Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 289-294

VANADIUM-DIASCORBATES ARE STRONG CANDIDATES FOR ENDOGENOUS OUABAIN-LIKE FACTORS IN HUMAN URINE:

Effects on Na-K-ATPase enzyme kinetics

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Received June 24, 1995

Summary. Recently, we isolated from the urine of salt-loaded healthy subjects a more polar ouabain-like factor OLF-1 and a more apolar OLF-2, the latter cross-reacted with a digoxin anti-body. They were purified to single compounds with dose-dependent Na-K-ATPase inhibition. Mass-spectroscopy (MS) showed a M_r of around 400 and ^{1}H-NMR- and IR-spectroscopy suggested diascorbic acid salts, i.e., vanadium (V) diascorbates (M_r 403) with similar elution times from RP-HPLC as OLFs. IC_{50} was 9×10^{-5}M for V^{IV}-diascorbate as compared to 2×10^{-6}M for V^{V}-diascorbate. Enzyme inhibition was non-competititive with respect to sodium and Mg-ATP; p-NPPase assay showed strong inhibition in its E2-configuration. We suggest that V-diascorbates represent endogenous OLFs excreted in human urine.

In 1969 we reported first experimental evidence for the existence of a natriuretic plasma fraction containing a ouabain-like factor (OLF), i.e. an endogenous compound which inhibited the Na-K-ATPase enzyme in vitro in a manner similar to that of ouabain (g-strophanthin) (1). We and others (e.g., 2,3) suggested that endogenous OLFs may play an important role in body fluid and blood pressure regulation, especially in essential hypertension. More recently, we found that the urine of salt-loaded subjects contains at least three OLFs with different polarities and cross-reaction with a digoxin antibody (4). Spectroscopic (UV, MS, NMR, IR) criteria of the more polar OLF-1 were similar to those of the more apolar OLF-2 and required a $-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-$ conformation. Since MS gave signals at around 400, these criteria strongly suggested that the urinary OLFs are identical with diascorbic acid salts (5), e.g., of vanadium which, when absorbed, is normally eliminated from the body by the kidney and excreted in the urine (6,7). Vanadium (V) is present inside the cell mainly in its tetravalent state (V^{IV}), i.e. as vanadyl or oxovanadium (VO^{2+}), which easily forms V-complexes with other molecules. The pentavalent state of V (V^{V}), i.e. vanadate, can be detected by NMR and is present in plasma and urine. Vanadium^{V} is a potent ATPase inhibitor from the luminal surface of the renal tubular epithelium (6). It is easily reduced, e.g., by ascorbate (7). In the present study we

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Abbreviations: EGTA: ethylene glycol bis(ß-aminoethyl ether)-N,N' -tetraacetic acid;IR: infra red; NMR: nuclear magnetic resonance; RP-HPLC: reversed-phase high performance liquid chromatography; TCA: trichloroacetic acid.

0006-291X/95 $12.00

therefore investigated the potency and mode of action of Na-K-ATPase inhibition of ascorbic acid and various diascorbates including V-diascorbic acid salts.

METHODS

In vitro-effects on Na-K-ATPase

In these experiments a purified Na-K-ATPase from hog cerebral cortex was employed which was purchased from Sigma Chemical (St. Louis, Mo., USA). Enzyme activity was determined as previously described (8). In short, incubation tubes contained 0.5 ml substrate solution to provide final concentrations of 1 mM ATP, 1 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 0.1 mM EGTA, and 10 mM imidazole-HCl buffer, pH 7.2. Inhibitor or buffer (0.3 ml) were added and the tubes were placed in a water bath at 37°C. The reaction was started by adding 0.1 ml enzyme preparation and was stopped after 5 min by adding 1.0 ml ice-cold 10% TCA. After centrifugation at 1,700 g for 10 min 1.0 ml supernatant was assayed for inorganic phosphate. All determinations were performed in triplicate and enzyme activities were expressed as μmoles of inorganic phosphate liberated per mg protein per hour (μmol P_i/mg protein x h). Na-K-ATPase activity averaged 26.9 ± 0.3 μmol P_i/mg protein x h. Since Mg-ATPase activity was less than 1.5% of total ATPase in this enzyme preparation when assayed in the presence of 1 mM ouabain, only total ATPase activities were determined in the absence or presence of inhibitors. For determination of IC_{50} serial dilutions of inhibitors were prepared. For comparison, the effects of ouabain were studied at final concentrations ranging from 10^{-9} to 10^{-4} M. To further characterize the enzyme inhibition kinetics studies were performed at concentrations of Na^+ ranging from 5 to 100, of K^+ from 0.5 to 20, and from Mg-ATP from 0.05 to 3.0 mM in the presence of constant IC_{50} of inhibitors (ouabain, 5×10^{-6}M; V^{IV}-diascorbate, 9×10^{-5}M; V^{V}-diascorbate, 2×10^{-6}M).

