Neurochemical Research, Vol. 28, No. 6, June 2003 (© 2003), pp. 903–910

A Comparative Study between a Brain Na^+,K^+-ATPase Inhibitor (Endobain E) and Ascorbic Acid

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(Accepted December 23, 2002)

In the search of Na^+,K^+-ATPase modulators, we have reported the isolation by gel filtration and HPLC of a brain fraction, termed endobain E, which highly inhibits Na^+,K^+-ATPase activity. In the present study we compared some properties of endobain E with those of ascorbic acid. Kinetic experiments assaying synaptosomal membrane K^+-*p*-nitrophenylphosphatase (K^+-*p*-NPPase) activity in the presence of endobain E or ascorbic acid showed that in neither case did enzyme inhibition prove competitive in nature versus K^+ or *p*-NPP concentration. At pH 5.0, endobain E and ascorbic acid maximal UV absorbance was 266 and 258 nm, respectively; alkalinization to pH 14.0 led to absorption drop and shift for endobain E but to absorbance disappearance for ascorbic acid. After cysteine treatment, endobain E absorbance decreased, whereas that of ascorbic acid remained unaltered; iodine treatment led to absorbance drop and shift for endobain E but to absorbance disappearance for ascorbic acid. HPLC analysis of endobain E disclosed the presence of two components: one eluting with retention time and UV spectrum indistinguishable from those of ascorbic acid and a second, as yet unidentified, both exerting Na^+,K^+-ATPase inhibition.

KEY WORDS: Endobain; K^+-*p*-nitrophenylphosphatase inhibitor; Na^+,K^+-ATPase inhibitor; ascorbic acid.

INTRODUCTION

Na^+,K^+-ATPase is the enzymatic version of the sodium pump, involved in several physiological functions such as cell volume regulation, cell differentiation, and maintenance of sodium/potassium equilibrium through membranes (1,2). The enzyme concentrates at nerve ending membranes (3), where sodium exit and potassium entry occur during nervous impulse transmission and diverse efforts have been devoted to the search of Na^+,K^+-ATPase modulators (4,5).

Ascorbic acid behaves as a potent inhibitor of Na^+,K^+-ATPase activity (6–8) and has been proposed as a candidate for ouabain-like activity of tissues and biological fluids (9,10). In this connection, bovine adrenal gland extracts have been found to inhibit isolated Na^+,K^+-ATPase and their active principle, identified as ascorbic acid, though it is incapable of inhibiting the sodium pump in intact cells (11). On seeking an inhibitor of Na^+,K^+-ATPase from beef brain, ascorbic acid has been isolated and proved to inhibit [^{3}H]ouabain binding, an effect attributed to a decrease in ouabain binding sites by reduction of a group within the ATP binding site of the enzyme (12).

We have already reported the isolation from rat cerebral cortex by gel filtration in Sephadex G-50 of

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two fractions, peaks I and II, able to stimulate and inhibit Na^+,K^+-ATPase activity, respectively (13). Besides inhibiting the activity of Na^+,K^+-ATPase (but not of other membrane-associated enzymes such as Mg^{2+}-ATPase, acetylcholinesterase, and 5′-nucleotidase), peak II induces diuresis and natriuresis (14), blocks high-affinity ^{3}H-ouabain binding (15), and induces neurotransmitter release (16). Taken jointly, these findings indicate that the fraction behaves much like ouabain, so that the term *endobain* has been coined (17). From peak II, by anionic exchange HPLC in a Synchropak AX-300 column, a subfraction (II-E) has been separated (18), which not only inhibits Na^+,K^+-ATPase activity but also shares other properties with ouabain, and hence is termed II-E (*endobain E*) (19).

An endogenous ligand of the glycoside binding sites on Na^+,K^+-ATPase may exert a wide spectrum of physiological functions, including modulation of neurotransmitter release. In support, we have observed that endobain E enhances norepinephrine release in rat hypothalamus (20).

In the present study we compared some properties of commercial ascorbic acid with those of brain endobain E, including kinetics of synaptosomal membrane K^+-*p*-NPPase interaction, UV absorbance profiles under diverse experimental conditions as well as chromatographic behavior.

