpha_3$-subunits are present, remained unchanged, and no significant increment in aluminum content was observed (data not shown). Conversely, the ($Na^+/K^+$)ATPase activity was significantly reduced (42%) after in vivo exposure to AlCl$_3$ (Fig. 3). The dose–response curves for ouabain inhibition of the enzyme activity overlapped in the range of high concentrations ($5 \times 10^{-5}$ M) of ouabain (Fig. 4C) and the $IC_{50}$ remained almost unchanged during exposure to AlCl$_3$, exhibiting values of $6.1 \times 10^{-5}$ M (control conditions) and $3.7 \times 10^{-4}$ M (in vivo exposure) (Fig. 4D). In contrast, in vivo exposure to AlCl$_3$ completely abolished the inhibition of ($Na^+/K^+$)ATPase activity by ouabain in the range of concentrations below $10^{-6}$ M.

These results strongly suggest that aluminum accumulation in nerve terminals during chronic dietary AlCl$_3$ administration decreases the ($Na^+/K^+$)ATPase activity without compromising the expression of $\alpha$-subunit isoforms, and it does this before any significant damage to the synaptic plasma membrane or perturbation of energy levels occurs.

**DISCUSSION**

The cause of ($Na^+/K^+$)ATPase inhibition by aluminum remains controversial (Caspers et al., 1994; King et al., 1983; Lai et al., 1980; Lal et al., 1993; Rao, 1992; Sarin et al., 1997; Silva and Gonçalves, 2003). Some authors have proposed stabilization of the phosphorylated form of the enzyme changing in the structure of plasma membrane (Caspers et al., 1994; Lal et al., 1993; Rao, 1992; Sarin et al., 1997). Others have suggested that failure of the hydrolytic activity results from concurrent ATP depletion as a result of mitochondrial impairment and/or formation of non-hydrolysable Al-ATP complexes (Kohila et al., 2004; Panchalingam et al., 1991). Aluminum appears to act as a noncompetitive inhibitor of the ATP hydrolysis by the ($Na^+/K^+$)ATPase (Caspers et al., 1994; Silva and Gonçalves, 2003), and total inhibition has never been reported in the range of micromolar concentrations. The functional activity of ($Na^+/K^+$)ATPase in brain homogenates (Fig. 4A). In accordance with data obtained in brain synaptosomes, a decrease in ($Na^+/K^+$)ATPase activity was observed in kidney homogenates after chronic exposure to aluminum (Fig. 3). Nevertheless, no significant changes in ($Na^+/K^+$)ATPase $\alpha_1$-subunit expression were observed in the AlCl$_3$-treated group, compared to the control (Fig. 4B). It is interesting to note that the isoform pattern in AlCl$_3$-exposed rat heart and brain total homogenates, in which the $\alpha_1$, $\alpha_2$, and $\alpha_3$-subunits are present, remained unchanged, and no significant increment in aluminum content was observed (data not shown). Conversely, the ($Na^+/K^+$)ATPase activity was significantly reduced (42%) after in vivo exposure to AlCl$_3$ (Fig. 3). The dose–response curves for ouabain inhibition of the enzyme activity overlapped in the range of high concentrations ($5 \times 10^{-5}$ M) of ouabain (Fig. 4C) and the $IC_{50}$ remained almost unchanged during exposure to AlCl$_3$, exhibiting values of $6.1 \times 10^{-5}$ M (control conditions) and $3.7 \times 10^{-4}$ M (in vivo exposure) (Fig. 4D). In contrast, in vivo exposure to AlCl$_3$ completely abolished the inhibition of ($Na^+/K^+$)ATPase activity by ouabain in the range of concentrations below $10^{-6}$ M. These results strongly suggest that aluminum accumulation in nerve terminals during chronic dietary AlCl$_3$ administration decreases the ($Na^+/K^+$)ATPase activity without compromising the expression of $\alpha$-subunit isoforms, and it does this before any significant damage to the synaptic plasma membrane or perturbation of energy levels occurs.

