Biochimica et Biophysica Acta, 558 (1979) 99—107

BBA 78558

EFFECTS OF VANADATE ON OUABAIN BINDING AND INHIBITION OF $(Na^+ + K^+)$-ATPase

TIMOTHY D. MYERS, ROBERT C. BOERTH and ROBERT L. POST

The Departments of Pharmacology, Pediatrics and Physiology, Vanderbilt University School of Medicine, Nashville, TN 37232 (U.S.A.)

(Received March 28th, 1979)

Key words: Vanadate; Ouabain; $(Na^+ + K^+)$-ATPase

Summary

Effects of vanadate on ouabain binding and inhibition of sodium and potassium adenosine triphosphatase ($(Na^+ + K^+)$-ATPase) were investigated under various ionic conditions.

1. Vanadate facilitated ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and this facilitation was partially reversed by catechol.

2. Vanadate antagonized the ability of high concentrations of NaCl to inhibit ouabain binding in the presence of magnesium.

3. Ouabain binding to the vanadate-enzyme complex, formed from magnesium and vanadate, was more sensitive to depression by potassium than that to the phosphoenzyme formed from magnesium and inorganic phosphate.

4. Preincubation of $(Na^+ + K^+)$-ATPase with vanadate in the presence of magnesium initially formed a potassium-insensitive complex as shown by a rapid initial rate of ouabain binding. However, within 5 min potassium overcame the vanadate potentiation of ouabain binding regardless of the order in which it was added to the reaction mixture.

5. Under conditions of enzyme turnover, vanadate failed to antagonize the inhibitory power of ouabain despite the presence of a high concentration of potassium. This suggests a possible relationship between the sensitivity of the sodium pump in various tissues to the cardiac glycosides and intracellular vanadate concentrations.

Introduction

Ouabain binds to $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and inhibits ATP hydrolysis [1]. Ouabain binds preferentially to the phosphorylated enzyme whether formed from ATP or from inorganic phosphate

[2]. Binding requires Mg^{2+}. Ouabain binding in the presence of magnesium and inorganic phosphate is antagonized by K^+ and this antagonism is thought to represent competition between potassium and ouabain for the same conformation of the enzyme [3].

Recently, Cantley et al. presented evidence that vanadate, with vanadium in the pentavalent state, is a potent inhibitor of ($Na^+ + K^+$)-ATPase [4]. Several lines of evidence point to a vanadium compound as an inhibitor in ATP (Sigma) derived from equine muscle [5,6]. Vanadate binding to a high affinity site on ($Na^+ + K^+$)-ATPase is facilitated by Mg^{2+} and K^+ and is inhibited by ATP [7]. Based on their binding studies, Cantley and coworkers proposed that vanadate produced inhibition of ($Na^+ + K^+$)-ATPase by acting as a transition state analog of inorganic phosphate at a site on the enzyme which is also a low affinity site for ATP [7].

The possibility that ouabain and vanadate produce inhibition of ($Na^+ + K^+$)-ATPase by the same mechanism seems remote in the light of previous experiments. Vanadate produces inhibition from the cytoplasmic side of the plasma membrane while ouabain inhibits from the extracellular solution [8]. K^+ facilitates vanadate binding and antagonizes ouabain binding [3,7]. However, both vanadate and ouabain binding to ($Na^+ + K^+$)-ATPase are facilitated by Mg^{2+}. Thus, if vanadate acts like a phosphate transition-state analog, it could facilitate ouabain binding to the enzyme in the presence of Mg^{2+}. Hansen [9] has made a preliminary report of facilitation of ouabain binding to ($Na^+ + K^+$)-ATPase by vanadate. The purpose of our experiments was to test various ionic ligands on the effects of vanadate on ouabain binding and inhibition ($Na^+ + K^+$)-ATPase.