EXPERIMENTAL PROCEDURE

Animals and Drugs. Adult male Wistar rats weighing 130–150 g were employed. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA. Reagents were analytical grade. Ouabain, disodium *p*-NPP, and 2,6-dichlorophenolindophenol sodium salt were from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of Synaptosomal Membranes. Synaptosomal membranes were prepared according to the method developed in our laboratory (3). For each preparation, cerebral cortices from five rats were pooled and homogenized at 10% (w/v) in 0.32 M sucrose (neutralized to pH 7 with Tris base solution) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenate was processed by differential centrifugation to separate the nuclear and mitochondrial fractions. The crude mitochondrial pellet was resuspended at 10% (w/v, original tissue) in redistilled water (pH 7 with Tris base) and homogenized during 2 min for osmotic shock. A pellet containing mitochondria, nerve ending membranes, and myelin was separated by centrifugation at 20,000 × *g* for 30 min, then resuspended in 0.32 M sucrose and layered on top of a gradient containing 0.8, 0.9, 1.0, and 1.2 M sucrose. The gradient was centrifuged at 50,000 × *g* for 2 h in a SW 28 rotor of an L8-Beckman ultracentrifuge; the fraction at 1.0 M sucrose level was separated, diluted with 0.16 M sucrose, and spun down at 100,000 × *g* for 30 min. The pellet was stored at −70°C and, prior to enzyme assay, resuspended by brief homogenization in redistilled water to reach a final concentration of 8.5–10.5 mg protein per ml, stored frozen and used for 3 weeks without appreciable change in enzyme activities.

Preparation of II-E Fraction (Endobain E). Peak I and II fractions from rat cerebral cortex were prepared as previously described (18,21). Thus, for each preparation, cerebral cortices from five rats were pooled, homogenized at 25% (w/v) in redistilled water, and centrifuged at 100,000 × *g* for 30 min in a 70.1 rotor of an L8-Beckman ultracentrifuge. A 5-ml supernatant sample (brain soluble fraction) was taken to pH 7.4 with 0.1 M NH_4HCO_3 and loaded on a Sephadex G-10 column (1 × 20 cm) and a single 11-ml fraction collected. This filtrate was then applied to a column packed with Sephadex G-50 (1.8 × 25 cm). For gel equilibration and elution, 0.01 M NH_4HCO_3 was used. Fractions of 1.4 ml each at a flow rate of 0.3 ml per min were collected in a Gilson Fraction Collector 202. The absorbance profile was recorded at 280 nm. Peaks I and II were made up with the fractions presenting maximal UV absorbance. Peak I was made up with fractions 19–23 but not used in this study; peak II was made up with fractions 48–52. Peak II was adjusted to pH 2.0 with 2 M HCl, lyophilized, and stored at −20°C. The following day, peak II samples were processed by anionic exchange HPLC on a Synchropak AX-300 column, 250 × 4.6 mm (Synchrom, Inc., Lafayette, IN, USA), and eluted at a flow rate of 0.5 ml per min with a 20-min gradient from 0.001 to 0.010 M NH_4HCO_3 to separate fractions II-A to II-H. Fractions were collected by visual inspection of absorbance curves at 230 nm; II-E was collected at 12–13 min, then lyophilized, and used within 20 days (18). Hereafter, the II-E fraction is termed *endobain E.*