In vitro-effects on K^+-pNPPase

The enzyme assay was modified from the procedure described by Phillips et al (9) utilizing purified hog cerebral cortex Na-K-ATPase as the enzyme source. The incubation tubes contained 5 mM Mg^{2+}, 10 mM K^+, 100 mM Tris-HCl buffer (pH 7.4) and 0.05 ml of purified Na-K-ATPase. The reaction was started by adding p-nitrophenylphosphate (pNPP), yielding a final concentration of 5 mM pNPP. The reaction was stopped after 15 min by adding 1.0 ml of 1 M ice-cold NaOH. After centrifugation (1,700 g for 5 min) the supernatant was read at 410 nm against a standard p-nitrophenol (pNP) curve. To assess the inhibitory mechanisms of V^{IV}- and V^{V}-diascorbates on the E2 configuration of the Na-K-ATPase enzyme, kinetic studies were performed with concentrations of K^+ ranging from 0.125 to 5.0 mM and of pNPP ranging from 0.1 to 5.0 mM in the presence of constant amounts of inhibitors (see above). For K^+-pNPPase studies, a molar ratio of 1:1 for Mg and pNPP was maintained. Michaelis-Menten kinetic constants were calculated for Na-K-ATPase and for K^+-pNPPase from the conventional Lineweaver-Burk plot.
Diascorbate salts were synthesized in the Institute for Pharmaceutical Pharmacy by G.K.jr., University of Bonn. The structure of V^{IV}-diascorbate (V^{IV}-oxoascorbate) is shown in Fig.1.

RESULTS

Preliminary studies showed that ascorbic acid itself and its precursor L-gulono-y-lactone or the diascorbic acid salts of the divalent cations Ca, Mg, and Zn had no effects on Na-K-ATPase from

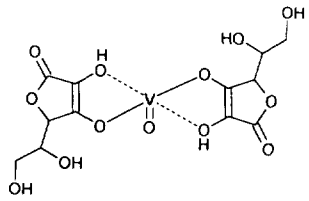

Figure 1. Structure of vanadium^{IV}-diascorbate (V^{IV}-oxoascorbate).

hog cerebral cortex. In contrast, strong enzyme inhibition by V-diascorbates was observed which showed similar elution patterns from RP-HPLC and the same UV-maximum as did OLF-2. For comparison Mo^{IV}-diascorbate did not inhibit the enzyme (Table 1). IC_{50}-values for V^{IV}- and V^{V}-diascorbates were 9 x 10^{-5}M and 2 x 10^{-6}M, respectively, as compared to 5 x 10^{-6}M for ouabain. The dose-response curves are shown in Fig. 2A. V-diascorbates dose-dependently inhibited K^+-pNPPase (Fig. 2B). IC_{50}-values for V^{IV}- and V^{V}-diascorbates were 5 x 10^{-7}M and 3 x 10^{-8}M, respectively.

The effects of V^{IV}- and V^{V}-diascorbates on Na-K-ATPase enzyme kinetics with respect to sodium and potassium are shown in Fig. 3A and B. Note that enzyme inhibition by V is enhanced by increasing K^+-concentration. Lineweaver-Burk plots revealed non-competitive inhibition with respect to sodium and Mg-ATP as substrates with K_i-values of approximately 0.7 mmol/L (Fig. 4A and 4B).

The kinetics of inhibition of the K^+-pNPPase enzyme by V-diascorbates with respect to p-NPP revealed non-competitive inhibition with K_i-values of approximately 2.2 mmol/L each (Fig. 5A). With respect to potassium uncompetitive inhibition was observed with K_i-values for V^{IV}- and V^{V}-diascorbate of 0.6 and 0.9 mmol/L, respectively, as compared to 1.2 mmol/L for the enzyme alone (Fig. 5B).