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The cause of ($Na^+/K^+$)ATPase inhibition by aluminum remains controversial (Caspers et al., 1994; King et al., 1983; Lai et al., 1980; Lal et al., 1993; Rao, 1992; Sarin et al., 1997; Silva and Gonçalves, 2003). Some authors have proposed stabilization of the phosphorylated form of the enzyme changing in the structure of plasma membrane (Caspers et al., 1994; Lal et al., 1993; Rao, 1992; Sarin et al., 1997). Others have suggested that failure of the hydrolytic activity results from concurrent ATP depletion as a result of mitochondrial impairment and/or formation of non-hydrolysable Al-ATP complexes (Kohila et al., 2004; Panchalingam et al., 1991). Aluminum appears to act as a noncompetitive inhibitor of the ATP hydrolysis by the ($Na^+/K^+$)ATPase (Caspers et al., 1994; Silva and Gonçalves, 2003), and total inhibition has never been reported in the range of micromolar concentrations. The functional activity of ($Na^+/K^+$)ATPase in brain homogenates (Fig. 4A). In accordance with data obtained in brain synaptosomes, a decrease in ($Na^+/K^+$)ATPase activity was observed in kidney homogenates after chronic exposure to aluminum (Fig. 3). Nevertheless, no significant changes in ($Na^+/K^+$)ATPase $\alpha_1$-subunit expression were observed in the AlCl$_3$-treated group, compared to the control (Fig. 4B). It is interesting to note that the isoform pattern in AlCl$_3$-exposed rat heart and brain total homogenates, in which the $\alpha_1$, $\alpha_2$, and $\alpha_3$-subunits are present, remained unchanged, and no significant increment in aluminum content was observed (data not shown). Conversely, the ($Na^+/K^+$)ATPase activity was significantly reduced (42%) after in vivo exposure to AlCl$_3$ (Fig. 3). The dose–response curves for ouabain inhibition of the enzyme activity overlapped in the range of high concentrations ($5 \times 10^{-5}$ M) of ouabain (Fig. 4C) and the $IC_{50}$ remained almost unchanged during exposure to AlCl$_3$, exhibiting values of $6.1 \times 10^{-5}$ M (control conditions) and $3.7 \times 10^{-4}$ M (in vivo exposure) (Fig. 4D). In contrast, in vivo exposure to AlCl$_3$ completely abolished the inhibition of ($Na^+/K^+$)ATPase activity by ouabain in the range of concentrations below $10^{-6}$ M. These results strongly suggest that aluminum accumulation in nerve terminals during chronic dietary AlCl$_3$ administration decreases the ($Na^+/K^+$)ATPase activity without compromising the expression of $\alpha$-subunit isoforms, and it does this before any significant damage to the synaptic plasma membrane or perturbation of energy levels occurs.
different tissues and cell types is closely related to the existence of multiple isozymes consisting of different subunit isoforms and displaying different kinetic properties (see Blanco and Mercer, 1998; Cornelius and Mahmoud, 2003; Jorgensen et al., 2003; Kaplan, 2002; Mobasheri et al., 2000, for recent reviews).

We demonstrated that the protein expression levels of the (Na+/K+)ATPase catalytic subunit is not diminished in rat kidney, heart, or brain after chronic exposure to dietary aluminum (daily supply of 0.03 g of AlCl3 during 4 months). The quantitative analysis of ouabain dose–response curves and Western blotting analysis of immunopositive bands using antibodies directed against specific amino acid sequences of the α1-, α2-, and α3-subunit isoforms of the (Na+/K+)ATPase (Figs. 2A and 2B and 4A and 4B) revealed that aluminum exposure did not significantly modify both the relative expression level of each catalytic subunit isoform and the characteristic isoform expression profile of the examined tissues. The decrease of expression level of one of the (Na+/K+)ATPase α-subunit isoforms is usually associated with a shift from one isoform to another or accompanied by a parallel decrease of all isoform levels, despite each isoform being encoded by a different gene (Charlemagne et al., 1994; Serluca et al., 2001).

Comparison of the α-subunit isoform expression profile did not reveal any insightful differences that accounted for the observed differences in (Na+/K+)ATPase activity (Figs. 1 and 3). Therefore, this is the first experimental demonstration that an aluminum-induced decrease of maximal enzyme activity cannot be attributed to the reduction of the expression levels of the catalytic subunits. These results, combined with outstanding similarity of in vivo and in vitro aluminum effects on the enzyme activity previously reported by Silva and Gonçalves (2003), support the hypothesis that aluminum either acts on post-transcriptional regulatory mechanisms or directly interacts with the enzyme. Under our experimental conditions, aluminum inhibited all three α-subunit isoforms of the (Na+/K+)ATPase, which does not support FXYD protein-mediated inhibitory action of aluminum. The FXYD regulatory subunits act in a tissue- and αβ-protomer–specific manner, modifying the Na+ and/or K+ affinities of the enzyme (Cornelius and