Enzyme Assay. Lyophilized endobain E samples were dissolved in 0.006 M HCl (at a concentration of 4 mg original tissue per μl) and immediately before assay neutralized with 0.2 M Tris base solution. *p*-NPPase activity was determined by measuring *p*-nitrophenol (*p*-NP) release (22). Mg^{2+}, K^+-*p*-NPPase activity was assayed in a medium containing 0.20 M Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 20 mM KCl, and 10 mM *p*-NPP, unless otherwise stated. Mg^{2+}-*p*-NPPase activity was determined in a similar medium without K^+ addition and containing 1 mM ouabain. The difference between activities was taken to correspond to K^+-*p*-NPPase. Before performing *p*-NPPase assay, synaptosomal membrane samples were preincubated with 0.16 M Tris-HCl buffer (pH 7.4) or with endobain E or ascorbic acid at 37°C for 10 min. During preincubation, samples contained 0.85–1.05 mg membrane protein and endobain E equivalent to 750 μg original tissue per μl or 5 mM ascorbic acid final concentration. Aliquots of preincubated fractions (10 μl) were distributed in two series of microtubes containing the respective medium (40 μl) for the assay of total- and Mg^{2+}-*p*-NPPase activities and incubated at 37°C for 20 min. The reaction was stopped with 0.1 M NaOH and released *p*-NP determined spectrophotometrically at 420 nm. In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0°C throughout the incubation period were processed to serve as blanks.

Protein Determination. Protein content in synaptosomal membranes was determined by the method of Lowry et al. (23) using bovine serum albumin as standard.

UV Absorbance of Endobain E and Ascorbic Acid after Several Treatments. Freshly prepared solutions were immediately treated as indicated below and UV absorbance profiles recorded in a Shimadzu UV-160A spectrophotometer.

pH Change. UV absorbance of endobain E and ascorbic acid solutions in distilled water was recorded in two conditions: (i) at pH 5.0 and (ii) at pH 14.0 (taken with 0.1 M NaOH); in the last case,

alkalinized samples were taken to pH 5.0 and 1.0 with 0.1 M HCl and UV absorbance recorded again.

Cysteine Treatment. Endobain E and ascorbic acid samples were prepared in 10 mM phosphate buffer, pH 6.8, containing 0.3% cysteine, and left at room temperature for 15 min; UV absorbance recorded against 0.3% cysteine solution.

Iodine Treatment. Endobain E and ascorbic acid solutions in distilled water were treated with 500-fold molar excess of 0.1 M iodine solution and UV absorbance recorded against the reagent.

Thin Layer Chromatography. Endobain E and ascorbic acid samples were applied onto 20 × 20 cm silica gel G plates and developed with ethyl acetate/pyridine/acetic acid/water (30:10:3:5) or butanol/ethyl acetate/acetic acid/water (1:1:1:1) as solvent systems. Thereafter, plates were air-dried and sprayed with 0.1% 2,6-dichlorophenolindophenol in ethanol.

Column Chromatography. HPLC was carried out on a 30 × 0.78 cm Bio Rad Aminex HPX-87H column (9 μm particle size) using 3 mM HCl as mobile phase at 0.6-ml/min flow rate. Elution was monitored with a 2140 LKB rapid spectral detector; peaks visualized at 210 nm were collected as separate fractions. Aliquots of each fraction were lyophilized and tested on enzyme assay.

RESULTS

To compare properties of ascorbic acid versus endobain E, K^+-*p*-NPPase kinetic experiments were run, UV absorbance profiles were recorded after diverse treatments and chromatographic behavior analyzed.

Synaptosomal membrane *p*-NPPase activities were assayed in the absence or presence of endobain E or commercial ascorbic acid. Basal K^+- and Mg^{2+}-*p*-NPPase activities assayed with 10 mM KCl and 20 mM *p*-NPP were respectively 12.0 ± 1.4 and 1.4 ± 0.4 μmol *p*-NP released per mg protein per hour (mean values ± SD, $n = 5$).

In the presence of variable K^+ concentration, the extent of K^+-*p*-NPPase inhibition by endobain E or ascorbic acid remained unaltered over the 1.25–40 mM range; results plotted according to Hanes-Woolf indicated that the slope of the plot [S]/v versus [S] was increased by endobain E or ascorbic acid and that the lines intercepted the horizontal axis at the same point (Fig. 1A and B).