DISCUSSION

Recently, we isolated from the urine of salt-loaded healthy subjects by gel chromatography and reverse-phase HPLC a more polar OLF-1 which eluted in the water phase and a more apolar OLF-2 that eluted at 20% acetonitrile and cross-reacted with a digoxin antibody (4). By two-dimensional TLC both were purified to single compounds which dose-dependently inhibited Na-K-ATPase. Mass spectroscopy suggested a M_r of around 400 and ^{1}H-NMR- and IR-spectra of OLF-1 and OLF-2 suggested them to be identical with ascorbic acid or its salts (5) such as Ca-, Zn-, or V-diascorbate with M_r of 392, 419 and 403, respectively, whose IR-spectra, however, may differ slightly. Each of the synthetic preparations of ascorbic acid salts yields signals in the 126 to 181 range, thus exhibiting the fragment character of MS signals found with the original OLFs.

In the present study we found that neither ascorbic acid and L-gulono-y-lactone, its precursor in non-human mammals, nor the synthetic divalent metal salts Ca-, Mg-, and Zn-diascorbates, nor the tetravalent Mo^{IV}-diascorbate did affect the Na-K-ATPase enzyme. In contrast, V^{IV}- and, ten-fold stronger, V^{V}-diascorbate reveal a significant and dose-dependent inhibition of Na-K-ATPase. IC_{50} of 2 x 10^{-6}M for V^{V}-diascorbate is slightly lower than the IC_{50} of 1.5 x 10^{-5}M which we found for OLF-

Table 1. Na-K-ATPase by L-gulono-y-lactone, ascorbic acid, and salts of diascorbic acid at a final concentration of 2 x 10^{-4}M

Inhibitors	Na-K-ATPase activity (μmol P_i/mg protein/h)	(percent)
control	26.9	100
L-gulono-y-lactone	26.7	100
ascorbic acid	26.8	100
Ca^{++}-diascorbate	26.3	100
Mg^{++}-diascorbate	26.5	100
Zn^{++}-diascorbate	26.2	100
Mo^{IV}-diascorbate	26.3	100
V^{IV2+}-diascorbate	7.7	29
V^{V}- -diascorbate	0.8	3
ouabain	0.5	2

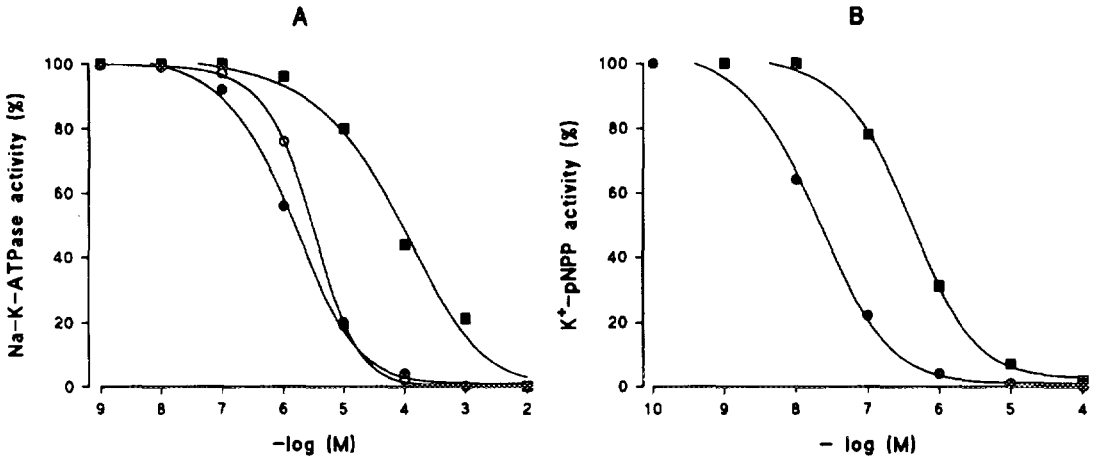

Figure 2. **(A)** Dose-response curves of Na-K-ATPase inhibition by V^{IV}- (closed squares) and V^{V}- (closed circles) diascorbates as compared to ouabain (open circles). **(B)** Dose-dependent inhibition of K^+-pNPPase by V-diascorbates.

