Biochimica et Biophysica Acta, 568 (1979) 265—269

BBA Report

BBA 61349

FACILITATION OF OUABAIN BINDING TO (Na^+ + K^+)-ATPase BY VANADATE AT IN VIVO CONCENTRATIONS

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(Received November 11th, 1978)

Keywords: (Na^+ + K^+)-ATPase; Vanadate interaction; Ouabain binding; (Facilitation of ouabain binding)

Summary

In the presence of Mg^{2+} vanadate was shown to facilitate ouabain binding to (Na^+ + K^+)-ATPase in much the same way as P_i does. Thus the hypothesis that vanadate interacts with the phosphate site of the enzyme seems to be supported by ouabain binding experiments.

At given ouabain concentrations maximum binding is achieved at μM concentrations of vanadate whereas mM concentrations of P_i are needed. Na^+ as well as K^+ counteract ouabain binding but some cardiac glycoside binding is still possible at in vivo concentrations of these cations. A minor contamination of the enzyme preparations with vanadate could explain the in vitro binding of ouabain that can be obtained with Mg^{2+} and in the absence of P_i.

Vanadate has a structural similarity to phosphate but whereas the latter hardly interferes with the hydrolysis of ATP by (Na^+ + K^+)-ATPase [1,2] vanadate is a potent inhibitor of the enzyme [3,4]. That inorganic phosphate interacts with the ATPase is shown in ^{18}O exchange reactions [5] and in its promotion of cardiac glycoside binding. In the presence of Mg^{2+} binding of these specific inhibitors of (Na^+ + K^+)-ATPase takes place when the substrate ATP or the hydrolysis product P_i is added to the incubation medium. Rather high concentrations of inorganic phosphate are needed for ATPase inhibition [2] and for facilitation of glycoside binding [6]. Attempts to characterize the enzyme-glycoside complex created in vivo indicate that the ATP-supported complex predominates [7], an observation which seems reasonable considering that Na^+ inhibits formation of the P_i-supported complex but facilitates the ATP-supported complex.

Since vanadate is a potent inhibitor of ATPase in the nM—μM concen-

tration range and seems to be present in many tissues in this same concentration range [4,8] it appears to be a likely canditate for the facilitation of the interaction of $(Na^+ + K^+)$-ATPase with cardiac glycosides in vivo.

For this reason a preliminary study on [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase was initiated.

$(Na^+ + K^+)$-ATPase from ox brain was prepared as described by Klodos et al. [9]. The preparations had specific activities of 197—226 μmol P_i/mg protein per h and were practically devoid of ATPase activity not dependent upon $Na^+ + K^+$.

[^{3}H]Ouabain obtained from New England Nuclear Corp. was purified as described elsewhere [10] and diluted with unlabelled ouabain (Merck) to specific activities in the range 25—200 Ci/mol.

Vanadium salt was obtained as metavanadate, $NaVO_3$ (Merck). A 400 mM stock solution of orthovanadate was prepared from the metavanadate in 1 N NaOH, (the nomenclature used in analogy with similar phosphorous compounds).

Determination of [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase and characterization of the enzyme-ouabain complex formed were carried out as described previously [10].

In the presence of Mg^{2+} some binding of ouabain to $(Na^+ + K^+)$-activated ATPase takes place even in the absence of P_i or any other 'substrate'. This is seen in Fig. 1 A as a slow binding, which terminates at a low level. On addition of vanadate, a remarkable effect on the rate and final level of ouabain binding is noticed. From the figure it is seen that 133 nM vanadate affects both parameters, and a further increase is seen with 1 μM vanadate. Almost no further binding is achieved in the presence of 10 μM vanadate. Fig. 1A also shows that an incubation medium containing 3 mM P_i appears to give a somewhat higher rate of binding and a higher equilibrium binding level than are reached with vanadate.

If the concentration of $(Na^+ + K)$-ATPase is defined as the number of ouabain binding sites [10,11] and if the simultaneous binding of one molecule of vanadate is necessary for the binding of one molecule of ouabain, it can be seen from Fig. 1B that there is no great excess of vanadate in the experiment in which 133 nM vanadate were used. The concentration of ouabain binding sites was 57 nM at the enzyme concentration used for binding as seen from the ordinate intercept in Fig. 1B which shows Scatchard plots of equilibrium binding data obtained with 3 incubation media. It is seen that the number of ouabain binding sites seems to be the same irrespective of whether 3 mM P_i, 1 mM *p*-nitrophenyl phosphate or 10 μM vanadate facilitate ouabain binding.