Likewise, the extent of enzyme inhibition remained unaltered in the presence of 0.625–20 mM *p*-NPP substrate concentration; the evaluation of Hanes-Woolf plot for enzyme activity also indicated an increase in the slope by endobain E or ascorbic acid and the lines intercepted the horizontal axis at the same point for the former (Fig. 2A) but crossed quite near the vertical axis, roughly at 0 mM *p*-NPP for the latter (Fig. 2B). Present results indicated that endobain E and ascorbic acid exert, respectively, noncompetitive and uncompetitive inhibition versus *p*-NPP concentration, whereas both inhibitors show noncompetitive interaction versus K^+ concentration. Both endobain E and ascorbic acid decreased V_{max} values either versus K^+ or *p*-NPP concentration, whereas they failed to alter K_M values.

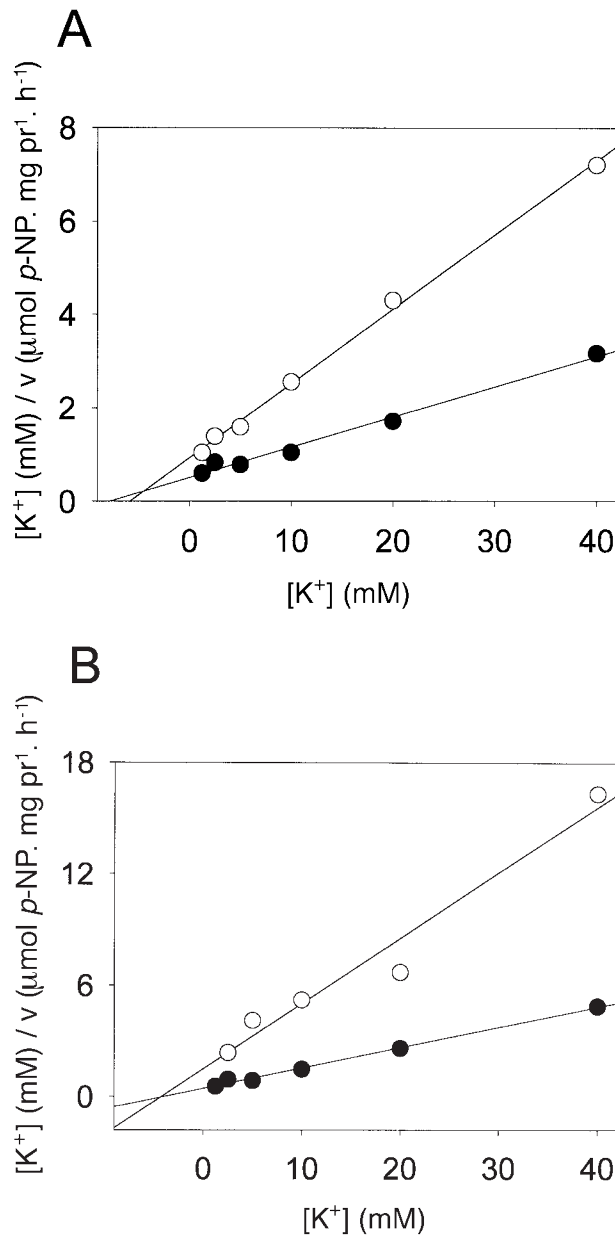

Fig. 1. Hanes-Woolf plots for synaptosomal membrane K^+-*p*-NPPase activity as a function of K^+ concentration. Enzyme activity was determined in the absence (●) or presence (○) of endobain E equivalent to 150 μg original tissue per μl (**A**) or 1mM ascorbic acid final concentration (**B**).

At pH 5.0, endobain E and ascorbic acid maximal UV absorbance was respectively 266 and 258 nm. Al-