FIG. 4. Effect of aluminum exposure on endogenously expressed kidney (Na+/K+)ATPase and on isozyme activity. At the end of the experimental period (120 days), tissue homogenates were prepared from kidney of control rats (●) and rats that received 0.03 g/day of AlCl3 (■). Panel A. Kidney proteins (100 µg/lane) were separated by SDS-PAGE and subsequently immunoblotted with anti-NASE (α1-subunit), anti-HERED (α2-subunit), anti-TED (α3-subunit), and actin (α-actin) antibodies, as described in the text. Data shown are typical for seven independent experiments. Panel B. Densitometric analysis of the Western blots. Panel C. Dose–response curves for the ouabain inhibition of the ATPase activity. Experimental conditions are similar to those described in the legend of Figure 3, except that reactions were performed in the absence and in the presence of increasing ouabain concentrations (0–0.001 M). Data are expressed as nmol Pi/min/mg protein, and are the mean ± S.E. of 8–16 independent determinations. Panel D. Normalized dose–response curves for the ouabain inhibition of the (Na+/K+)ATPase activity of experimental data plotted in Panel C and expressed as a percentage of maximal activity in control conditions (○) and in vivo exposure to AlCl3 (●), respectively. The curves represent the fitting by Equation 2, assuming the presence of one saturable inhibitory process by ouabain.
that favor maximal activation of the (Na\(^+\)/K\(^+\))ATPase, which makes it improbable that the noncovalent αβ-protomer will reassemble during in vivo exposure to AlCl\(_3\). Typically, different αβ-subunit combinations with the same catalytic subunit isoform differ with regard to control of biosynthesis and properties, such as Na\(^+\), K\(^+\) and ATP affinities (Geering, 2001). Furthermore, the binding sites for ATP and cations are localized in the α-subunit and only the β-protomers are able to carry out (Na\(^+\)/K\(^+\))ATPase activity. The reduction by AlCl\(_3\) of ATPase activity under conditions that favor maximal activation of the (Na\(^+\)/K\(^+\))ATPase reported here (Figs. 1 and 3) has been consistently observed by other investigators (Caspers et al., 1994; King et al., 1983; Lai et al., 1980; Lal et al., 1993; Rao, 1992; Sarin et al., 1997), and it is difficult to reconcile with simple aluminum-induced dissociation of αβ-protomeric units, because the aluminum inhibitory effect requires the presence of high ATP concentrations and does not occur when uridine triphosphate (UTP), a poor ATP substitute at low-affinity nucleotide binding sites, is used as a hydrolytic substrate (Silva and Gonçalves, 2003).

As evidenced from our data, aluminum-induced inhibition of (Na\(^+\)/K\(^+\))ATPase activity is resistant to cell fractionation (isolation of synaptosomes) and freeze-thaw procedures, suggesting that it remains trapped on the membrane fraction. It is interesting to note that when assessed by in vitro approaches, much higher aluminum chloride concentrations were required (at micromolar range), and the extent of the inhibition appeared to be time-dependent (Caspers et al., 1994; Lai et al., 1980). Taking together, these results seem to implicate high-affinity and low accessibility of aluminum binding sites that are responsible for the inhibition of (Na\(^+\)/K\(^+\))ATPase. In fact, it was previously suggested that after in vivo aluminum exposure, substantial amounts of aluminum remain bound to high molecular weight proteins (Julka et al., 1996). Although the results presented in this work do not define the mechanism responsible for AlCl\(_3\)-induced alteration of (Na\(^+\)/K\(^+\))ATPase activity, they are in agreement with the hypothesis that aluminum induces impairment of the interprotomeric interaction within the oligomeric ensemble of membrane-bound (Na\(^+\)/K\(^+\))ATPase (Silva and Gonçalves, 2003). It remains to be clarified whether aluminum interacts directly with the subunits of the enzyme or interferes indirectly with the oligomerization of the membrane-bound enzyme’s protomeric functional units of the (Na\(^+\)/K\(^+\))ATPase, after aluminium-induced alterations of membrane fluidity, enhancement of free radical production, and impairment of the phosphorylation cascade (Boldyrev, 2001; Laughery et al., 2004).