1 and IC_{50} of 9 x 10^{-5}M for V^{IV}-diascorbate is close to the IC_{50} of 1.5 x 10^{-4}M which we found previously for in vitro-inhibition of the enzyme by OLF-2 (5). Inhibition is non-competititve with respect to sodium and Mg-ATP. K^+-pNPPase inhibition indicates inhibition of Na-K-ATPase in its E2-configuration. It is to note that the water solubility of the individual ascorbic acid salts of metals, including trace elements, varies remarkably. V-diascorbates elute from RP-HPLC at similar elution times and acetonitrile gradients as OLFs. V^{IV}-diascorbate also shows the same UV-maximum as we reported for OLF-2 (5). Thus, V^{IV}- and V^{V}-diascorbates, with their different water solubilities, are strong candidates for the urinary OLFs which we described recently (5).

As a trace metal V is diuretic, natriuretic, and causes vasoconstriction (6,7,10). It is a potent inhibitor of Na-K-ATPase and has previously been investigated intensively as a potential regulator of Na-K-ATPase activity. Its biological role has been reviewed by Nechay et al. (6). It strongly

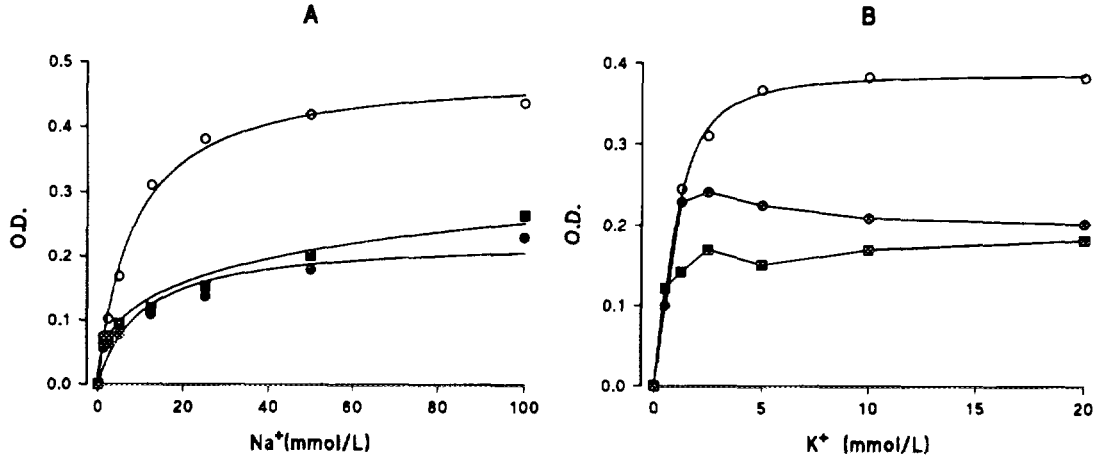

Figure 3. Na-K-ATPase enzyme kinetics in the absence (open circles) and presence of VI^{V}-(closed squares) and V^{V}- (closed circles) diascorbates with respect to sodium (**A**) and potassium (**B**).

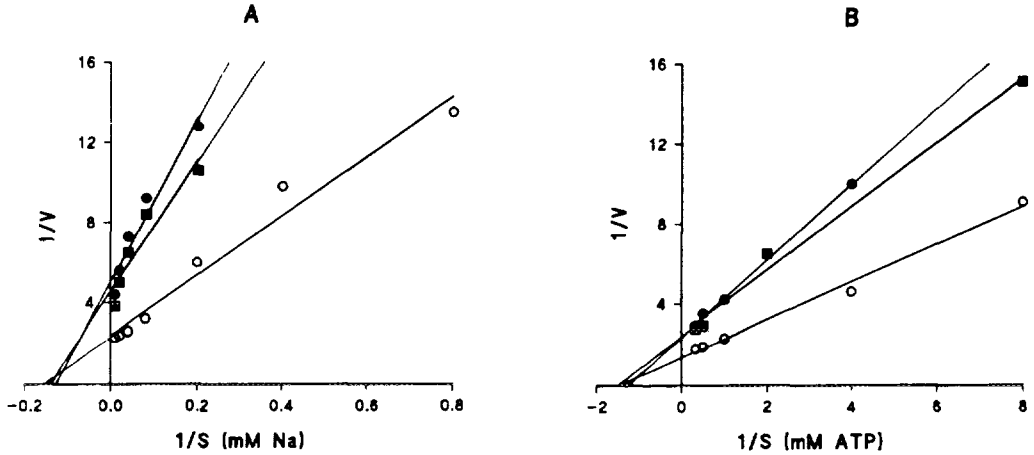

Figure 4. Lineweaver-Burk plot of the effects of V^{IV}- (closed squares) and V^{V}- (closed circles) diascorbates on Na-K-ATPase enzyme (enzyme alone: open circles) kinetics with respect to sodium (**A**) and Mg-ATP (**B**).

inhibits Na-K-ATPase in its E_2-configuration (6,7) as we observed in the present study for V-diascorbates. Interestingly, this trace metal also sensibilizes the enzyme against ouabain (11).