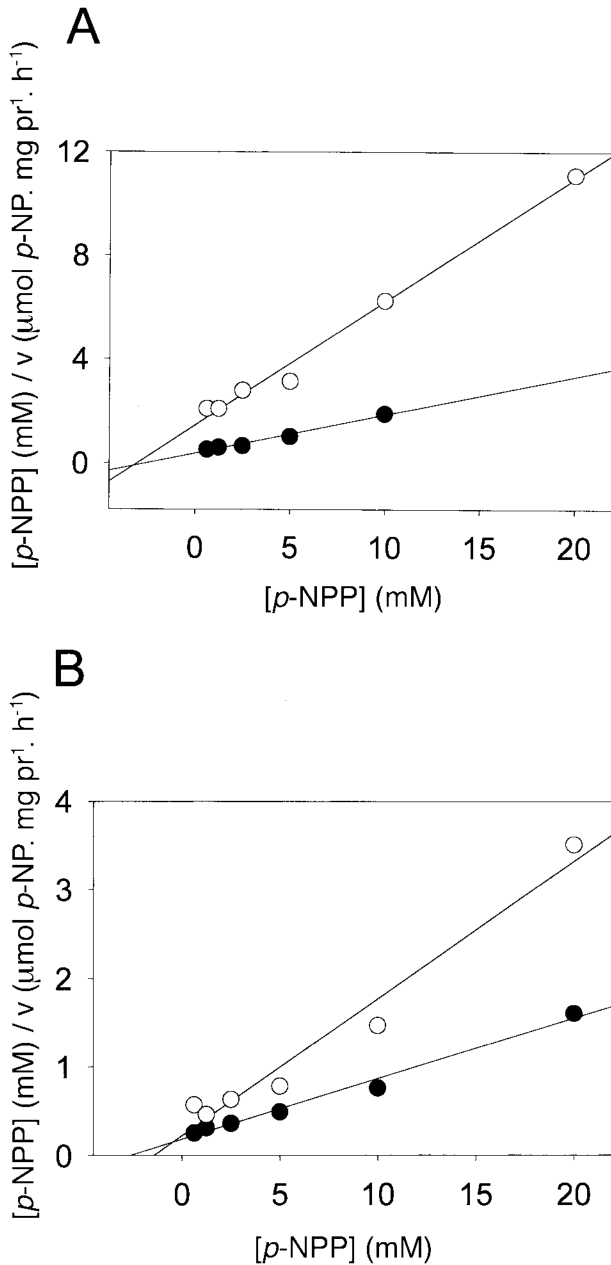

Fig. 2. Hanes-Woolf plots for synaptosomal membrane K^+-*p*-NPPase activity as a function of *p*-NPP concentration. Enzyme activity was determined in the absence (●) or presence (○) of endobain E equivalent to 150 μg original tissue per μl (**A**) or 1 mM ascorbic acid final concentration (**B**).

kalinization to pH 14.0 led to absorption drop and shift (→ 297 nm) for endobain E but to absorbance disappearance for ascorbic acid; initial profiles were not recovered after acidification to pH 5 (data not shown) or pH 1 (Fig. 3).

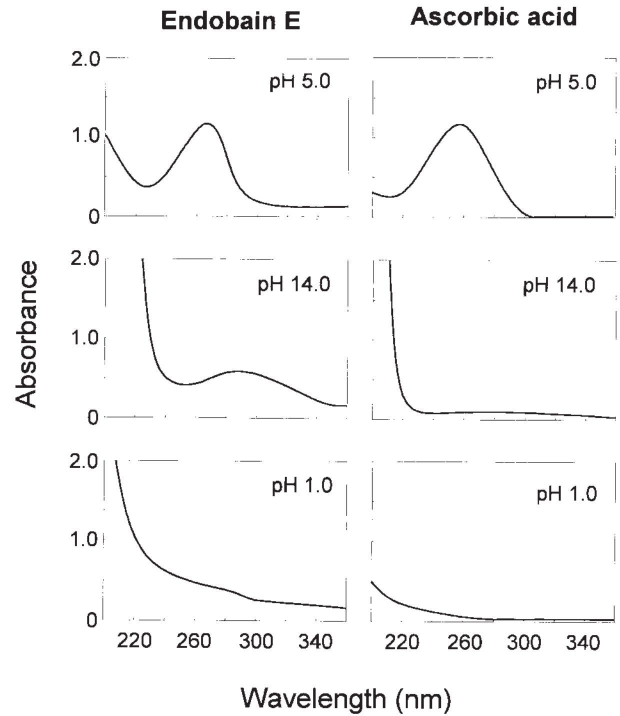

Fig. 3. UV absorbance profiles of endobain E and ascorbic acid at three pH values. Samples were taken to pH 5.0, 14.0, or 1.0, and absorbance spectra for endobain E (left) or ascorbic acid (right) recorded.