Methods

Enzyme preparation and assay for inhibition by ouabain

The enzyme preparation consisted of crude membranes from guinea-pig kidney. These membranes were prepared according to the method of Post and Sen [10]. The protein content of the stock solution was 6.3 mg/ml and the specific activity was 3.7 μmol P_i/min per mg protein at 37° C.

The assay for inhibition of ATPase activity by ouabain closely followed the procedure of Matsui and Schwartz [11]. The stock enzyme solution was diluted 20-fold in the buffer used for the ouabain-binding assay (see below) and 50 μl was used in each reaction tube. The enzyme was preincubated for 20 min at 37°C in a buffer containing 100 mM NaCl, 20 mM KCl, 50 mM Tris-HCl (pH = 7.4), 1.0 mM EDTA, and 5.0 mM $MgCl_2$. Ouabain (Sigma Chemical Co.) and $NaVO_3$ when added, were present during the preincubation period. Reactions were started by adding the disodium salt of ATP (Sigma Chemical Co., nominally vanadium-free) to a final concentration of 2 mM. The volume of the reaction mixture was 1.0 ml. Reactions were stopped and inorganic phosphate was assayed as previously described [11].

[^{3}H]Ouabain binding assay

[^{3}H]Ouabain (New England Nuclear, 14.4 Ci/mmol) was received in an ethanol/benzene solvent which was evaporated. The [^{3}H]ouabain was redis-

solved in water or 0.9% NaCl. Assays were conducted in 5-ml polypropylene test tubes at room temperature. Reactions usually were started by combining a 20- or 40-fold dilution of the stock enzyme solution in 20.2 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, and 5.2 mM $MgCl_2$ with the radioactive ouabain. KCl, NaCl, NH_4Cl, NH_4VO_3, and $NaVO_3$ were of reagent grade and were added as indicated in each experiment. The final volume of the reaction mixture was 0.1 ml.

Binding reactions were terminated by addition of 2 ml of ice-cold water and immediate vacuum filtration through 0.22 μm filters (Millipore Corp.). The filters were rinsed with an additional 5 ml of cold water and placed in scintillation vials. The filters were dried, 8 ml of PPO-POPOP-toluene fluor were added to each vial, and they were counted in a Beckman LS-250 counter. The counts per minute illustrated in the Figures represent total counts per minute. Nonspecific binding determined by either potassium or sodium suppression of ouabain binding in the presence of Mg^{2+} represented less than 5—10% of the total binding values.

Results

Vanadate facilitation of ouabain binding and partial reversal by catechol

Our initial observation of a vanadate effect on ouabain binding was made in the presence of 100 mM NaCl. This concentration of NaCl added before or simultaneously with [^{3}H]ouabain (1 μM) routinely reduced the binding level at 25°C to less than 7% of the total binding in the absence of sodium. Inorganic phosphate (1.0 mM) had no effect on the sodium antagonism of ouabain binding in the presence of magnesium. However, the addition of ammonium vanadate (88 μM) 30 min before ouabain caused a 10-fold increase in the

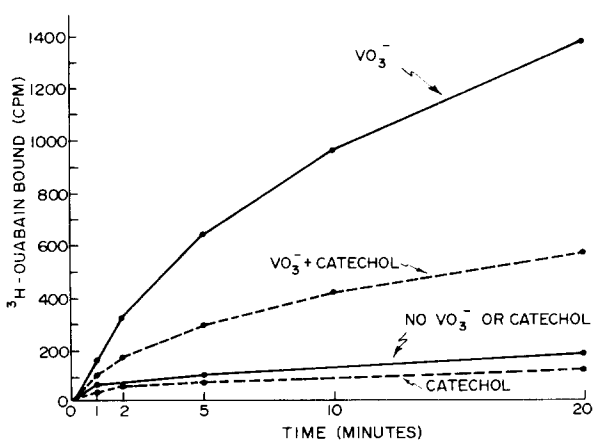

Fig. 1. Vanadate potentiation of ouabain binding and partial reversal by catechol. Preincubation times were 30 min at room temperature (25° C) with or without vanadate (9 μM). Catechol (20 mM) was then added to designated reaction mixtures (dashed lines). All reaction mixtures were incubated for another 120 min at 37° C in a water bath. At time 0 [^{3}H]ouabain (10 nM) with NaCl (15.4 mM) was added and the time course of binding was followed at room temperature. The enzyme dilution was 20-fold with a binding level at saturation of 148 · 10^3 cpm per reaction tube.

amount of ouabain bound to the enzyme after 120 min with almost no effect on the total binding level seen in the absence of sodium.