Concentrations of V in plasma and extracellular fluid are in the (low) nanomolar range (6,7). Concentration of total V in human tissue is in the order of 10^{-7}M (12) with highest concentration in the renal cortex, especially in proximal tubular cells (10,13). Vanadium, when absorbed by the body, is excreted mainly in the urine (6,7). It may form a number of complexes with biogenic and related ligands including V^{IV}-complex with ascorbic acid (14) which shows a pK for binding of VO^{2+} of 3.3 (7,14). Twenty-four h urinary excretion of V should be in the micromolar range (10^{-6}M), thus close to the IC_{50} found in the present study for inhibition of Na-K-ATPase by V^{VI}-diascorbate. Nechay et al. (15) reported an IC_{50} for V in the order of 10^{-7}M as compared to 10^{-6}M for ouabain. It was also found that ascorbic acid reduced concentration-dependently the enzyme inhibition by V between

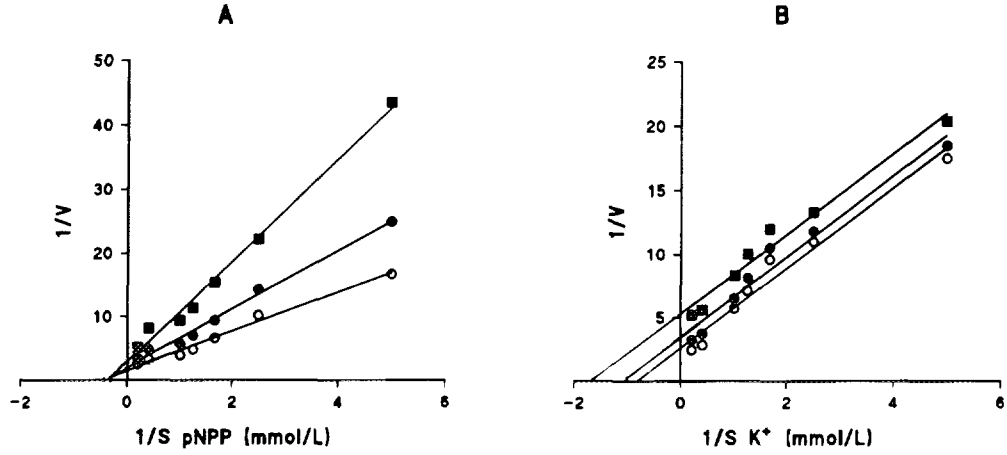

Figure 5. Lineweaver-Burk plot of the effects of V^{IV}- (closed squares) and V^{V}- (closed circles) diascorbates on K^+-pNPPase enzyme (enzyme alone: open circles) kinetics with respect to p-NPP (**A**) and potassium (**B**).

approximately 5% and 10% at 10^{-5} and 10^{-4}M, respectively (15). These data are well compatible with our present findings.

Since we and others (3,4) found that the activities of OLFs in the urine vary with sodium intake or high blood pressure, it may be reasoned that changes in V-diascorbate excretion are related to urinary changes, e.g., urine flow rate, sodium or potassium excretion or urinary pH. In this context, it is interesting to note that urinary excretion of V was found to be increased in patients with essential hypertension (16). However, the exact physiologic and pathologic meanings of the present findings remain to be elucidated. Especially, the questions of whether the presence of the inhibitor(s) in the urine truly reflect endogenous circulating regulator(s) of Na-K-ATPase and whether they are related to mechanisms governing body salt and water balance and blood pressure regulation await further investiga-tion. Nonetheless, since V is eliminated from the body via the kidney and is a potent inhibitor of membrane-bound renal and vascular Na-K-ATPase, our previous (5) and present data strongly suggest that V-diascorbates represent endogenous OLFs excreted in human urine. Interestingly, whereas ouabain is not truly natriuretic, V possesses all properties postulated for OLF, i.e., it is a diuretic and natriuretic as well as vasoconstrincting agent (6). Its multiple (intra-) cellular signalling mechanisms are compatible with our previous observations with the urinary OLF (17).

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