Endobain E maximal UV absorbance decreased by cysteine treatment whereas dropped and shifted by iodine treatment. Ascorbic acid UV spectrum presented maximal absorbance was unaltered by cysteine treatment but disappeared by iodine treatment (Fig. 4).

In TLC separations and testing for organic acids (with 2,6-dichlorophenolindophenol as developer), endobain E gave a spot with Rf values of 0.39 and 0.79 respectively using ethyl acetate/pyridine/acetic acid/water (30:10:3:5) and butanol/ethyl acetate/acetic acid/water (1:1:1:1) as solvent systems. Under these conditions, ascorbic acid Rf values failed to differ from those of endobain E.

Samples of endobain E, as well as commercial ascorbic and dehydroascorbic acids, were applied to an Aminex column for the analysis of small-size carbohydrates, organic acids, or other organic compounds. The elution profile at 210 nm showed that endobain E rendered several components, two of them, with retention times of 9.5 and 10.3 min, fully inhibited K^+-*p*-NPPase activity; the latter component, though showing, at 210 nm, higher UV absorbance than the former, presented almost no absorbance at 280 nm (Fig. 5). In turn,

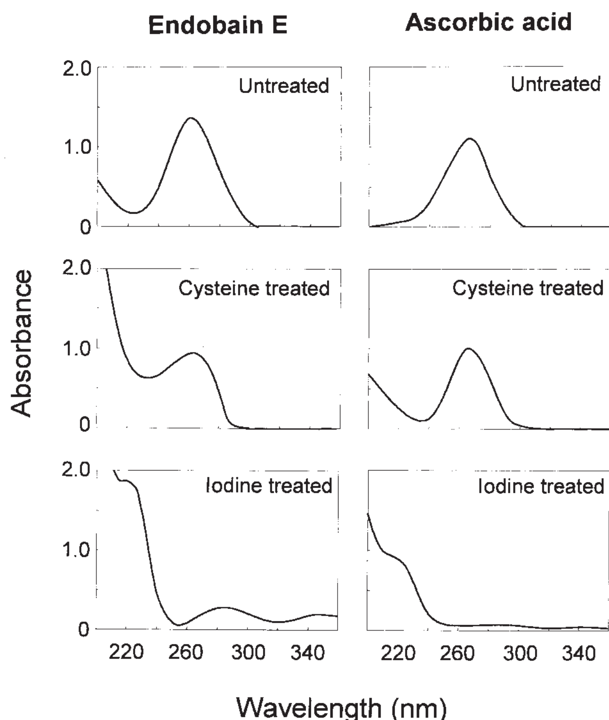

Fig. 4. UV absorbance profiles of untreated and cysteine- or iodine-treated endobain E and ascorbic acid. Samples were treated with cysteine or iodine and absorbance spectra for endobain E (left) or ascorbic acid (right) recorded.