In order to follow the time course of the effect of vanadate the concentrations of ouabain, vanadate, and NaCl were lowered (Fig. 1). When vanadate was preincubated with the enzyme, the rate of ouabain binding was noticeably faster than the rate observed in the absence of vanadate. When catechol was included with vanadate, the effect of vanadate was partially reversed. Preincubation of the enzyme with catechol alone had no effect on the rate of ouabain binding. This slower rate of binding found with addition of catechol to vanadate was expected since it is known that catechols lower the free vanadate concentration by reducing the vanadate and forming a complex with it [7].

Effects of potassium on vanadate potentiation of ouabain binding

Observations by other investigators that potassium facilitates vanadate binding and antagonizes ouabain binding to $(Na^+ + K^+)$-ATPase prompted experiments to determine the effect of potassium on ouabain binding to the enzyme in the presence of vanadate [3,7]. When the enzyme was preincubated with potassium either in the presence or absence of vanadate for 30 min prior to addition of ouabain or when potassium was added simultaneously with ouabain in the absence of vanadate, the same 'slow' rate of ouabain binding was observed (Fig. 2). A faster rate of ouabain binding was observed in the absence of potassium, and the fastest rate of binding was seen with preincubation with vanadate in the absence of potassium. Preincubation of the enzyme with vanadate followed by simultaneous addition of potassium and ouabain yielded an initial rapid rate of ouabain binding which then slowed over several minutes to become as slow as the 'slow' rate described above. These observations suggested that the initial rate of ouabain binding was dependent on the duration

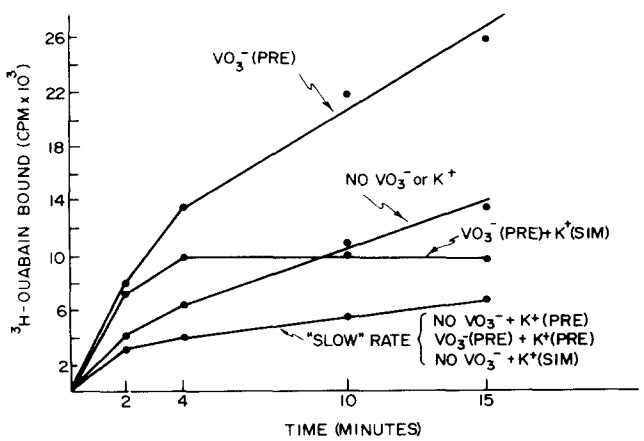

Fig. 2. Effects of potassium on vanadate-enhanced ouabain binding. Vanadate (0.9 μM), when added, was preincubated for 30 min at room temperature with the enzyme with or without KCl (0.5 mM). Reactions were started by addition of [^{3}H]ouabain (500 nM) with or without KCl at time 0. The enzyme dilution was 40-fold with a saturation binding level of 80 · 10^3 cpm per reaction mixture tube. The 'slow' rate was identical for the three incubation conditions. SIM, potassium added simultaneously with [^{3}H]ouabain; PRE, potassium preincubated with the enzyme preparation for 30 min at room temperature prior to addition of [^{3}H]ouabain.