The facilitating effect of even very low concentrations of vanadate on binding of ouabain to $(Na^+ + K^+)$-ATPase thus fits well with the hypothesis that vanadate interacts with the phosphate site of the enzyme [4]. The remarkable effect of vanadate on ouabain binding at concentrations similar to that of enzyme, seems compatible with a 1 : 1 vanadate: enzyme interaction and the reported K_i of 40 nM for vanadate inhibition of $(Na^+ + K^+)$-activated hydrolysis of ATP [4]. Vanadate-supported ouabain binding at in vivo concentrations of vanadate [4,8] thus seems to be a reasonable possibility pro-

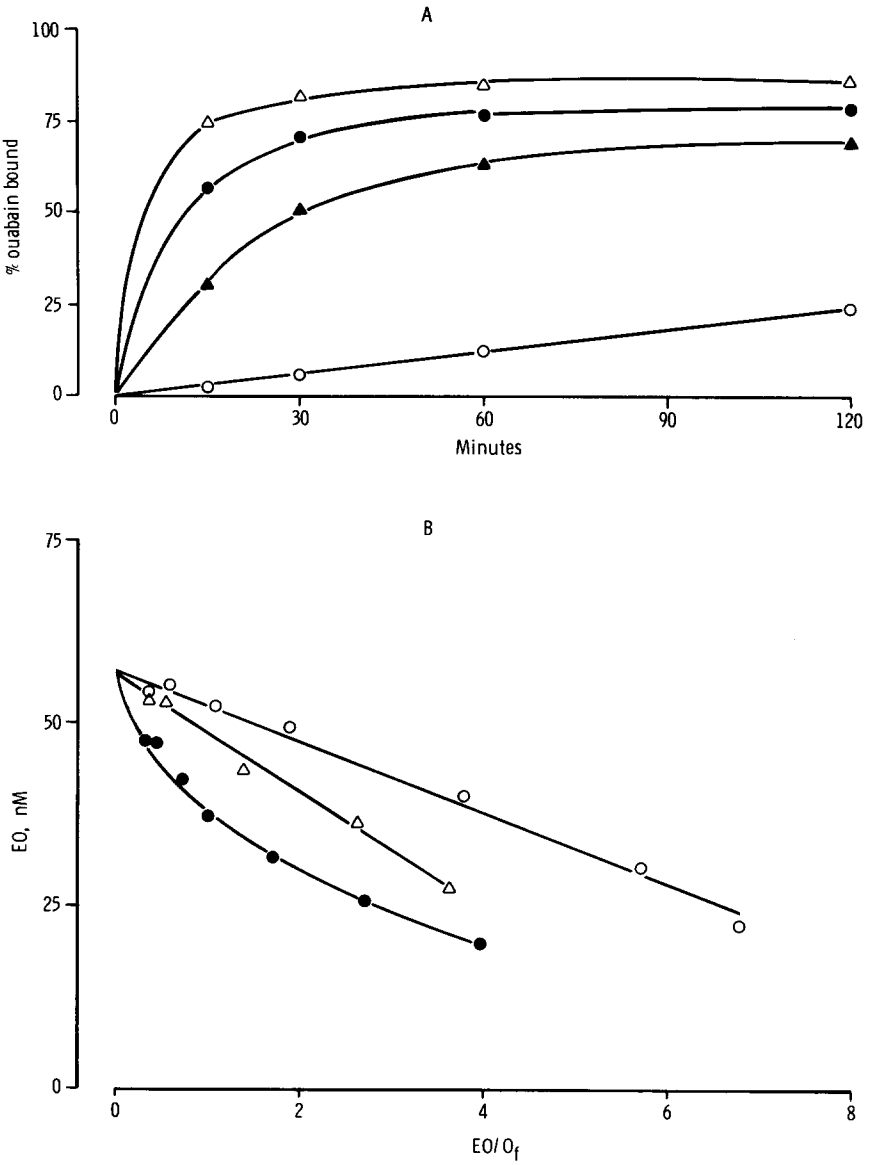

Fig. 1(A) Time course of [^{3}H] ouabain binding in the presence of Mg^{2+}, (Mg^{2+} + vanadate) or (Mg^{2+} + P_i). Enzyme (0.14 mg protein/ml, ouabain-sensitive activity 226 μmol P_i/mg protein per h) was incubated with 3 mM Mg^{2+}, 40 mM Tris (pH 7.25), $2.5 \cdot 10^{-8}$ M [^{3}H] ouabain (○——○); plus 0.133 μM vanadate (▲——▲) or 1 μM vanadate (●——●) or 3 mM P_i (△——△). Ouabain binding was determined as described elsewhere [11]. (B) Scatchard plots of ouabain binding data obtained with (Mg^{2+} + P_i), (Mg^{2+} + vanadate) and (Mg^{2+} + *p*-nitrophenyl phosphate). Equilibrium binding data of bound [^{3}H] ouabain (EO) in nM obtained after 2 h incubation at 37°C are plotted against bound ouabain/free ouabain (EO/O_f). Incubation medium 3 mM Mg^{2+}, $2.5 \cdot 10^{-8}$ M—$2 \cdot 10^{-7}$ M [^{3}H] ouabain and 3 mM P_i + 40 mM Tris (pH 7.25) (○——○) or 10 μM vanadate + 40 mM Tris (△——△) or 1 mM *p*-nitrophenyl phosphate + 5 mM Tris (●——●), enzyme preparation 0.14 mg protein/ml, ouabain sensitive activity 226 μmol P_i/mg protein per h.