The present study provides data related to the analysis of membrane integrity (Table 1) and energy status (Table 2) of synaptic nerve terminals obtained from rat brains chronically exposed to aluminum. Because no significant changes were observed in LDH leakage, ECP values, or ATP/ADP levels, the results are consistent with the idea that these parameters reflect the maintenance of cell viability (Hardie and Hawley, 2001). Moreover, an adequate energy supply seems to be assured during the aluminum exposure protocol reported in this work, because the overall high-energy phosphate content, reflected by the ECP, was maintained. In fact we may consider that, inhibition of (Na\(^+\)/K\(^+\))ATPase (the major energy consumer in a cell) would be beneficial for preserving high intracellular ATP level. It is increasingly recognized that, depending on intensity/duration parameters of the insult, aluminum can produce cell death either by apoptosis or by necrosis (Johnson et al., 2005), and maintenance of ATP is required for apoptotic cell death pathways (Kim et al., 2003). Accordingly, Savory et al. (2003) demonstrated that agents interfering with the mitochondrial and/or the endoplasmic reticulum–mediated apoptosis cascade have the ability to prevent aluminum-induced apoptosis in rabbit brain, which is in line with the idea that modifications in the intracellular calcium homeostasis and potentiation of transition metal pro-oxidant action could mediate toxic effects during chronic aluminum exposure. Moreover, Julka and Gill (1996) observed that exogenous addition of glutathione, an endogenous antioxidant, could only partially reverse Ca\(^2+\)-ATPase inhibition after in vivo administration of aluminum, whereas desferrioxamine, an aluminum chelator, produced total reversion. Thus inhibition of (Na\(^+\)/K\(^+\))ATPase could be interpreted as a way the cells signal leads to eventual cell death through apoptosis (Yu, 2003).

Apparently, in vivo aluminum-induced partial inhibition of (Na\(^+\)/K\(^+\))ATPase activity should promote a continuous reduced enzyme activity irrespective of subtle differences in adapting cellular enzyme activity to specific physiological requirements. Instead, in vivo exposure to AlCl\(_3\) seemed to specifically reduce the (Na\(^+\)/K\(^+\))ATPase sensitivity to ouabain at submicromolar concentrations (Figs. 2C and 4C), and to prevent the stimulatory effect produced by ATP binding with low affinity (Silva and Gonçalves, 2003). According to several investigators, both features—the ouabain very highly sensitive inhibition and the stimulatory ATP effect—are even more pronounced when the membrane-bound (Na\(^+\)/K\(^+\))ATPase exhibits high levels of oligomerization (Boldyrev, 2001; Laughery et al., 2004). Moreover, high-affinity cardiotonic steroid binding capacity of the enzyme is relevant for (Na\(^+\)/K\(^+\))ATPase–mediated signal transduction within caveolae (Xie and Cai, 2003). Chemical cross-linking between neighbouring active α-β protomers (oligomerization) is a specific process, giving rise to the formation of complexes highly sensitive to changes in microviscosity and signaling molecules (Boldyrev, 2001). The presence of (Na\(^+\)/K\(^+\))ATPase within caveolae provides a means of explaining the importance of multimeric interactions between active protomeric ensembles, since these membrane morphological and functional units are involved in signaling pathways, starting from the plasma membrane. It is
clear that inhibition of the (Na\(^+/K^+\))ATPase leads, in a cell-specific manner, to activation of multiple signaling pathways (i.e., MEK/MAPK-ERK and Src family kinase pathways), to endoplasmic reticulum inositol 1,4,5-trisphosphate receptor-mediated intracellular Ca\(^{2+}\) oscillations and to mitochondrial production of reactive oxygen species (see Xie and Cai, 2003 for review).

In conclusion, the present study shows that during aluminum exposure, inhibition of (Na\(^+/K^+\))ATPase activity occurs, preceding possible alterations of expression of catalytic subunits, cellular energy depletion, and disturbances in cellular membrane integrity. The decrease in total (Na\(^+/K^+\))ATPase activity is assured by partial inhibition of isoforms containing \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\)-subunits. In addition, aluminum-induced inhibition of the enzyme activity was maintained post-exposure; therefore the preparative procedures and activity assays might have contributed to underestimates of the degree of inhibition of (Na\(^+/K^+\))ATPase in in vivo aluminum-exposed rats. These data also support the concept that (Na\(^+/K^+\))ATPase inhibition occurs at early stages of the neurotoxic action, because no evidence of cell death/membrane disruption or energy depletion were observed in synaptosomes isolated from AlCl\(_3\) chronically exposed rats.

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