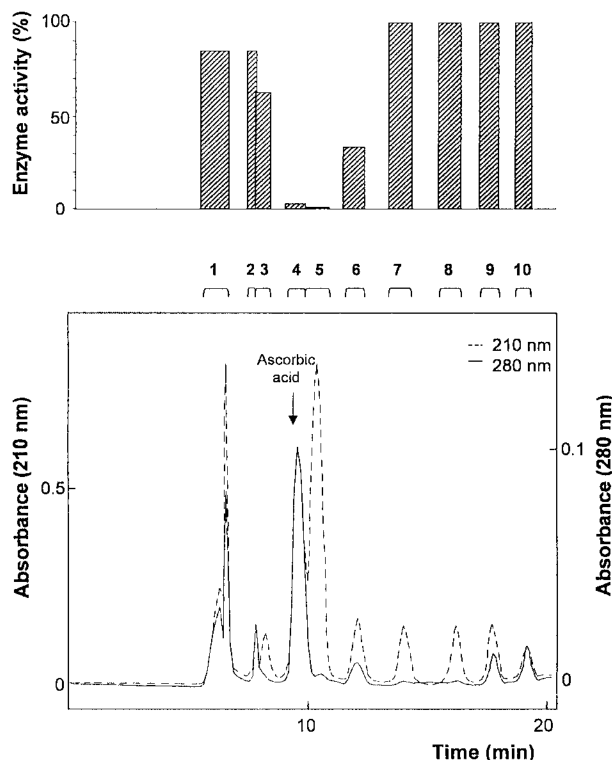

Fig. 5. Elution profile at 210 and 280 nm of endobain E chromatographed on a Bio Rad Aminex HPX-87H column (30 × 0.78 cm) and eluted with 3 mM HCl at 0.6-ml/min flow rate; arrow indicates standard ascorbic acid elution time. Synaptosomal membrane K^+-*p*-NPPase activity in the presence of added fractions is expressed as percentage enzyme activity taking as 100% values obtained without additions.

dehydroascorbic and ascorbic acid standards eluted, respectively, at 6 and 9.5 min.

DISCUSSION

In the present study, properties of a brain Na^+,K^+-ATPase inhibitor, endobain E, versus commercial ascorbic acid were compared. Their inhibitory action was assayed on synaptosomal membrane Na^+, K^+-ATPase activity by testing interaction kinetics on E-2 conformation using *p*-NPP as substrate. In addition, inhibitor UV absorbance profiles at diverse pH values and after treatment with cysteine or iodine were determined and chromatographic behavior studied.

Kinetic assays of synaptosomal membrane K^+-*p*-NPPase activity versus K^+ or *p*-NPP concentrations in the presence of endobain E or ascorbic acid showed that in neither case did enzyme inhibition prove competitive in nature. Uncompetitive inhibition was observed in the presence of ascorbate versus *p*-NPP, whereas noncompetitive interaction occurred with either inhibitor versus K^+ or with endobain E versus *p*-NPP. Both endobain E and ascorbic acid decreased V_{max} value, but they failed to alter K_M value.

UV absorbance profile was very similar for endobain E and ascorbic acid, though sample alkalinization produced a marked drop with a maximal shift for the former but absorbance disappearance for the latter. Further acidification failed to recover initial profiles; thus changes were most likely irreversible.

There were differences in endobain E and ascorbic acid UV absorbance profiles after cysteine treatment, with a decrease in maximal absorbance for the former but no change for the latter, indicating a dissimilar redox potential. The treatment with iodine, which changes ascorbic acid into dehydroascorbic acid, modified UV absorbance profile of both samples, indicating the presence of oxidizable components.

Both ascorbic acid and endobain E samples were positive to 2,6-dichlorophenolindophenol, and there was hardly any difference between Rf values of spots given in TLC under the two-solvent systems employed.

HPLC analysis in an Aminex column indicated that although dehydroascorbic acid was not present in endobain E sample, ascorbic acid was detected. However, it was evident the presence of two different ATPase inhibitors, one of them eluting at 9.5 min, with UV spectrum indistinguishable from standard ascorbic acid, and the other with 10.3 min retention time, showed almost no absorbance above 220 nm. Likewise, an Aquapore column also disclosed several components in endobain E, one most likely corresponding to ascorbic acid (data not shown).

The first evidence of isolation to purity of a reducing agent (hexuronic acid) from ox adrenal cortex was provided by Szent-Györgyi (24); the chemical structure of such substance was later established in several laboratories and received ascorbic acid as its trivial name (25). The ability of ascorbic acid to inhibit Na^+,K^+-ATPase has been largely documented (6–8). Interestingly, extracellular ascorbic acid levels normally range from 200 to 400 μM in striatal tissue (26) but increase over 2-fold during lengthy increases in extracellular glutamate (27,28). In the present conditions, 1 mM commercial ascorbic acid inhibited 40%–50% Na^+,K^+-ATPase present in synaptosomal membranes, assayed as K^+-*p*-NPPase activity.