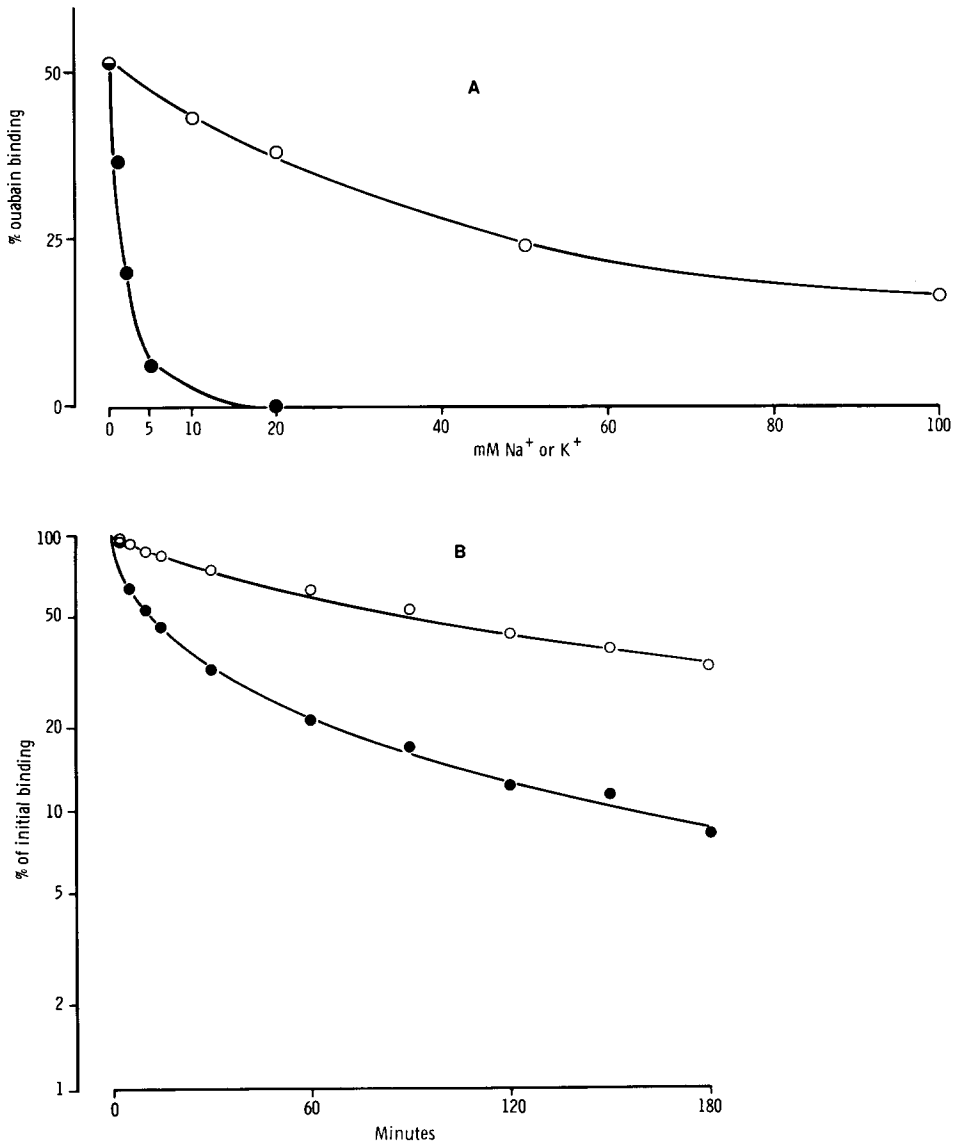

Fig. 2(A) Effect of the concentration of Na^+ or K^+ on the ouabain equilibrium binding level obtained with (Mg^{2+} + vanadate) at a fixed ionic strength. Enzyme (0.20 mg protein/ml, ouabain sensitive activity 197 μmol P_i/mg protein per h) was incubated for 180 min at 37°C with 3 mM Mg^{2+}, 1 μM vanadate, 0—100 mM Na^+, 25—150 mM Tris (pH 7.25) and $2.5 \cdot 10^{-8}$ M [^{3}H] ouabain (○—○) or 0—20 mM K^+, 125—150 mM Tris (pH 7.25) and $2.5 \cdot 10^{-8}$ M [^{3}H] ouabain (●—●). Ouabain binding was determined as described elsewhere [11]. (B) Effect of K^+ on the ouabain dissociation rate after (Mg^{2+} + vanadate)-facilitated ouabain binding with 150 mM Na^+ present during the binding. Enzyme (0.14 mg protein/ml, ouabain sensitive activity 226 μmol P_i/mg protein per h) was incubated for 90 min with 3 mM Mg^{2+}, 150 mM Na^+, 5 mM Tris (pH 7.25), 10 μM vanadate, and $2 \cdot 10^{-7}$ M [^{3}H] ouabain. Incubation was terminated and the enzyme-ouabain complex isolated at 0°C as described elsewhere [10]. The yield was divided into two fractions, one part was resuspended in 4 mM Tris (●), the other part was resuspended in 4 mM Tris + 2 mM K^+ (○). 10^{-3} M ouabain was present in both cases. The release of [^{3}H] ouabain at 37°C was determined as described [11] and expressed as percent of initial binding after wash and resuspension at 0°C.

vided that Na^+ does not abolish the glycoside binding in analogy with the inhibition of the (Mg^{2+} + P_i)-supported binding of ouabain [6]. K^+, on the other hand, does not exclude (Mg^{2+} + P_i)-supported binding although the apparent affinity of binding is reduced [6].