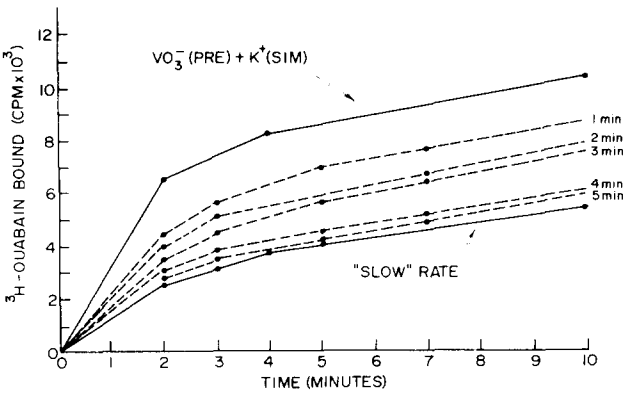

Fig. 3. Time dependence of potassium suppression of vanadate-enhanced ouabain binding. All reaction mixtures were allowed to equilibrate with vanadate (0.9 μM) at room temperature for 30 min before addition of KCl (0.5 mM). Dashed lines represent addition of KCl at 1 min, 2 min, 3 min, 4 min, or 5 min prior to addition of [^{3}H]ouabain (500 nM) at time 0. The 'slow' rate representing preincubation of the enzyme, vanadate, and KCl for 30 min prior to the addition of [^{3}H]ouabain has been replotted from Fig. 2 for the convenience of the reader. The enzyme dilution was 40-fold with a saturation binding level of 80 · 10^3 cpm per reaction tube. The rate constant for the action of potassium was about 0.41/min. PRE and SIM, see legend Fig. 2.

of exposure of the vanadate-enzyme complex to potassium. To investigate this phenomenon further, potassium was added at various times before reactions were initiated with ouabain (Fig. 3). Following the addition of potassium, the rate of ouabain binding gradually shifted down towards that of the 'slow' rate as the time of exposure to potassium increased. Preincubation of the enzyme with potassium for 5 min before addition of ouabain was sufficient to abolish the initial burst of ouabain binding in the presence of vanadate and produce the

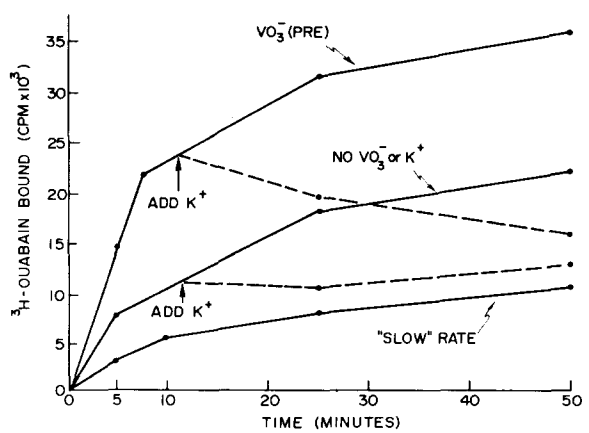

Fig. 4. Potassium reversal of vanadate potentiated ouabain binding. Vanadate (0.9 μM), when added, was preincubated for 30 min with the enzyme with or without KCl (0.5 mM). At time 0 reactions were started by addition of [^{3}H]ouabain (500 nM). To certain reactions preincubated without KCl plus or minus VO_3^-, the KCl was added at 12 min after the addition of ouabain (dashed lines). The 'slow' rate representing preincubation of the enzyme for 30 min with KCl alone or vanadate + KCl was identical to that observed in Fig. 3. The enzyme dilution was 40-fold with a saturation binding level of 80 · 10^3 cpm per reaction tube. PRE and SIM, see legend Fig. 2.

'slow' rate. From the information contained in Figs. 2 and 3 it appeared that exposure to potassium was an important determinant of vanadate's ability to potentiate ouabain binding to $(Na^+ + K^+)$-ATPase. When potassium was added 12 min after the addition of ouabain to the enzyme preincubated with vanadate it was able to completely antagonize the vanadate potentiation of ouabain binding (Fig. 4). Thus, it appeared that regardless of when potassium was added to the reaction mixture the same 'slow' rate of ouabain binding was eventually observed.