A potential role of ascorbate as neuromodulator, together with evidence indicating that its release into extracellular brain fluid regulates dopaminergic and glutamatergic transmission has been advanced (29). Although ascorbic acid is liable to reduce dopamine uptake after prolonged incubation, it is routinely included in incubation media to prevent dopamine oxidation during uptake; when present in physiological concentrations (100–500 μM), it decreases dopamine uptake and Na^+,K^+-ATPase activity of striatal synaptosomes, an effect most likely due to lipid peroxidation involving oxidation of thiol groups (30). In this connection, it should be recalled that Na^+,K^+-ATPase activity is essential for dopamine transporter (31).

Extracellular levels of ascorbic acid in striatum fluctuate in relation to behavioral activation (32,33); thus the proposal that the neuromodulatory action of ascorbic acid contributes to behaviorally relevant changes in sensorimotor responsiveness has been advanced (34). In fact, electrophysiological studies performed in striatal neurons have shown that, similar to dopamine (35), ascorbic acid potentiates excitation induced by glutamate (34).

Although ascorbic acid enhances glutamate excitation, it is also able to attenuate glutamate response in striatal neurons according to dose and application period employed (34). This inhibitory effect may be related to the observation that ascorbic acid proved to decrease [^{3}H]glutamate and [^{3}H]thienylcyclohexylpiperidine binding to glutamatergic NMDA receptors (36). In this connection, endobain E behaves as a negative allosteric modulator of [^{3}H]dizocilpine, binding to cerebral cortex membranes (37,38).

On studying the effect of brain extracts on isolated Na^+,K^+-ATPase, the inhibitory agent has been proposed to be ascorbic acid (9,10), whose effect has been attributed to peroxidative degradation of unsaturated phospholipids essential for Na^+,K^+-ATPase activity (10,39).

Membrane lipoperoxidation has been involved in ascorbate-induced inhibition of brain Na^+,K^+-ATPase, an effect prevented by EDTA, at variance with that achieved by vanadyl (VO^{2+}) (40). However, under experimental conditions able to carefully remove contaminants, enzyme inhibition by ascorbic acid was achieved without peroxidation of membrane lipids metal ions, and inhibitory activity of adrenal extracts (proved to be ascorbic acid) is antagonized by EDTA. Whereas the inhibitory effect of adrenal endogenous compound on guinea pig brain Na^+,K^+-ATPase activity is significantly reduced by 0.1 mM EDTA (11), such a chelating concentration fails to alter endobain E modulatory effect on ligand binding to NMDA receptors (42).

Whereas adrenal extracts or ascorbic acid fail to inhibit the sodium pump, most likely indicating that the inhibitory site is not accessible in intact cells (11), endobain E has proven effective as a norepinephrine releaser in hypothalamic tissue (20) or as a phosphoinositide hydrolysis stimulator in neonatal brain prisms (43).

Ascorbic acid is widely distributed in several tissues, and the same Na^+,K^+-ATPase inhibitor would seem to be present and further isolated from adrenal gland, brain, and skeletal muscle guinea pig tissue. Therefore the enzyme inhibitory activity of endogenous substances reported by diverse authors may well be due to ascorbic acid. Whether as the major principle or merely as a contaminant in tissue extracts, ascorbic acid fails to meet the criteria for digitalis-like activity regarding its interaction with Na^+,K^+-ATPase (11).

Another difference between endobain E and ascorbic acid is related to their stability. Biological activity of endobain E decays over time even when stored at -20°C either dried or in acid solution (44). As is widely known, ascorbic acid is stable in powder form but not in solution.

In sumary, on comparing properties of endobain E versus ascorbic acid, similar Rf values but slight dif-

ferences in K^+-*p*-NPPase kinetics, UV absorbance at various pH values and after cysteine treatment was recorded. However, dissimilarities were found in UV absorbance after sample alkalinization, as well as in biological activity decay. HPLC analysis demonstrated the presence of ascorbic acid in endobain E samples, as well as that of a second Na^+,K^+-ATPase inhibitor, distinct to ascorbic acid, which merits further study.

ACKNOWLEDGMENTS

G. R. de L. A. and C. P. and Chief Investigators from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Financial support was provided from Universidad de Buenos Aires, CONICET and Agencia de Promoción Cientifica, Argentina.

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