From Fig. 2A it can be seen that with 1 μM vanadate ouabain binding can still take place in the presence of 100 mM Na^+. On the other hand, 20 mM K^+ abolishes ouabain binding. We have reasons to believe, however, that this effect of K^+ takes place on the extracellular side of the membrane fragment bearing the ATPase since high concentrations of Na^+ do not neutralize the inhibitory effect of K^+ in concentrations as low as 5 mM. Thus in vivo concentrations of Na^+ and K^+ would probably not prevent the binding of cardiac glycosides by the (Mg^{2+} + vanadate)-induced pathway.

The dissociation properties of the enzyme-ouabain complex formed in the presence of (Mg^{2+} + P_i) have also been used as an argument against this complex being formed in vivo [7]. The enzyme-ouabain complex obtained in the presence of (Mg^{2+} + P_i) has a slow rate of decay after isolation from the ligands promoting binding and the dissociation is insensitive to K^+. On the contrary, the complex formed in the perfused heart has a fairly fast dissociation rate that is K^+ sensitive [7]. This argument will not hold since it appears that the minute quantity of enzyme-ouabain complex formed in the presence of (Mg^{2+} + P_i + Na^+) behaves like the complex formed in the presence of (Mg^{2+} + Na^+ + ATP): it has a fast rate of decay which is affected by K^+ [10]. Fig. 2B shows that the vanadate-induced complex obtained in the presence of Na^+ also has a fast rate of dissociation that is K^+ sensitive. The vanadate-induced complex formed in the absence of Na^+ has a much slower rate of dissociation (not shown). The nature of the complexes obtained in the presence of vanadate reflects the conditions that existed during their formation. Thus the possibility of in vivo formation of an enzyme-ouabain complex involving vanadate is compatible with the properties of that complex which is formed in the presence of Na^+.

The author gratefully acknowledges the technical assistance of Ms. Kirsten Pedersen. Thanks are due to Dr. P. Leth Jorgensen for helpful discussions during the preparation of the manuscript.

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