Comparison of the vanadate and phosphate preincubated enzymes: effects on ouabain binding

Since potassium decreases the rate of ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of magnesium plus phosphate (see Ref. 3) and magnesium plus vanadate (see Figs. 2, 3 and 4), experiments were conducted comparing the potassium sensitivity of the phosphoenzyme to that of the vanadate-enzyme complex. The time course and amount of [^{3}H]ouabain bound in the presence of magnesium and inorganic phosphate (1.0 mM) was virtually identical to the curve for VO_3^-(PRE) shown in Fig. 4. Preincubation of the enzyme with potassium (0.5 mM) and vanadate (0.9 or 9 μM) before the addition of [^{3}H]ouabain (500 nM) reduced the level of ouabain binding to 15% of the control level in the absence of potassium for periods longer than 10 min. The ouabain binding level of the enzyme preincubated with inorganic phosphate (1.0 mM) was reduced by potassium (0.5 mM) to 55% of the control level at the same time points. These observations suggested that the vanadate-enzyme complex had a higher affinity for potassium than did the phosphoenzyme.

In other experiments a concentration of phosphate (5.0 mM) producing a maximal rate of ouabain binding to the enzyme was combined with a concentration of vanadate (90 μM) producing the same maximal rate. The rate produced by a combination of these concentrations of phosphate and vanadate was the same as the rate obtained with either ligand alone. We took this as evidence that vanadate was acting on the dephosphoenzyme since vanadate produced no potentiation of ouabain binding once the enzyme was maximally phosphorylated.

Inhibition by ouabain and vanadate under enzyme turnover conditions

The observation of vanadate facilitation of ouabain binding together with the high affinity for potassium of the vanadate-enzyme complex led to experi-

TABLE I

LACK OF VANADATE EFFECT ON INHIBITORY POWER OF OUABAIN

Enzyme activity was assayed as described in Methods. The values represent percent inhibition of contr activity (i.e., no inhibitors present). The inhibitors were preincubated for 20 min before reactions wer started with disodium ATP and reactions were stopped after 21 min at 37°C.

Inhibitor	Control inhibition (%)	Additional ouabain (2.0 μM)(%)
Ouabain (1.0 μM)	37	60
Vanadate (62 nM)	37	60

ments to find out if vanadate modified the ability of ouabain to inhibit ATPase activity. A concentration of ouabain (2 μM), previously determined to produce inhibition, was added to a concentration of vanadate (62 nM) or to a concentration of ouabain (1 μM) producing inhibition equal to that of vanadate. The total inhibition of the enzyme produced by the addition of ouabain (2 μM) in the presence of either vanadate or the lower concentration of ouabain was equal (Table I). This result suggested that the inhibitory power of ouabain under enzyme turnover conditions was not altered by the presence of vanadate.

Discussion

Vanadate inhibits a variety of enzymes that hydrolyze phosphate ester bonds and appears to function as a transition-state analog of phosphate [7,12,13,14]. Our results support this theory since it acted like phosphate in facilitating ouabain binding to ($Na^+ + K^+$)-ATPase (Fig. 1). It was effective at concentrations roughly 1000-fold less than those of phosphate producing a comparable facilitation suggesting that vanadate is an extremely potent phosphate analog. The ability of vanadate to overcome the inhibitory power of 100 mM NaCl further supports this conclusion. Hansen [15] reported that in the presence of magnesium (5 mM) and phosphate (0.3 mM) 100 mM NaCl antagonized ouabain binding. Under similar conditions high concentrations of sodium prevent formation of the phosphoenzyme from inorganic phosphate [16]. Therefore, we conclude that 100 mM NaCl impairs ouabain binding by preventing formation of the phosphoenzyme but is much less effective at preventing formation of a vanadate-enzyme complex which avidly binds ouabain.

The effects of potassium on vanadate supported ouabain binding are interesting because potassium antagonizes ouabain binding competitively and yet vanadate facilitates the binding of both potassium and ouabain [7,17]. When the enzyme preincubated with vanadate was added simultaneously to potassium and ouabain an initial burst of ouabain binding was observed (Fig. 2). This suggests that the vanadate-enzyme complex is initially insensitive to potassium but gradually acquires a sensitivity to potassium over a period of minutes. Magnesium alone is not responsible for the initial potassium insensitivity since preincubation of the enzyme in the presence of magnesium for 30 min with the simultaneous addition of potassium and ouabain yielded the same 'slow' rate of ouabain binding as preincubation of the enzyme with magnesium and potassium followed by addition ouabain (Fig. 2). Since vanadate binds poorly to ($Na^+ + K^+$)-ATPase in the absence of magnesium [7], it is logical to assume that the potassium-insensitive enzyme is a ternary complex with both magnesium and vanadate. Magnesium has been shown to convert the potassium-sensitive phosphoenzyme to potassium-insensitive phosphoenzyme, each form displaying a similar response to ouabain [16]. It is possible that magnesium is acting in an analagous manner on the vanadate-enzyme complex to produce initially a potassium-insensitive complex. The sensitivity to potassium ultimately acquired in the presence of vanadate, however, is greater than in the presence of phosphate (see Results section comparing phosphate- and vanadate-preincubated enzymes).

Our observations of vanadate potentiation of ouabain binding and the increased affinity of the vanadate-enzyme complex for potassium coupled with previous observations that vanadate alone is a potent inhibitor of the plasma membrane ($Na^+ + K^+$)-ATPase suggest a possible role for vanadate in the binding of the cardiac glycosides and their pharmacological potency. Vanadium concentrations of 0.1 μM to 1 μM in mammalian tissue have been previously reported [18,19]. If free intracellular vanadate binds to the enzyme, this inhibition should mimic the actions of the cardiac glycosides alone since other agents which inhibit ($Na^+ + K^+$)-ATPase by different mechanisms produce cardiac glycoside-like effects [20]. The vanadate-enzyme complex ultimately formed displays an increased affinity for ouabain or potassium leading us to conclude that the response to a concentration of cardiac glycosides at a given concentration of potassium should vary with the concentration of free vanadate capable of interacting with ($Na^+ + K^+$)-ATPase. Since the vanadate-enzyme complex has a higher affinity for potassium than the phosphoenzyme and a slower rate of ouabain binding in the presence of potassium one might suppose that free vanadate could antagonize the effects of the cardiac glycosides. This is unlikely because the vanadate-enzyme complex is already inhibited whether ouabain binds or not and the inhibitory power of ouabain is not affected by vanadate under conditions of enzyme turnover (Table I). The latter observation coupled with the binding studies (Fig. 4) suggests that the vanadate potentiation of ouabain binding to ($Na^+ + K^+$)-ATPase (Fig. 1) is seen only in the absence of potassium. When potassium was present (i.e., under enzyme turnover conditions) the vanadate potentiation of ouabain binding was completely antagonized resulting in no potentiation of ouabain inhibition. These results lead us to conclude that inhibition of ($Na^+ + K^+$)-ATPase by ouabain is simply added to an existing inhibition of the enzyme by intracellular vanadate. Therefore, preexisting inhibition of ($Na^+ + K^+$)-ATPase by intracellular vanadate could be an important determinant of the response in vivo to a given dose of ouabain. Since digitalis toxicity is a serious problem, the role of intracellular vanadate as a modulator of the sensitivity of the sodium pump in the heart to the cardiac glycosides should be explored further.

Acknowledgements

This work was supported in part by National Institutes of Health Training Grant GM-00058, Biomedical Research Support Grant RR-05424 and Grant No. 5R01 HL-01974 from the National Heart, Lung, and Blood Institute of the N.I.H.

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